ON MYOCARDIAL PROTECTION BY BRIEF PERIODS OF ISCHEMIC AND NON-ISCHEMIC STRESS

Over de bescherming van ischemisch hartweefsel door korte perioden van ischemische en niet-ischemische stress

Proefschrift

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Aan
mijn ouders
en
grootouders

Contents

Chapter 1

Introduction

Less than a decade ago Murry *et al* reported that infarct size in dogs subjected to a 40 min coronary artery occlusion was 29% of the anatomical area at risk. When the 40 min occlusion was preceded by 4 cycles of 5 min coronary artery occlusion and 5 min of reperfusion infarct size was only 7% [1] (Figure 1). This protective effect of brief periods of reversible myocardial ischemia was termed "ischemic preconditioning" and has now been confirmed in a large number of other laboratory animals including pigs, rabbits and rats [2].

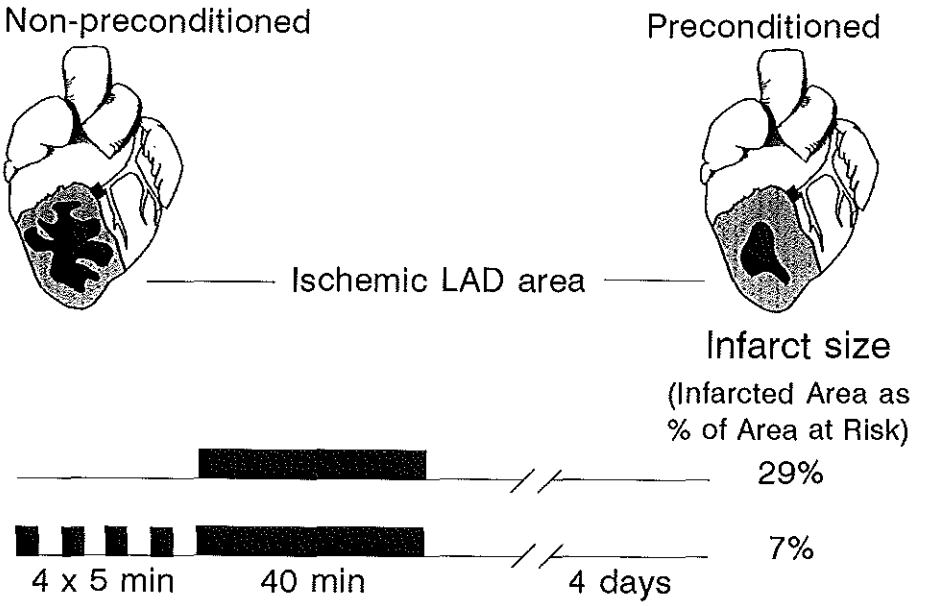

Figure 1. Ischemic preconditioning protocol used by Murry *et al* [1] in an in vivo dog model. Closed bars are periods of ischemic stress applied by occluding the coronary artery of which the distibution zone became the area at risk (gray and black area in the heart). Infarct size (black area in the heart) was determined by histochemical techniques and is expressed as the percentage infarcted area of the area at risk.

In chapter 2 we will review the experimental evidence of the protective effect of ischemic preconditioning and outline the problems that exist in obtaining clinical evidence for this phenomenon.

It has been reported that not only total[1, 3-10] but also partial[11, 12] coronary artery occlusions can precondition the myocardium. Thus, Ovize *et al*[11] observed in dogs myocardial necrosis after a 60 minute total coronary artery occlusion was less when coronary blood flow was reduced by 50% for 15 minute preceding that 60 minute coronary artery occlusion. In that study 15 minute of complete reperfusion between the partial coronary artery occlusion and the sustained total coronary occlusion was necessary to obtain the reduction in infarct size. In contrast, we observed earlier 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 intervening

reperfusion.[12] These findings appear contradictory but could also suggest that the severity and duration of the flow reduction play a critical role in eliciting protection. Although ischemic preconditioning has been the topic of many studies, none of these investigated whether the protection by ischemic preconditioning was the same for the inner and outer myocardial layers across the left ventricular wall. It is well established that a partial coronary artery occlusion affects perfusion of the subendocardial layers more severely than that of subepicardial layers.[13-18] 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 in chapter 3 (*i*) whether the protection by partial coronary artery occlusions depends on the severity and(or) duration of the flow reduction and (*ii*) the transmural distribution of this protection. 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 partial and total coronary artery occlusion stimuli we also analyzed the subendocardial and subepicardial distribution of infarct size of pigs preconditioned with a brief total coronary artery occlusion.[11]

Myocardial protection can be produced by a variety of stimuli causing myocardial ischemia. Thus, one or more brief total[2] or partial[8, 11, 12] coronary artery occlusion(s) limit(s) infarct size produced by a sustained period of ischemia. Moreover, infarct size can also be limited by brief ischemia in adjacent myocardium[19]. Thus a temporary interruption of oxygen supply either within or outside the myocardial region of interest can lead to protection. Recent studies suggest that stimuli that do not cause ischemia may also protect the myocardium. Thus, Ovize *et al*[20] 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[21]. In chapter 4 we describe the effect of *rapid ventricular pacing* on infarct size development produced by a sustained coronary artery occlusion.

Ischemic preconditioning has been described not only for the myocardium,[1] but also for the kidney,[22] skeletal muscle,[23] brain[24] and liver.[25] Furthermore, Przyklenk *et al*[19] reported that a brief coronary artery occlusion preconditioned not only the myocardium within but also outside its perfusion territory ("remote" but intracardiac ischemic preconditioning). It is unknown, however, whether remote organ ischemia can protect the myocardium against infarction. Therefore, we examined in chapter 5 whether brief remote organ ischemia prior to a 60-minute coronary artery occlusion limited myocardial infarct size. For this purpose, we produced transient ischemia in the small intestine or left kidney by occluding the anterior

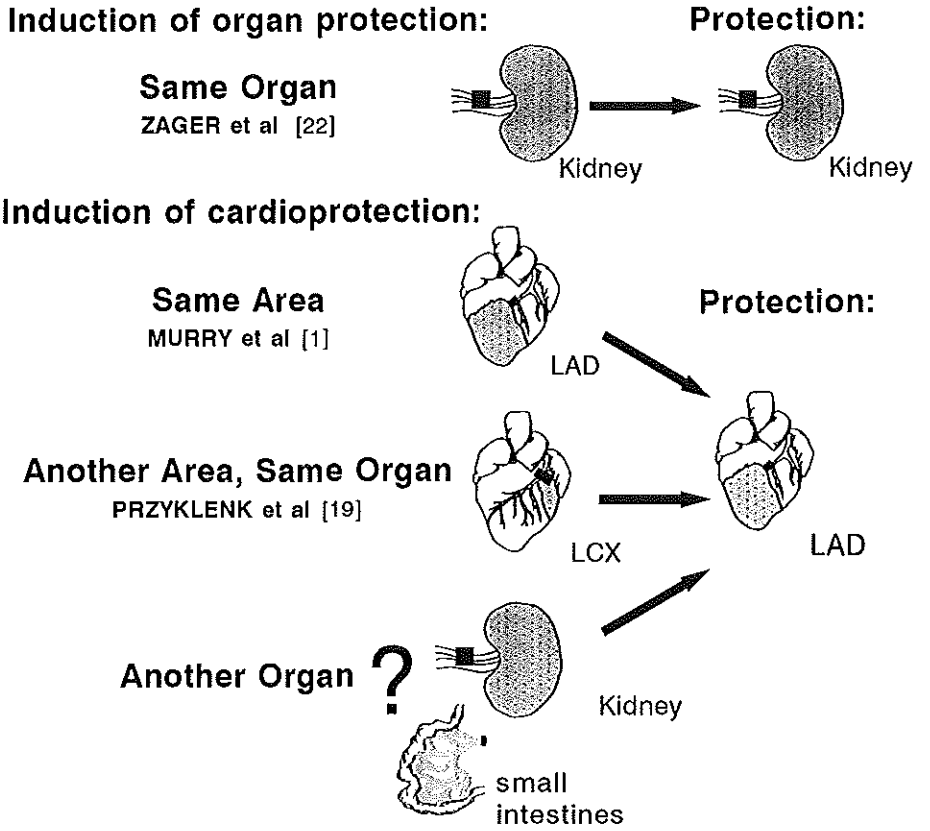

Figure 2. Background of protection by ischemia in organs other than the heart. Ischemic preconditioning is not an organ specific phenomenon and its protective effect is not limited to the area that has become ischemic but also expands to the 'virgin' adjacent area. In chapter 5 we investigated the effect of brief ischemia in other organs on myocardial infarction. LAD=left anterior descending coronary artery; LCX=left circumflex coronary artery.

mesenteric artery or the left renal artery in rats and examined its effect on myocardial infarct size (Figure 2). Since body temperature may influence infarct size[26, 27] and cardioprotection by the adenosine deaminase inhibitor pentostatin was only observed in the presence of mild hypothermia[28] studies were performed at two temperatures. Because results indicated that brief mesenteric artery occlusion provided cardioprotection at both temperatures, mesenteric artery occlusion was selected to examine the mechanism of protection by remote organ ischemia. To investigate whether a neurogenic pathway was involved we repeated the studies after ganglion blockade with hexamethonium. To determine whether activation of the neurogenic pathway occurred during remote organ ischemia or the subsequent 10 minutes of reperfusion, we also determined infarct size after 60-minute coronary artery occlusion in the presence of permanent mesenteric artery occlusion.

In chapter 5, we showed that myocardial infarct size produced by a 60-minute coronary artery occlusion in rats was not different when the experiments were performed at normothermia (36.5°C-37.5°C) or hypothermia (30°C-31°C) [29]. This finding is at variance with previous studies in rabbits [26] and swine [27] in which infarct size produced by 30-[26] and 45-minute [27] coronary artery occlusion coronary artery occlusion was smaller at lower body temperatures. Reasons for the apparent discrepancy could be differences in species or the duration of the coronary artery occlusion. In chapter 6 we therefore investigated the importance of the duration of coronary artery occlusion on the infarct size limiting effect of hypothermia. Since hypothermia was associated with bradycardia, which could possibly limit infarct size per se [30], we studied an additional group of hypothermic rats in which heart rates were increased to heart rates at baseline of the normothermic animals.

Initially, studies searching for the mechanism of ischemic preconditioning focussed on time characteristics and on extracellular endogenous and exogenous factors that either mimicked or inhibited the phenomenon. Protection proved to occur during two distinct episodes: a classical preconditioning period (first window of protection (FWOP)) that lasted 2 to 3 hours after the preconditioning stimulus was applied [1, 2], and a second window of protection (SWOP) between 24 and 72 hours [31-33]. The mechanisms of protection for these two windows are most likely not the same. Endogenous factors rapidly produced agonists may activate intracellular pathways during FWOP, while the slower process of induction of heat-shock proteins may be involved during the SWOP. Initially, attention focussed on activation of adenosine A_1-receptors [34, 35] or K^+_{ATP} channel opening [36, 37], as the mechanisms for protection during FWOP. More recently activation of protein kinase C has received wide attention [38] (Figure 3). Prior to reviewing the role of protein kinase C in chapter 7, we introduce the biochemical properties of protein kinase C and discuss the limitations of the techniques used to investigate its role in ischemic preconditioning. Finally, the evidence that activation of protein kinase C and the intracellular signalling pathways leading to its activation play a pivotal role in the mechanism of ischemic preconditioning is summarized.

In chapter 8 we investigated the role of protein kinase C in ischemic preconditioning and the protection obtained by remote organ ischemia (chapter 5).

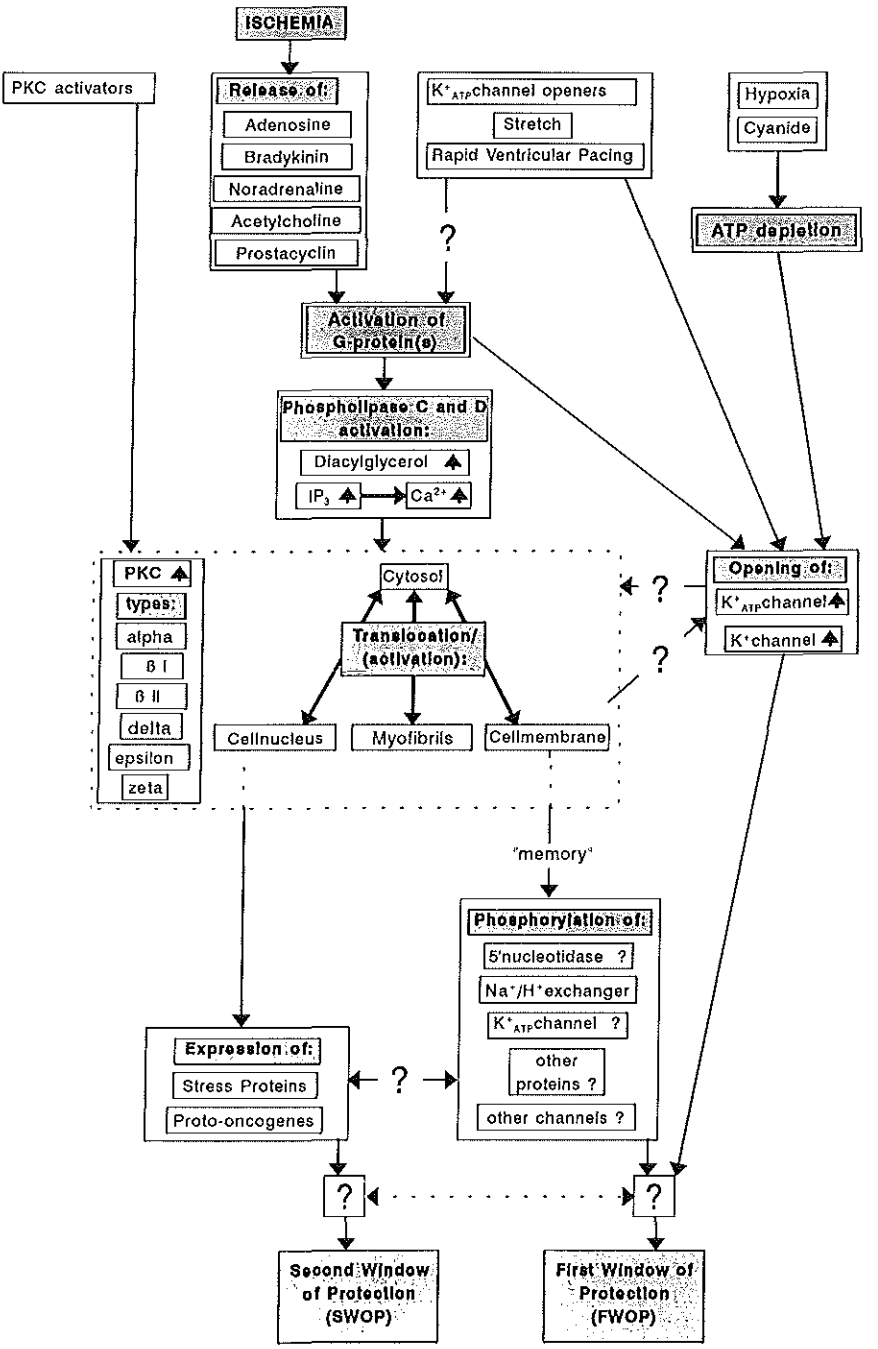

Figure 3. Scheme depicting possible mechanisms in (ischemic) preconditioning.

References

1. Murry CE, Jennings RB, Reimer KA. Preconditioning with ischemia: a delay of lethal cell injury in ischemic myocardium. *Circulation* 1986;75:1124-1136.
2. Lawson CS, Downey JM. Preconditioning: state of the art myocardial protection. *Cardiovasc Res* 1993;27:542-550.
3. Cohen MV, Lui GS, Downey JM, Preconditioning causes improved wall motions as well as smaller infarcts after transient coronary occlusion in rabbits. *Circulation* 1991;84:341-349.
4. Gross GJ, Auchampach JA, Blockade of ATP-sensitive potassium channels prevents myocardial preconditioning in dogs. *Circ Res* 1992;70:223-233.
5. Schott RJ, Rohmann S, Braun ER, Schaper W. Ischemic preconditioning reduces infarct size in swine myocardium. *Circ Res* 1990;66:1133-1142.
6. Sack S, Mohri M, Arras M, Schwarz ER, Schaper W. Ischaemic preconditioning - time course of renewal in the pig. *Cardiovasc Res* 1993;27:551-555.
7. Rohmann S, Weygandt H, Schelling P, Soei LK, Becker KH, Verdouw PD, Lues I, Haüsler G. Effect of Bimakalim (EMD 52692), an opener of ATP-sensitive potassium channels, on infarct size, coronary blood flow, regional wall function and oxygen consumption in swine. *Cardiovasc Res* 1994;28:858-863.
8. Iwamoto T, Miura T, Adachi T, Noto T, Ogawa T, Tsuchida A, Iimmura O. Myocardial infarct size-limiting effect of ischemic preconditioning was not attenuated by oxygen free-radical scavengers in the rabbit. *Circulation* 1991;83:1015-1022.
9. Yellon DM, Alkhulaifi AM, Browne EE, Pugsley WB. Ischaemic preconditioning limits infarct size in the rat heart. *Cardiovasc Res* 1992;26:983-987.
10. Liu Y, Downey JM. Ischemic preconditioning protects against infarction in rat heart. *Am J Physiol* 1992;263(*Heart Circ Physiol* 32):H1107-H1112.
11. Ovize M, Przyklenk K, Kloner RA. Partial coronary stenosis is sufficient and complete reperfusion is mandatory for preconditioning the canine heart. *Circ Res* 1992;71:1165-1173.
12. Koning MMG, Simonis LAJ, De Zeeuw S, Nieukoop S, Post S, Verdouw PD. Ischaemic preconditioning by partial occlusion without intermittent reperfusion. *Cardiovasc Res* 1994;28:1146-1151.
13. Gewirtz H, Gross SL, Williams DO, Most AS. Contrasting effects of nifedipine and adenosine on regional myocardial flow distribution and metabolism distal to a severe coronary arterial stenosis: observations in sedated, closed-chest, domestic swine. *Circulation* 1984;69:1048-1057.
14. Most AS, Williams DO, Gewirtz H. Elevated coronary vascular resistance in the presence of reduced resting blood flow distal to a severe coronary artery stenosis. *Cardiovasc Res* 1985;19:599-605.
15. Pantely AJ, Bristow JD, Swenson LJ, Ladley HD, Johnson B, Anselone CG. Incomplete coronary vasodilatation during myocardial ischemia in swine. *Am J Physiol.* 1985;249 (Heart Circ Physiol. 18):H638-H647.
16. Sassen LMA, Soei LK, Heere TJM, van Woerkens LJ, Saxena PR, Verdouw PD. Nicorandil and cardiovascular performance in anesthetized pigs with a concentric coronary artery stenosis. *Arch Pharmacol* 1989;340:733-739.
17. Pantely GA, Malone SA, Rhen WS, Anselone CG, Arai A, Bristow J, Bristow JD. Regeneration of myocardial phosphocreatine in pigs despite continued moderate ischemia. *Circ Res* 1990;67:1481-1493.
18. Arai AE, Pantely GA, Anselone CG, Bristow J, Bristow JD. Active downregulation of myocardial energy requirements during prolonged moderate ischemia in swine. *Circ Res* 1991;69:1458-1469.
19. Przyklenk K, Bauer B, Ovize M, Kloner RA, Whittaker P. Regional ischemic "preconditioning" protects remote virgin myocardium from subsequent sustained coronary occlusion. *Circulation* 1993;87:893-899.
20. Ovize M, Kloner RA, Przyklenk K. Stretch preconditions canine myocardium. *Am J Physiol (Heart Circ Physiol 35)* 1994;266:H137-H146.
21. Vegh A, Szekeres L, Parratt JR. Transient ischaemia induced by rapid cardiac pacing results in myocardial preconditioning. *Cardiovasc Res* 1991;25:1051-1053.
22. Zager RA, Baltes LA, Sharma HM, Jurkowitz MS. Responses of the ischemic acute renal failure kidney to additional ischemic events. *Kidney Int* 1984;26:689-700.
23. Mounsey RA, Pang CY, Forrest C. Preconditioning: a new technique for improved muscle flap survival. *Otolaryngology-Head and Neck Surg* 1992;107:549-552.
24. Kitagawa K, Matsumoto M, Tagaya M, Hata R, Ueda H, Niinobe M, Handa N, Fukunaga R, Kimura K, Mikoshiba K, Kamada T. 'Ischemic tolerance' phenomenon found in the brain. *Brain Res* 1990;528:21-24.

25. Lloris-Carsi JM, Cejalvo D, Toledo-Pereyra LH, Calvo MA, Suzuki S. Preconditioning: effect upon lesion modulation in warm liver ischemia. *Transplant Proc* 1993;25:3303-3304.
26. Chien GL, Wolff RA, Davis RF, Van Winkle DM. "Normothermic range" temperature affects myocardial infarct size. *Cardiovasc Res* 1994;28:1014-1017.
27. Duncker DJ, Klassen CL, Herrlinger SH, Pavek TJ, Ishibashi Y, Bache RJ. Effect of temperature on myocardial infarction in swine. *Am J Physiol (Heart Circ Physiol 39)* 1996;270:H1189-H1199.
28. McClanahan TB, Mertz TE, Martin BJ, Gallagher KP. Pentostatin reduces infarct size in pigs only when combined with mild hypothermia. *Circulation* 1994;90:I478(Abstract)
29. Gho BCG, Schoemaker RG, van den Doel MA, Duncker DJ, Verdouw PD. Myocardial protection by brief ischemia in noncardiac tissue. *Circulation* 1996;94:000-000.
30. Nienaber Ch, Gottwik M, Winkler B, Schaper W. The relationship between the perfusion deficit, infarct size and time after experimental coronary artery occlusion. *Basic Res Cardiol* 1983;78:210-225.
31. Kuzuya T, Hoshida S, Yamashita N, Fuji H, Oe H, Hori M, Kamada T, Tada. Delayed effects of sublethal ischemia on the acquisition of tolerance to ischemia. *Circ Res* 1993;72:1293-1299.
32. Marber MS, Latchman DS, Walker M, Yellon DM. Cardiac stress protein elevation 24 hours after brief ischemia or heat stress is associated with resistance to myocardial infarction. *Circulation* 1993;88:1264-1272.
33. Baxter GF, Goma FM, Yellon DM. Duration of the "second window of protection" following ischaemic preconditioning in the rabbit (Abstract). *J Mol Cell Cardiol* 1995;27:A162 (Abstract).
34. Thornton J, Liu GS, Olsson RA, Downey JM. Intravenous pretreatment with A1-selective adenosine analogues protects the heart against infarction. *Circulation* 1992;85:659-665.
35. Toombs CF, McGee DS, Johnston WE, Vinten-Johanson J. Myocardial protective effects of adenosine: infarct size reduction with pretreatment and continued receptor stimulation during ischemia. *Circulation* 1992;86:986-994.
36. Gross GJ, Auchampach JA. Blockade of ATP-sensitive potassium channels prevents myocardial preconditioning in dogs. *Circ Res* 1992;70:223-233.
37. Rohmann S, Weygandt H, Schelling P, Soei LK, Verdouw PD, Lues I. Involvement of ATP-sensitive potassium channels in preconditioning protection. *Basic Res Cardiol* 1994;89:563-576.
38. Downey JM, Cohen MV, Ytrehus K, Liu Y. Cellular mechanisms in ischemic preconditioning: The role of adenosine and protein kinase C. In: Cellular, Biochemical, and Molecular Aspects of Reperfusion Injury. Ed: Das DK; *Ann NY Acad Sci* 1994;723:882-898.

Chapter 2

Ischaemic preconditioning: is it clinically relevant?

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Less than a decade ago Murry *et al* reported that infarct size in dogs subjected to a 40 min coronary artery occlusion was 29% of the anatomical area at risk. When the 40 min occlusion was preceded by 4 cycles of 5 min coronary artery occlusion and 5 min of reperfusion infarct size was only 7% [1]. This protective effect of brief periods of reversible myocardial ischemia was termed "ischemic preconditioning" and has now been confirmed in a large number of other laboratory animals including pigs, rabbits and rats [2].

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 [3,4]. Because in large animal species 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, reperfusion arrhythmias and recovery of contractile function are usually studied in separate models. The anti-arrhythmic component of preconditioning has been convincingly demonstrated only in the rat; in other animal species this effect is less prominent [1].

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 ischaemia will also be discussed.

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

Four distinct phases can be characterized in ischemic preconditioning experiments (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.

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 (threshold) to elicit preconditioning is not sharply defined but no data are available to suggest that occlusions less than 2 min can trigger preconditioning. 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 [5]. Not only total coronary artery occlusions, but clinically even more relevant, also partial coronary artery occlusions can precondition the myocardium [6,7]. Ovize *et al* have described that cyclic coronary flow variation produced by a fixed stenosis and endothelial injury also preconditioned myocardium [8]. Most investigators have used an impaired blood supply to induce preconditioning, but preconditioning

Table 1. **Important features of preconditioning in experiments with infarct size as endpoint and the problems obtaining evidence in man.**

Features of preconditioning in experiments	*Problems in man in obtaining evidence*
Preconditioning stimulus	
Ischemic	
- single or multiple total coronary artery occlusions lasting from 2 to 10 min (supply ischemia) [1,2,7]	- unknown if the duration and severity of anginal attacks are sufficient to precondition the myocardium - unknown if silent ischemia can precondition the myocardium
- severe partial coronary artery occlusions (supply ischemia) [6,7]	- unknown if hibernation leads to preconditioning
- moderate partial coronary artery occlusions + adrenergic stimulation (demand ischemia) [9]	- unknown if exercise stopped at the earlieast sign of ischemia can precondition myocardium
- moderate fixed stenosis + endothelial injury [8]	
Non-ischemic	
- heat stress (heat shock proteins)	
- brief total occlusion of coronary artery supplying adjacent myocardium (remote preconditioning) [10]	
- left ventricular volume loading [13]	- unknown if dilated hearts in heart failure are or can be preconditioned
- transient hypoxia	
- transient occlusion of renal artery? (remote 'preconditioning') [11, 12]	
Pharmacological substances	
- adenosine, K^+_{ATP} channel openers, protein kinase C activators [27,30-33]	- 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 angina pectoris or treatment with K^+_{ATP} channel blockers (Glibenclamide) for diabetes mellitus type II]
- decreased threshold for ischemic preconditioning by subthreshold K^+_{ATP} channel activation [5]	
Intervening (reperfusion) period	
- mandatory following total occlusion (self evident) 1 min - 2 hours first window of protection [1,18] 24 hours - ? second window of protection [15,16]	- reperfusion following anginal attacks may be incomplete and variable in duration
- not necessary following severe partial occlusions [7]	
Sustained coronary artery occlusion	
- duration limited to 90 min (species specific) [1]	- 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
Sustained reperfusion	
- mandatory following the sustained coronary artery occlusion (self evident)	- onset of reperfusion cannot be accurately defined - reperfusion may be incomplete due to pre-existent coronary artery stenosis
Other aspects of preconditioning	
- controversy about the loss of the protective effect of preconditioning during first window (gradual decrease or "all or nothing" phenomenon) [20,21]	- 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
- preconditioning can be reinstated after the protection is lost [22]	
- tolerance develops with chronically applied preconditioning stimuli [24]	
- preconditioning has been demonstrated in hypertrophic hearts [26]	

can also be obtained by increasing oxygen in the face of a maintained oxygen supply[9]. Evidence is accumulating that the myocardium can also be preconditioned by ischemia in a remote region of the left ventricle. 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 [10]. Another example of such remote ischemic preconditioning is the reduction in myocardial infarct size when a renal artery is transiently occluded prior to the coronary artery occlusion [11, 12]. The concept of cardiac protection without the need for ischemia is forwarded. Ovize *et al* [13] described that stretching the myocardium by volume loading reduces infarct size during a subsequent coronary artery occlusion. This could provide an explanation for the remote ischemic preconditioning experiments by Przyklenk *et al* as severe regional ischemic contractile dysfunction leads to stretching of the adjacent non-ischemic myocardium [10]. We have shown that 30 min of rapid ventricular pacing also preconditions the myocardium (Chapter 5). This protection did not involve ischemia as 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 involved activation of K^+_{ATP} channels as pretreatment with the K^+_{ATP} channel inhibitor glibenclamide prevented the protection by ventricular pacing.

The intervening reperfusion period. It is self evident that an intervening reperfusion period is mandatory when myocardium is preconditioned with a total coronary artery occlusion as otherwise the period of sustained ischemia would merely be prolonged. Ischemic preconditioning is a transient phenomenon as with reperfusion periods exceeding two hours the protective effect has dissipated [14]. However, when the duration of the intervening reperfusion period is increased to 24 hours the myocardium may again become preconditioned [15,16]. The evidence for this reappearance of protection is yet not as convincing as that for the classical preconditioning [17].

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 with a 50% flow reduction lasting 15 min unless a period of complete reperfusion was allowed [6]. In contrast, we found that a 70% flow reduction which was maintained for 30 min preconditioned myocardium without an intervening reperfusion period [7].

Whether myocardium can be preconditioned when reperfusion during the intervening period after a brief total coronary artery occlusion is incomplete has not yet been investigated. These data would be of significant clinical importance as they are likely to mimic more closely the clinical situation 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 [1]. The protective effect of preconditioning should therefore be considered to be a shift in the time course of infarct size development [1,18].

The sustained reperfusion period. Ischemic preconditioning could merely postpone myocardial

cell death during the early reperfusion period similarly to what has been described for a number of pharmacological agents. That the protective effect of preconditioning persists and is not only a delay of cell death during the reperfusion period follows from studies that allowed 3-4 days of reperfusion prior to infarct size determination [1,19].

Other features of preconditioning. Some studies suggest a gradual disappearance of the protection effect already during the first hour of the intervening reperfusion period [20]. Our studies support the hypothesis that in the individual animal the protection does not warn off gradually but is more consistent with an "all or nothing" phenomenon [21].

A stimulus given immediately after preconditioning is lost will reinstate the cardioprotective action [22], but a stimulus given while the myocardium is still protected does not prolong the protective effect beyond that produced by the initial stimulus [23]. 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 awake rabbits subjected to 5 min occlusions at 30 min intervals for 8 hours [24]. After 3 to 4 days these repetitive occlusions did not protect the myocardium but after a stimulus- free period preconditioning could be recaptured with a single 5 min occlusion. Although it is unlikely that such a large array of multiple occlusions occurs in patients these data indicate that repetitive ischemia in patients could lose its protective effect, particularly as it is possible that preconditioning may already be lost after a smaller number of ischemic episodes.

Many patients that encounter myocardial infarction are older than 50 years and are likely to have hearts which are quite different from the normal hearts in which ischemic preconditioning has been demonstrated. The Framingham study revealed that left ventricular hypertrophy occurs in 12-40% of subjects older than 50 years [25]. This would increase its potential clinical relevance if ischemic preconditioning could be demonstrated in hearts with left ventricular hypertrophy. Speechly-Dick *et al* indeed showed that rats with left ventricular hypertrophy can be preconditioned with a single 5 min coronary artery occlusion [26].

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 [27]. Murry *et al* [1] and others [28] reported reduced rates of glycolysis and high energy phosphate depletion, better preservation of pH and myocardial ultrastructure during the sustained coronary artery occlusion of preconditioned hearts. 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 in stunned myocardium was recruited by infusion of dobutamine within the allocated 2 hours interval between the short and the longlasting coronary artery occlusions [29], indicating that myocardial stunning is not a prerequisite for triggering cardioprotection.

It was not until Downey and co-workers proposed a role for adenosine that a large number of studies began to focus on (sub)cellular mechanisms [30]. Mechanisms that are currently ascribed a pivotal role in ischemic preconditioning include adenosine, K^+_{ATP} channel activation, and G-protein/Protein kinase C activation. For a detailed description of these mechanisms the reader is referred to one of many extensive reviews [2,30-33].

Evidence for the existence of ischemic preconditioning in patients

No clinical study can meet 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 is not available. 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 these do 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. Another 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. The area at risk is most often not determined which makes it impossible to relate the amount of necrosis to the area at risk. Finally, patients may be on medication that is capable of mimicking or inhibiting ischemic preconditioning (aminophylline, K^+_{ATP} channel modulators). Because reperfusion is mandatory following the sustained period of ischemia for preconditioning to be able to limit infarct size, studies performed during the pre- and thrombolytic eras should be differentiated (Table 2) [34-45].

Because of these limitations we also review studies in which multiple periods of ischemia have been investigated usually as part of a diagnostic or therapeutic intervention. If 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.

Several patient categories have been identified in which myocardial adaptation might exist [46,47]. These include patients who have experienced two or more episodes of reversible ischemia, secondary to exercise stress tests, atrial pacing stress tests and percutaneous transluminal coronary angioplasty (Table 3) [48-65]. Patients undergoing arterial cross-clamping as part of

cardiac surgery, and *in-vitro* studies in samples obtained from human hearts may provide additional evidence for ischemic preconditioning in man [66-68].

Table 2. **Evidence for ischemic preconditioning to irreversible damage in man using indirect measurements of infarct size as endpoint.**

Angina preceding infarction	*References*
Positive results	
Without reperfusion	
- global/regional wall function, ejection fraction	[34-36]
With reperfusion (acute PTCA, thrombolysis)	
- enzyme leakage	[37], [38] (TIMI-4)
- hypokinetic area/area beyond the stenosis (=IA/AR)	[37]
- heart failure, shock	[38] (TIMI-4)
- reocclusions	[39] (TAMI)
- short term survival	[39] (TAMI)
Negative results	
Without reperfusion	
- incidence of subendocardial infarction	[40]
- recurrent ischemia, infarct extension	[46] (MILIS)
- recurrent myocardial infarction	[41] (Framingham)
- heart failure	[41] (Framingham)
- short term survival	[40]
- long term survival	[41] (Framingham)
With reperfusion (acute PTCA, thrombolysis)	
- recurrent angina and infarction	[42] (TIMI II)
- residual stenosis	[42] (TIMI II)
- heart failure	[43]
- Killip class	[44] (ITPA/SMT)
- short term survival	[42] (TIMI II), [43, 44] (ITPA/SMT), [45]
- long term survival	[43]

TAMI = Thrombolysis and Angioplasty in Myocardial Infarction

MILIS = Multicenter Investigation of the Limitation of Infarct Size

TIMI = Thrombolysis in Myocardial Infarction

ITPA/SMT = International Tissue Plasminogen Activator / Streptokinase Mortality Trial

PTCA = percutaneous transluminal coronary angioplasty

IA = infarcted area

AR = area at risk

Angina prior to an acute myocardial infarction. Positive as well as negative results, which cannot be explained by the absence or presence of reperfusion by thrombolysis or acute percutaneous transluminal coronary angioplasty, have been reported in patients with angina prior to infarction (Table 2). Importantly, none of the studies reported positive results on long 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 in the non-survivors. Because of the frequency of ischemic episodes prior to the occurrence of myocardial infarction it cannot be excluded that in a number of patients tolerance to ischemic preconditioning had developed [24]. 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. 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 may explain this observation [69], but 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. Exercise-induced ischemia is also less severe during the second of two identical exercise tests when these exercise tests are separated by a short recovery period [48-50]. 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 a subsequent myocardial infarction.

Atrial pacing stress test. 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. Several studies showed that the time to onset of angina was prolonged, while other signs of ischemia were less during the second test (Table 3), which is compatible with the hypothesis of myocardial adaptation. It is unknown whether pacing-induced ischemia may be too brief in duration and not severe enough to elicit ischemic preconditioning.

Angioplasty procedures. Several studies have reported that during the second balloon inflation signs of ischemia are less severe than during the first inflation (Table 3). Failure to observe adaptation might be due to the myocardium already being in an adapted state because of previous ischemic episodes [23] or had become resistant [24]. In addition, the duration of inflation might have been too short (60 sec) or the intervening reperfusion too long to trigger adaptation. Kerensky *et al* [62] reported that after pretreatment with intracoronary adenosine, the magnitude of ST-segment changes during the first and second inflation was no longer different. Their findings could suggest that adenosine pretreatment already adapted the myocardium prior to the first inflation analogous to its protective effect on infarct size. Pretreatment with glibenclamide abolished the attenuation of intracoronary electrocardiographic changes during the second

Table 3. **Myocardial adaptation to repeated episodes of reversible ischemia in man.**

Adaptation to angina	*References*
Exercise stress test ("Warm up" and "Walk through" phenomenon)	
- decreased intensity of pain	[48]
- increased time to onset of pain	[49,50]
- less ST segment changes	[49,50]
- decreased anaerobic myocardial metabolism	[50]
- decreased myocardial O_2-demand (systemic hemodynamics)	[48,49]
- decreased myocardial O_2-consumption	[50]
- increased coronary bloodflow	[49]
Atrial pacing stress test	
Positive results	
- decreased intensity of pain	[51, 52]
- increased time to onset of pain	[51]
- less ST segment changes	[52-54]
- decreased anaerobic myocardial metabolism	[52-54]
- decreased myocardial O_2-consumption	[52]
- decreased myocardial O_2-demand (systemic hemodynamics)	[53, 54]
Negative results	
- similar intensity of pain	[55]
- similar time to onset of pain	[55]
- similar ST segment changes	[51]
- similar anaerobic myocardial metabolism	[55]
- similar myocardial O_2-consumption	[55]
- similar myocardial O_2-demand (systemic hemodynamics)	[51]
Angioplasty procedures	
Positive results	
- decreased intensity of pain	[56-60]
- less ST segment changes	[56-58], [60-62]
- decreased anaerobic myocardial metbolism	[57]
- improved global hemodynamics	[57,58]
Negative results	
- similar ST segment changes	[63-65]
- similar global hemodynamics	[59]
- similar anaerobic myocardial metabolism	[65]
Aortic cross clamp during cardiac surgery	
- slowing of high energy phosphates degradation	[66]
In vitro studies	
Isolated human right atrial trabeculae	
- less deterioration of contractile function	[67]
Human ventricular myocytes	
- decreased cell death, decreased H^+, preserved aerobic myocardial metabolism	[68]

balloon inflation [60], suggesting that activation of K^+_{ATP} channels was involved in this study.

Studies in human myocardial samples. Yellon *et al* [66] randomly assigned patients to a sustained period of global ischemia consisting of 10 min cross-clamping without (control) and with two preceding sequences of 3 min cross-clamping and two min of reperfusion (preconditioning). ATP levels were decreased after the preconditioning protocol when the 10 min cross-clamping period was started but by the end of this period ATP levels in the preconditioned group were higher than in the control group. These observations are in agreement with the original observations by Murry *et al* who showed that ischemic preconditioning slows energy metabolism during a sustained coronary artery occlusion [1].

Walker *et al* [67] suspended 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. The preconditioned group and a control group were then subjected to pacing (180 pulses/min) for 90 min during superfusion with a hypoxic and substrate-free buffer and by 120 min of reoxygenation and pacing at 60 pulses/min. At the end of this period recovery of function was twice as in the preconditioned group period large as in the control group .

Summary and Conclusion

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 atrial pacing stress tests, percutaneous transluminal coronair 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 these 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 maybe 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. 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, but with the recognition and better understanding of the different subtypes possibly involved in preconditioning, new protein kinase C activators may become available without these side-effects. On the other hand, 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 or have

developed tolerance. In this situation it is not unlikely that (additional) protection by a pharmacological agent cannot be accomplished at that time. It is reassuring, however, that in the animal 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.

Finally, 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.

References

1. Murry CE, Jennings RB, Reimer KA. Preconditioning with ischemia: a delay of lethal cell injury in ischemic myocardium. *Circulation* 1986;75:1124-1136.
2. Lawson CS, Downey JM. Preconditioning: state of the art myocardial protection. *Cardiovasc Res* 1993;27:542-550.
3. Cohen MV, Lui GS, Downey JM, Preconditioning causes improved wall motions as well as smaller infarcts after transient coronary occlusion in rabbits. *Circulation* 1991;84:341-349.
4. Parratt J, Vegh A. Pronounced antiarrhythmic effects of ischemic preconditioning. *Cardioscience* 1994;5:9-18.
5. Yao Z and Gross GJ. Activation of ATP-sensitive potassium channels lowers threshold for ischemic preconditioning in dogs. *Am J Physiol* 1994;267:H1888-H1894.
6. Ovize M, Przyklenk K, Kloner RA. Partial coronary stenosis is sufficient and complete reperfusion is mandatory for preconditioning the canine heart. *Circ Res* 1992;71:1165-1173.
7. Koning MMG, Simonis LAJ, De Zeeuw S, Nieukoop S, Post S, Verdouw PD. Ischaemic preconditioning by partial occlusion without intermittent reperfusion. *Cardiovasc Res* 1994;28:1146-1151.
8. Ovize M, Kloner RA, Hale SL, Przyklenk K. Coronary cyclic flow variations "precondition" ischemic myocardium. *Circulation* 1992;85:779-789.
9. Iwamoto T, Bai XJ, Downey HF. Preconditioning with supply-demand imbalance limits infarct size in dog heart. *Cardiovasc Res* 1993;27:2071-2076.
10. Przyklenk K, Bauer B, Ovize M, Kloner RA, Whittaker P. Regional ischemic "preconditioning" projects remote virgin myocardium from subsequent sustained coronary occlusion. *Circulation* 1993;87:893-899.
11. McClanahan TB, Nao BS, Wolke LJ, Martin BJ, Metz TE, Gallagher KP. Brief renal occlusion and reperfusion reduces myocardial size in rabbits. *The FASEB Journal* 1993;7:A118,682.
12. Gho BCG, Schoemaker RG, Van der Lee C, Sharma HS, Verdouw PD. Cardioprotection by transient renal ischemia, an in vivo study in rats. *Circulation* 1994;90,4-2:I-476,2557.
13. Ovize M, Kloner RA, Przyklenk K. Stretch preconditions canine myocardium. *Am J Physiol* 1994;266:H137-H146.
14. Li YW, Whittaker P, Kloner RA. The transient nature of the effect of ischemic preconditioning on myocardial infarct size and ventricular arrhythmia. *Am Heart J* 1992;123:346-353.
15. Kuzuya T, Hoshida S, Yamashita N, Fuji H, Oe H, Hori M, Kamada T, Tada. Delayed effects of sublethal ischemia on the acquisition of tolerance to ischemia. *Circulation Res* 1993;72:1293-1299.
16. Marber MS, Latchman DS, Walker M, Yellon DM. Cardiac stress protein elevation 24 hours after brief ischemia or heat stress is associated with resistance to myocardial infarction. *Circulation* 1993;88:1264-1272.
17. Tanaka M, Fujiwara H, Yamasaki K, Miyamae M, Yokota R, Hasegawa K, Fujiwara T, Sasayama S. Ischemic preconditioning elevates cardiac stress protein but does not limit infarct size 24 or 48 h later in rabbits. *Am J Physiol* 1994;267:H1476-H1482.
18. Miura T, Iimura O. Infarct size limitation by preconditioning: its phenomenological features and the key role of adenosine. *Cardiovasc Res* 1993;27:36-42.
19. Miura T, Goto M, Urabe K, Endoh A, Shimamoto K, Iimura O. Does myocardial stunning contribute to infarct size limiation by ischemic preconditioning? *Circulation* 1991;84:2504-2512.
20. Miura T, Adachi T, Ogawa T, Iwamoto T, Tsuchida A, Iimura O. Myocardial infarct size-limiting effect of ischemic preconditioning: its natural decay and the effect of repetitive preconditioning. *Cardiovasc Pathol* 1992;1:147-154.
21. Koning MMG, De Zeeuw S, Nieukoop S, De Jong JW, Verdouw PD. Is myocardial infarct size limitation by ischemic preconditioning an "all or nothing" phenomenon? In: Cellular, Biochemical and Molecular Aspects of Reperfusion Injury. Ed: Das DK. *Ann NY Acad Sci* 1994;723:336-336.
22. Yang X-M, Arnoult S. Tsuchida A, Cope D, Thornton JD, Daly JF, Cohen MV, Downey JM. The protection of ischaemic preconditioning can be reinstated in the rabbit heart after the initial protection has waned. *Cardiovasc Res* 1993;27:556-558
23. Sack S, Mohri M, Arras M, Schwarz ER, Schaper W. Ischaemic preconditioning - time course of renewal in the pig. *Cardiovasc Res* 1993;27:551-555.
24. Cohen MV, Yang XM, Downey JM. Conscious rabbits become tolerant to multiple episodes of ischemic preconditioning. *Circ Res* 1994;74:998-1004.
25. Savage DD, Garrison RJ, Kannel WB, Levy D, Anderson SJ, Stokes J 3d, Feinleib M, Castelli WP. The spectrum of left ventricular hypertrophy in a general population sample: the Framingham study. *Circulation* 1987;75:26-33.

26. Speechly-Dick ME, Baxter GF, Yellon DM. Ischaemic preconditioning protects hypertrophied myocardium. *Cardiovasc Res* 1994;28:1025-1029.
27. Rohmann S, Weygandt H, Schelling P, Soei LK, Becker KH, Verdouw PD, Lues I, Hausler G. Effect of Bimakalim (EMD 52692), an opener of ATP-sensitive potassium channels, on infarct size, coronary blood flow, regional wall function, and oxygen consumption in swine. *Cardiovasc Res* 1994;28:858-863.
28. Kida M, Fujiwara H, Ishida M, Kawai C, Ohura M, Miura I, Yabuuchi Y. Ischemic preconditioning preserves creatine phosphate and intra cellular pH. *Circulation* 1991;84:2495-2503.
29. Matsuda M, Catena TG, Vander Heide RS, Jennings RB, Reimer KA. Cardiac protection by ischemic preconditioning is not mediated by myocardial stunning. *Cardiovasc Res* 1993;27:585-592.
30. Downey JM, Cohen MV, Ytrehus K, Liu Y. Cellular mechanisms in ischemic preconditioning: The role of adenosine and protein kinase C. In: Cellular, Biochemical, and Molecular Aspects of Reperfusion Injury. Ed: Das DK; *Ann NY Acad Sci* 1994;723:882-898.
31. Walker DM, Yellon DM. Ischemic preconditioning: from mechanisms to exploitation. *Cardiovasc Res* 1992;26:734-739.
32. Parratt J. Protection of the heart by ischaemic preconditioning: Mechanisms and possibilities for pharmacological exploitation. *TiPS* 1994;15:19-25.
33. Gross GJ, Yao Z, Pieper GM, Auchampach JA. The ATP-regulated Potassium channel in ischemia-reperfusion injury. In: Cellular, Biochemical and Molecular Aspects of Reperfusion Injury. Ed: Das DK. *Ann NY Acad Sci* 1994;723;71-81.
34. Matsuda Y, Ogawa H, Moritani K, Matsuda M, Naito H, Matsuzaki M, Ikee Y, Kusukawa R. Effects of the presence or absence of preceding angina pectoris on left ventricular function after acute myocardial infarction. *Am Heart J* 1984;108:955-958.
35. Cortina A, Ambrose JA, Prieto-Granada J, Moris C, Simarro E, Holt J, Fuster V. Left ventricular function after myocardial infarction: clinical and angiographic correlations. *J Am Coll Cardiol* 1985;5:619-624.
36. Hirai T, Fujita M, Yamanishi K, Ohno A, Miwa K, Sasayama S. Significance of preinfarction angina for the preservation of left ventricular function in acute myocardial infarction. *Am Heart J* 1992;124:19-24.
37. Ottani F, Galvani M, Ferrini D, Morgagni GL, Pettini A, Sorbello F, Rusticali F. Ischemic preconditioning prodromal angina limits myocardial infarct size. *J Am Coll Cardiol* 1993;21:149A.
38. Kloner RA, Shook T, Przyklenk K, Davis V, Junio L, Matthews, Burstein S, Gibson CM, Poole K, Cannon CP, McCabe C, Braunwald E, for TIMI 4 Study Group. Previous angina alters in-hospital outcome in TIMI 4. *Circulation* 1993;88:I-49.
39. Muller DW, Topol EJ, Califf RM, Sigmon KN, Gorman L, George BS, Kereiakes, Lee KL, Ellis SG. Relationship between antecedent angina pectoris and short-term prognosis after thrombolytic therapy for acute myocardial infarction. Thrombolysis and Angioplasty in Myocardial Infarction (TAMI) Study Group. *Am Heart J* 1990;119:224-231.
40. Harper RW, Kennedy G, De Sanctis RW, Hutter AM Jr. The incidence and pattern of angina prior to acute myocardial infarction: a study of 577 cases. *Am Heart J* 1979;79:178-183.
41. Cupples LA, Gagnon DR, Wong ND, Ostfeld AM, Kannel WB. Pre-existing cardiovascular conditions and long term prognosis after initial myocardial infarction: the Framingham study. *Am Heart J* 1993;125:863-872.
42. Ruocco NA, Bergelson BA, Jacobs AK, Frederick MM, Faxon DP, Ryan TJ. Invasis versus conservative strategy after thrombolitic therapy for acute myocardial infarction in patients with antecedent angina. A report from thrombolysis in myocardial infarction phase II (TIMI II). *J Am Coll Cardiol* 1992;20:1445-1451.
43. Pierard LA, Dubois C, Smeets JP, Boland J, Carlier J, Kulbertus H. Prognostic significance of angina pectoris before first acute myocardial infarction. *Am J Cardiol* 1988;61:984-987.
44. Barbash GI, Harvey D, White HD, Modan M, Van der Werf F. Antecedent angina pectoris predicts a worse outcome after myocardial infarction in patients receiving thrombolytic therapy: experience gleaned from the international tissue plasminogen activator/streptokinase mortality trial. *J Am Coll Cardiol* 1992;20:36-41.
45. Behar S, Reicher-Reiss H, Abinader E, Agmon J, Friedman Y, Barzilai J, Kaplinsky E, Kauli N, Kishon Y, Palant A, Peled B, Rabinovich B, Reisin L, Schlesinger Z, Zahavi I, Zion M, Goldbourt U. The prognostic significance of angina pectoris preceding the occurance of a first acute myocardial infarction in 4166 consecutive hospitalized patients. *Am Heart J* 1992;123:1481-1486.
46. Kloner RA, Yellon D. Does ischemic preconditioning occur in patients? *J Am Coll Card* 1994;24:1133-1142.
47. Lawson CS. Does ischemic preconditioning occur in the human heart? *Cardiovasc Res* 1994:28:1461-1466.
48. MacAlpin RN, Kattus AA. Adaptation to exercise in angina pectoris. *Circulation* 1966;33:183-201.
49. Jaffe MD, Quinn NK. Warm-up phenomenon in angina pectoris. *Lancet* 1980;ii:934-936.

50. Okazaki Y, Kazuhisa K, Sato H, Kitakaze M, Hirayama A, Mishima M, Hori M, Inoue M. Attenuation of increased regional myocardial oxygen consumption during exercise as a major cause of warm-up phenomenon. *J Am Coll Cardiol* 1993;21:1597-1604.
51. Thadani U, Lewis JR, Mathew TM, West RO, Parker JO. Reproducibility of clinical and hemodynamic parameters during pacing stress testing in patients with angina pectoris. *Circulation* 1979;60:1036-1044.
52. Williams DO, Bass TA, Gewirtz H, Most AS. Adaptation to the stress of tachycardia in patients with coronary artery disease: insight into the mechanism of the warm-up phenomenon. *Circulation* 1985;71:687-692.
53. Remme WJ, Look MP, Bartels GL, Remme PA, Kruyssen HACM. Reproducibility of ischaemia-induced changes in myocardial lactate metabolism in humans. A prospective study comparing atrial pacing stress tests with varying intervals. In: Metabolic and neurohumoral aspects of acute myocardial ischemia in man. Remme WJ, PhD thesis; Erasmus University Rotterdam 1990;149-166.
54. Drobinski G, Voudris V, Lechat P, Eugene L, Canny M, Moussalem N, Fraysse JB, Grosgogeat Y. Reproducibility of myocardial ischemia induced by atrial stimulation. *Arch Mal Coeur Vaiss* 1988;81:1463-1471.
55. Bagger JP,Nielsen TT, Thomassen A. Reproducibility of coronary haemodynamics and cardiac metabolism during pacing-induced angina pectoris. *Clin Physiol* 1985;5:359-570.
56. Heibig J, Bolli R, Harris S. Initial coronary occlusion improves tolerance to subsequent prolonged balloon inflation. *Cathet Cardiovasc Diagn* 1989;16:99-102.
57. Deutsch E, Berger M, Kussmaul WG, Hirshfeld JW, Herrmann HC, Laskey MD. Adaptation to ischemia during percutaneous transluminal coronary angioplasty. Clinical, hemodynamic and metabolic features. *Circulation* 1990;82:2044-2051.
58. Cribier A, Korsatz L, Koning R, Rath P, Gamra H, Stix G, Merchant S, Chan C, Letac B. Improved myocardial ischemic response and enhanced collateral circulation with long repetitive coronary occlusion during angioplasty: a prospective study. *J Am Coll Cardiol* 1992;20:578-586.
59. De Bruyne B, Vantrimpont PJ, Goethals M, Stockbroeckx J, Paulus WJ. Can coronary angioplasty serve as a model of myocardial preconditioning? *Circulation* 1992;86:I-378.
60. Tomai F, Crea F, Gaspardone A, Versaci F, De Paulis R, Penta de Peppo A, Chiariello L, Gioffre PA. Ischemic preconditioning during coronary angioplasty is prevented by glibenclamide, a selective ATP-sensitive K^+ channel blocker. *Circulation* 1994;90;700-705.
61. Taggart PI, Sutton PMI, Oliver RM,Swanton RH. Ischemic preconditioning may activate potassium ATP channels in humans? *Circulation* 1993;88:I-568.
62. Kerensky RA, Kutcher MA, Braden GA, Applegate RJ, Solis GA, Little WC. The effect of intracoronary adenosine on preconditioning during PTCA. *Circulation* 1993;88:I-588.
63. Kober G, Buck T, Sievert H, Vallbracht C. Myocardial protection during percutaneous transluminal coronary angioplasty: effects of trimetazidine. *Eur Heart J* 1992;13:1109-1115.
64. Denys BG, Lee J, Reddy PS, Uretsky BF. Is there adaptation to ischemia during PTCA? [abstract] *Eur Heart J* 1992;13:246.
65. Oldroyd KG, Paterson JG, Rumley AG, Eteiba H, Rae AP, Shepherd J, Cobbe SM, Hutton I. Coronary venous lipid peroxide concentrations after coronary angioplasty: correlation with biochemical and electrocardiographic evidence of myocardial ischemia. *Br Heart J* 1992;68:43-47.
66. Yellon DM, Alkhulaifi AM, Pugsley WB. Preconditioning the human myocardium. *Lancet* 1993;342:276-277.
67. Walker DM, Walker JM, Pattison C, Pugsley W, Yellon DM. Preconditioning protects isolated human muscle. *Circulation* 1993;88, 4-2:I-138.
68. Ikonomidis JS, Tumiati LC, Mickle DAG, Weisel RD. Preconditioning protects human cardiac myocytes from ischemic injury. *Circulation* 1993;88, 4-2:I-570.
69. Hausmann D, Lichtlen PR, Nikutta P, Wenzlaff P, Daniel WG. Circadian variation of myocardial ischemia in patients with stable coronary artery disease. *Chronobiol Int* 1991;8:385-398.

Chapter 3

Endocardial and epicardial infarct size after preconditioning by a partial coronary occlusion without intervening reperfusion. Importance of the degree and duration of flow reduction

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Endocardial and epicardial infarct size after preconditioning by a partial coronary occlusion without intervening reperfusion. Importance of the degree and duration of flow reduction

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Background Recently, we reported that a partial coronary artery occlusion immediately preceding a sustained coronary artery occlusion limited infarct size. We now investigated whether the protection by partial coronary artery occlusions (*i*) depends on the severity and(or) duration of the flow reduction and (*ii*) varies in the different myocardial layers.

Methods and Results In 71 open-chest pigs (eight groups) left ventricular area at risk (AR) and infarct area (IA) were determined for the endocardial (IA_{endo} and AR_{endo}) and epicardial halves (IA_{epi} and AR_{epi}). In control animals [60 min total coronary artery occlusion (TCO) followed by 120 min reperfusion (Rep)] there were highly linear relations between IA and AR in the endocardium ($r=0.98$, $p<0.01$) and epicardium ($r=0.97$, $p<0.01$), which could be described by $IA_{endo} = 1.01AR_{endo} - 4.5$ and by $IA_{epi} = 0.88AR_{epi} - 3.6$, respectively. In animals that underwent a 10 min TCO + 15 min Rep prior to the 60 min TCO + 120 min Rep, IA in both myocardial layers were again highly linearly related with AR, with less steep slopes for both the endocardium (0.63) and epicardium (0.57) (both $p<0.01$). Two groups of pigs were subjected to either a 30 or 90 min 70% reduction in coronary blood flow (FR) immediately preceding the 60 min TCO + 120 min Rep, *without* intervening reperfusion. A 30 min 70% FR decreased IA to the same degree in the endo- and epicardial half. A 90 min 70% FR resulted in protection in the epicardium ($p<0.01$) but not in the endocardium, most likely because 90 min 70% FR without 60 min TCO already caused infarction which was more severe in the endo- than in the epicardium ($p<0.01$). Endocardial and epicardial IA after either a 30 or 90 min 30% FR prior to the 60 min TCO was not different from that in the control group, indicating that this mild flow reduction failed to limit irreversible damage.

Conclusions Thirty or ninety min of severe (70%) but not mild (30%) coronary flow reductions protected against myocardial infarction. The protection by a 70% FR was influenced by the duration of FR as a 30 min 70% FR similarly decreased IA in the endocardial and epicardial halves, while 90 min 70% FR preferentially limited IA in the epicardial half. These findings suggest that perfusion abnormalities immediately preceding an infarction could be an important source of infarct size variability in patients.

Keywords myocardial ischaemia, myocardial infarct size, systolic segment shortening, pig, anesthetized, coronary blood flow.

Recently, it has been reported that not only total[1-9] but also partial[10,11] coronary artery occlusions can precondition the myocardium. 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 intervening reperfusion.[11] These findings appear contradictory but could also suggest that the severity and duration of the flow reduction play a critical role in eliciting protection. Although ischaemic preconditioning has been the topic of many studies, none of these investigated whether the protection afforded was the same for the different myocardial layers across the left ventricular wall. It is well established that a partial coronary artery occlusion affects perfusion of subendocardial- more severely than of subepicardial layers.[12-17] Thus, differences in severity of ischaemia 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*) the transmural distribution of this protection. 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 partial and total coronary artery occlusion stimuli we also analyzed the subendocardial and subepicardial distribution of infarct size of pigs preconditioned with a single 10 min total coronary artery occlusion.[10]

Methods

Experimental Design

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 (figure 1)

The results of eight groups of animals are presented. A total of 79 animals entered the study, of which 20 animals underwent a single 60 min total coronary artery occlusion (TCO), followed by 120 min of reperfusion (Rep) (Group 1), while 11 animals underwent a 60 min TCO preceded by a single 10 min TCO + 15 min Rep (Group 2). Four groups underwent a partial coronary artery occlusion of either 30 min [Group 3 (n=10) and Group 6 (n=8)] or 90 min [Group 4 (n=9) and Group 7 (n=8)], before the artery was occluded completely for 60 min without intervening

reperfusion. The partial occlusions were chosen such that coronary blood flow was reduced by either 70% (Groups 3 and 4) or 30% (Groups 6 and 7) 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 ischaemic myocardium was reperfused for 120 min following the 60 min total coronary artery occlusion. Because it is conceivable that episodes of ischaemia lasting longer than 30 min already lead to necrosis before the 60 min TCO period, two groups of animals underwent 90 min of either 70% FR (Group 5, n=8) or 30% FR (Group 8, n=5) followed by 120 min Rep *without* the 60 min TCO. 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 a 30 min 70% FR have been presented in an earlier study.[11]

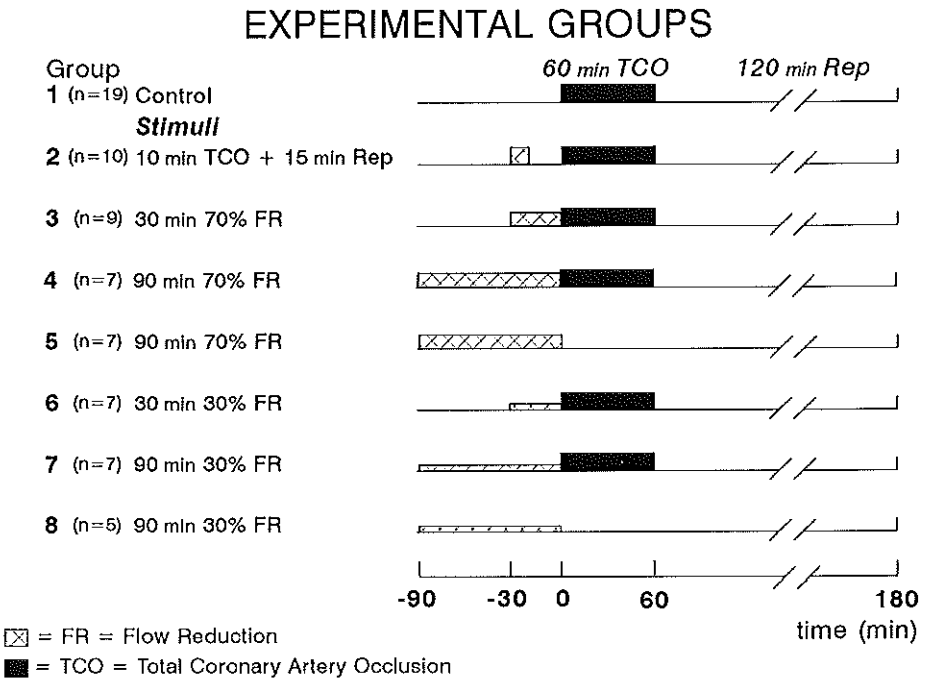

Figure 1. Experimental protocols of the 8 groups of animals in which the distribution of infarction size was determined. The 60 min total coronary artery occlusion (60 min TCO) has been indicated in black. Flow reductions have been indicated with a cross-hatch pattern. TCO = total coronary artery occlusion,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

Surgical Procedures

Domestic Yorkshire-Landrace pigs (n=79, 25-35 kg, HVC, Hedel, The Netherlands) were anaesthetised with pentobarbital and instrumented for measurement of arterial blood 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 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. In the animals of groups 4-8, the great cardiac vein was cannulated to allow selective sampling of venous blood draining the myocardium perfused by the LADCA.

Regional Myocardial Contractile Function

A 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). From the segment length tracings the segment length at the start of ejection (EL, 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 segment shortening during ejection was computed as:

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

while post-systolic segment shortening was computed as:

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

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 haemodynamic variables and regional segment length changes were recorded throughout the experimental protocols. When necessary, in the animals subjected to the partial coronary artery occlusions (Groups 3-8) 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 case of ventricular fibrillation defibrillation was started within 30 s, using DC countershocks (15-30 Watt). If defibrillation was successful within 1 min animals were allowed to complete the experimental protocol, since this procedure does not produce irreversible damage. Animals in which defibrillation could not be accomplished within 1 min were excluded from further study.

Area at Risk and Infarct Area

Validation of the methods to determine the area at risk and infarct area has been described extensively.[5,19,20] 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 (Bayer Dental, 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 onto a transparent acetate sheet under an ultraviolet light. The viable myocardium was then stained deeply purple by incubating the segments for 20 min in 0.125 g para-nitrobluetetrazolium (Sigma Chemicals Co., St. Louis, USA) per litre 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 was subsequently subdivided into a subendocardial (inner) half and a 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 basal and apical side 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

In earlier studies we observed that in pigs the relation between IA and AR is highly linear but not proportional i.e. the linear regression line has a positive intercept on the AR-axis.[11] Because this makes the IA/AR ratio dependent on the absolute AR, infarct size data are presented by plotting the IA as a function of AR. Linear regression analysis was performed to determine the relation between endocardial and epicardial IA and AR in the control group and the animals preconditioned with 10 min TCO + 15 min Rep. For the animals that underwent the two-stage coronary artery occlusion the individual data are presented. Intergroup differences in IA_{endo}, IA_{epi}, or total IA were analyzed, with AR_{endo}, AR_{epi} or total AR as respective covariates, by analysis

of covariance (ANCOVA) followed by modified Bonferroni procedure to correct for multiple comparisons.[21] Intragroup differences between IA_{endo} and IA_{epi} were, analyzed with AR_{endo} and AR_{epi} as respective covariates, using ANCOVA for repeated measures. The incidence of ventricular fibrillation was analyzed by Fisher's exact test. Haemodynamic data were analyzed with two-way (experimental group and time course) ANOVA followed by paired t-test (intragroup comparison) or unpaired t-test (intergroup comparison). A p value of less than 0.05 was considered statistically significant (two-tailed). Data are presented as mean(SEM).

Results

Mortality and Exclusions

In both the control group (60 min TCO, Group 1) and the 10 min TCO + 15 min Rep group (Group 2), one animal was excluded because defibrillation was unsuccessful during the 60 min TCO. Two animals which underwent a 30% FR [one pig for 30 min (Group 6) and the other for 90 min (Group 7)] could also not be defibrillated during the 60 min TCO period. Two animals died because defibrillation was unsuccessful during 70% FR (15 min and 20 min after onset of the flow reduction in Groups 4 and 5, respectively), while one animal in Group 5 was excluded from study because of technical failure of the balloon occluder. One animal was excluded from Group 4 because of incomplete reperfusion due to vasospasm after 60 min TCO. Infarct sizes have therefore been presented for 71 of the 79 animals that entered the study .

Ventricular Fibrillation (Table 1)

During the 60 min TCO ventricular fibrillation occurred in 8 of the 20 control animals, and in 5 of the 11 animals that had been subjected to 10 min TCO + 15 Rep (p=NS). Animals that fibrillated during 60 min TCO had larger areas at risk than animals that did not fibrillate (35±3% vs 19±2%, respectively, $p<0.01$). A 30 min 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%) but ventricular fibrillation was absent (0 of 8, $p<0.05$ vs control group) in the animals that were preconditioned with 90 min 70% FR (area at risk 38±3%). In contrast, the incidence of ventricular fibrillation during the 60 min TCO in the groups preconditioned with either a 30 or 90 min 30% FR (area at risk 35±2%) was not different from the control group. Thus, while the protective effect of a 90 min 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 ischaemic episode. Upon reperfusion ventricular fibrillation was rare, which is in agreement with studies showing that ventricular fibrillation occurs predominantly after TCO's with a duration between 10 min and 30 min.[22,23]

Table 1. **Ventricular fibrillation in all experimental groups**

Groups	*Preconditioning stimulus*	*60 min TCO*	*Reperfusion*
(1) Control	-	8(20)[a]	2(19)[a]
(2) 10 min TCO + 15 min Rep + 60 min TCO	2(11)[a, c]	5(11)[a]	3(10)[a, b]
(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	3(8)[c]	2(8)[c]	1(7)
(7) 90 min 30% FR + 60 min TCO	0(8)	6(8)	0(7)
(8) 90 min 30% FR	0(5)	-	0(5)

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, [a] one pig fibrillated during both 60 min TCO and Reperfusion, [b] one pig fibrillated during both the preconditioning stimulus and Reperfusion, [c] one pig fibrillated during both the preconditioning stimulus and 60 min TCO, *p<0.05 vs Control

Infarct Area - Area at Risk Relation in Control Pigs and Pigs Preconditioned with a Single 10 min TCO (figure 2)

In the control group there was a linear relation (r=0.98, p<0.001) between IA_{endo} and AR_{endo} which could be described by $IA_{endo} = 1.01AR_{endo} - 4.5$. In the epicardial half of the left ventricle we also observed a linear relation (r=0.97 , p<0.001) which could be described by $IA_{epi} = 0.88AR_{epi} - 3.6$. ANCOVA (with AR_{endo} and AR_{epi} as respective covariates) revealed that IA_{endo} was slightly larger than IA_{epi} (p<0.05).

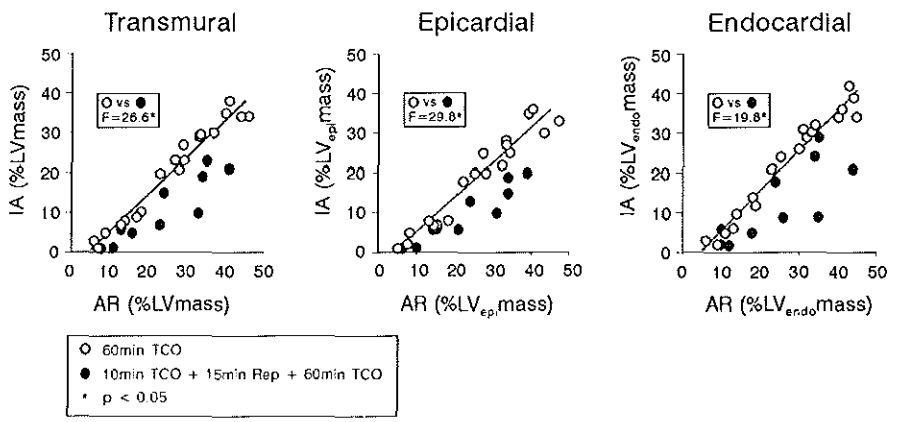

Figure 2. Individual data points determining the relation between infarcted area and area at risk in the transmural left ventricular wall (left panel), epicardium (middle panel) and endocardium (right panel), expressed as a percentage of left ventricular (LV), endocardial (LV_{endo}) or epicardial mass (LV_{epi}), respectively. Shown are the regression line and individual data points in the control group (60 min total coronary occlusion, TCO)(open circles) and the individual data points in the animals preconditioned with 10 min TCO + 15 min Rep prior to the 60 min TCO (closed circles). Infarct size limitation afforded by the 10 min TCO + 15 min Rep was of similar magnitude in the endo- and epicardium.

Preconditioning with 10 min TCO + 15 min Rep decreased infarct size in both the inner and outer half of the left ventricle. The infarct areas in the endocardium and epicardium were again linearly related with the areas at risk: $IA_{endo} = 0.63AR_{endo} - 3.19$ ($r=0.79$; $p<0.01$) and $IA_{epi} = 0.57AR_{epi} - 3.38$ ($r=0.94$; $p<0.01$). ANCOVA (with AR_{endo} or AR_{epi} as covariates) indicated that both IA_{endo} and IA_{epi} were smaller in the 10 min TCO + 15 min Rep group compared to the control group (both $p<0.01$). The degree of protection afforded by 10 min TCO + 15 min Rep was nearly identical for the inner and outer halves of the left ventricle ($F=0.68$; $p=0.42$).

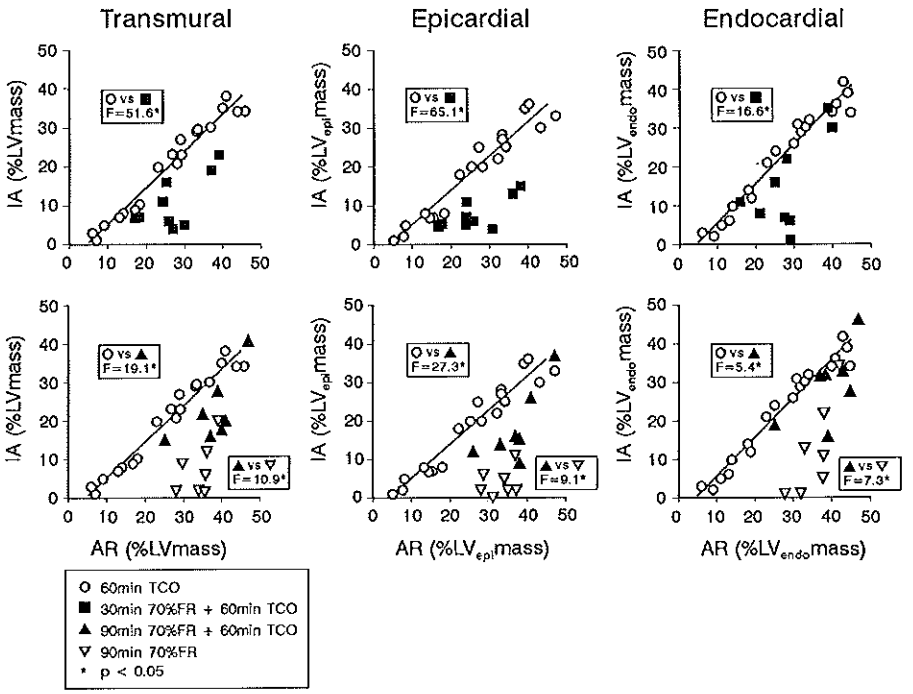

Figure 3. Individual data points determining the relation between infarcted area and area at risk in the transmural left ventricular wall (left panel), epicardium (middle panel) and endocardium (right panel), expressed as a percentage of left ventricular (LV), endocardial (LV_{endo}) or epicardial mass (LV_{epi}), respectively. Shown are 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 a 30 min 70% coronary flow reduction (FR) followed by 60 min TCO (closed squares), a 90 min 70% FR followed by 60 min TCO (closed triangles pointing upward), and a 90 min 70% FR without the 60 min TCO (open triangles pointing downward). Note that a 70% FR which itself resulted in significant infarction after 90 min, still provided protection against the irreversible damage produced by 60 min TCO.

Infarct Area - Area at Risk Relation in Pigs Undergoing Two Stage Coronary Artery Occlusion

70% Flow Reduction (figure 3)

Transmural Infarct Size. When the 60 min TCO was preceded by a 30 min 70% FR, transmural infarct size was significantly smaller compared to the control group. Extending the

duration of the 70% FR to 90 min produced similar protection against infarction compared to the 30 min 70% FR animals. A 90 min 70% FR without the 60 min TCO already resulted in some myocardial necrosis. Nonetheless the individual transmural data points of animals subjected to a 90 min 70% FR and 60 min TCO remained *below* the regression line of the control group ($p<0.01$) indicating protection against irreversible myocardial damage produced by the subsequent 60 min TCO.

Distribution of Infarct Size. The 30 min 70% FR decreased infarct size in both endo- and epicardial halves compared to the control group ($p<0.01$). The protection tended to be greater in the epicardium than in the endocardium but this failed to reach statistical significance ($p=0.16$). When the duration of the 70% FR was extended to 90 min, infarct size limitation in the outer half of the left ventricle was similar to that observed with the 30 min 70% FR. Infarct size was also reduced in the endocardium ($p<0.05$ vs control group), but the degree of protection produced by the 90 min 70% FR was greater in the epicardium than in the endocardium ($p<0.01$). The 90 min 70% FR alone resulted in infarction in both endo- and epicardium, with the greater infarct size in the endocardium ($p<0.01$ endocardium vs epicardium). The addition of a 60 min TCO increased infarct size slightly further in both endocardium ($p<0.05$) and epicardium ($p<0.01$), so that the amount of *additional* necrosis produced by the 60 min TCO was not different for the endocardium and the epicardium ($p=0.20$).

30% Flow Reduction (figure 4)

Transmural Infarct Size. Transmural infarct size in animals subjected to a 30 min 30% FR prior to the 60 min TCO was not different from that of the control group (figure 4). Extending the period of flow reduction to 90 min did also not alter infarct size produced by a 60 min TCO, indicating that exposure to such mild flow reductions fails to limit irreversible ischaemia damage during a subsequent 60 min TCO. A 90 min 30% FR without the 60 min TCO did not produce myocardial necrosis (figure 4).

Distribution of Infarct Size. Endocardial and epicardial infarct sizes in animals subjected to the 30 or 90 min 30% FR prior to the 60 min TCO were not different from those of the control group (figure 4). The 90 min 30% FR alone did not produce myocardial necrosis in either the outer or inner half of the left ventricle.

***Systemic Haemodynamic Variables and Segment Shortening** (Table II)*

Baseline values (n=71) of heart rate (110±2 beats min^{-1}), mean arterial blood pressure (89±1 mmHg), cardiac output (2.9±0.1 L min^{-1}) or the product of heart rate and systolic arterial pressure (12200±270 beats mmHg min^{-1}) were not statistically different between the eight experimental groups. In all groups the 60 min TCO tended to increase heart rate, but this reached levels of statistical significance only in groups 3,4 and 7. Although in most groups cardiac output was significantly lower at the end of the 60 min TCO compared to baseline levels, mean aortic

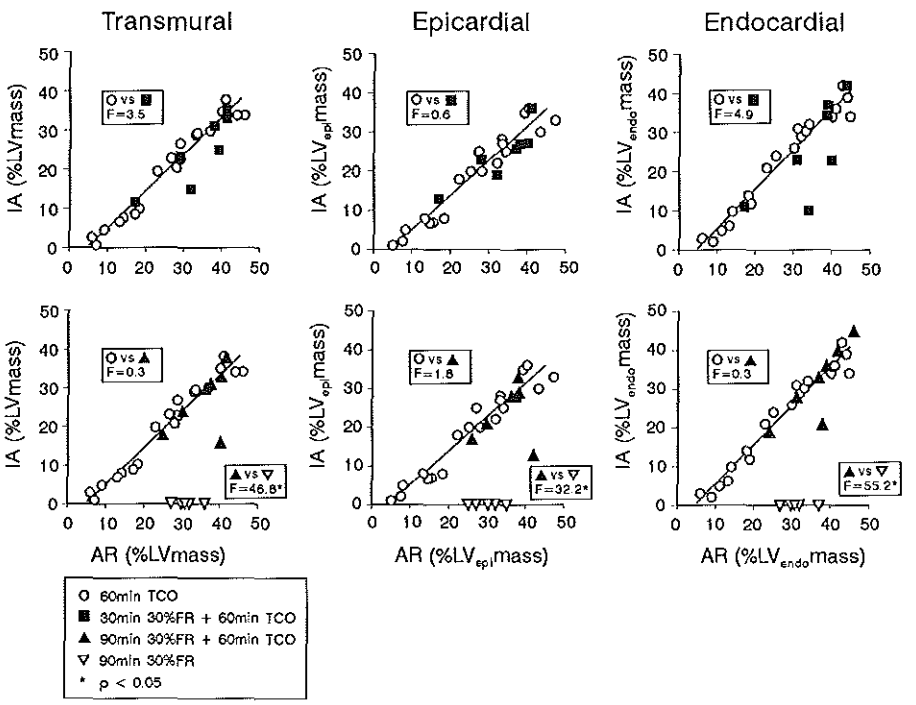

Figure 4. Individual data points determining the relation between infarcted area and area at risk in the transmural left ventricular wall (left panel), epicardium (middle panel) and endocardium (right panel), expressed as a percentage of left ventricular (LV), endocardial (LV_{endo}) or epicardial mass (LV_{epi}), respectively. Shown are 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 a 30 min 30% coronary flow reduction (FR) followed by 60 min TCO (closed squares), a 90 min 30% FR followed by 60 min TCO (closed triangles pointing upward), and a 90 min 30% FR without the 60 min TCO (open triangles pointing downward). Note that a 30% FR which itself did not result in significant infarction after 90 min, failed to protect against the irreversible damage produced by 60 min TCO.

pressure was generally maintained. The preconditioning stimuli had only minor effects on heart rate or mean aortic blood pressure. In the 10 min TCO + 15 min Rep group, the preconditioning stimulus produced significant decreases in cardiac output (14±3% from baseline, $p<0.01$) and a reduction in segment shortening in the LADCA perfused area (to 61±6% of baseline, ($p<0.01$)), with no effect on heart rate or central arterial blood pressure. Similarly, the 70%FR produced a 16±2% decrease in cardiac output and decreased segment shortening to 13±5% of baseline. In contrast, a 30% FR produced a significant decrease in segment shortening (to 63±8% of baseline), with negligible effect on global cardiac pump function.

Table II: **Systemic haemodynamics, global and regional myocardial function in groups 1-8.**

	Baseline	End-stimulus	60min TCO	120min Rep
Group 1 (n=19)				
HR	114±3	-	120±5	119±5
MAP	89±1	-	89±2	88±2
CO	3.0±0.2	-	2.5±0.1*	2.4±0.1*
SS	15.5±1.1	-	0.9±0.5*	1.8±0.5*
Group 2 (n=10)				
HR	107±6	108±4	113±4	117±7
MAP	90±2	90±2	86±3	83±3
CO	3.2±0.2	2.8±0.1*	2.3±0.1*	2.2±0.1*
SS	20.7±1.9	11.7±1.4*	1.7±1.2*	2.0±1.1*
Group 3 (n=9)				
HR	91±7	92±6	103±6*	134±7*
MAP	90±2	88±3	90±1	83±3
CO	2.7±0.2	2.4±0.2*	2.3±0.1*	1.8±0.1*
SS	18.4±1.4	2.7±1.3*	-0.4±0.8*	-1.2±0.7*
Group 4 (n=7)				
HR	111±11	119±13	126±13*	144±10*
MAP	90±2	86±3	81±4*	78±4*
CO	2.8±0.2	2.2±0.2*	2.1±0.2*	1.7±0.1*
SS	15.9±1.5	0.9±0.9*	-0.3±0.2*	0.0±0.6*
Group 5 (n=7)				
HR	100±4	102±4		126±11*
MAP	85±2	78±4		71±6
CO	2.7±0.2	2.2±0.2*		1.9±0.1*
SS	16.4±3.0	2.4±1.9*		1.4±1.2*
Group 6 (n=7)				
HR	127±11	120±12	131±12	155±11*
MAP	91±2	84±3*	78±4*	73±3*
CO	3.2±0.3	2.8±0.3*	2.4±0.2*	2.3±0.3+
SS	17.6±1.5	10.1±3.1*	1.7±1.1*	1.4±0.9*
Group 7 (n=7)				
HR	113±8	112±8	125±9*	133±9*
MAP	84±3	83±3	79±3	77±5
CO	2.4±0.2	2.3±0.1	1.9±0.9	1.7±0.2*
SS	17.7±1.1	14.5±1.0+	-0.1±0.3*	0.4±0.5*
Group 8 (n=5)				
HR	115±7	120±6		136±9*
MAP	89±4	85±6		90±5
CO	3.1±0.2	2.8±0.3		2.8±0.3
SS	13.1±2.7	7.1±3.3*		8.0±2.0

TCO=Total coronary artery occlusion, Rep=Reperfusion, HR=heart rate (bpm), MAP=mean aortic pressure (mmHg), CO=Cardiac output (L/min). SS=segment shortening during left ventricular ejection (%). Data are mean ±SEM, *p<0.05, +p=0.07 vs Baseline. See figure 1 for description of the groups.

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 product of heart rate and systolic arterial pressure and infarcted area ($p>0.10$). Similar results were obtained when heart rate or systolic arterial pressure were entered as separate covariates into the ANCOVA.

There was no correlation between the loss of systolic wall thickening produced by the preconditioning stimuli and the infarct size. Similarly, we did not observe a correlation between ejection segment shortening at the end of 120 min Rep and IA/AR ratio, indicating that ischaemic preconditioning did not lead to improved functional recovery during the first 120 min of reperfusion. In contrast, a modest but significant ($r=0.49$, $p<0.01$) inverse correlation was observed between the magnitude of post-systolic shortening (a marker of myocardial tissue viability)[24] of the anterior wall at the end of reperfusion and transmural IA/AR.

Responses of Regional Myocardial Perfusion, Metabolism and Contractile Function to Partial Coronary Artery Occlusions. *(figure 5)*

The 30% and 70% reductions in coronary blood flow resulted in decreases in myocardial oxygen consumption as the increase in oxygen extraction, which was significantly greater in the 70% FR than in the 30% FR animals, was clearly insufficient to compensate for the decreases in coronary flow. The decreases in flow were accompanied by a flow reduction dependent increase in the arterio-coronary venous pH difference, which is in this model highly linearly related to lactate release due to increased anaerobic metabolism.[25] Another indication of ischaemia was the flow reduction dependent loss of segment shortening and appearance of post-systolic shortening. All variables remained constant between 15 min and 90 min of 30% FR and 70% FR, with the exception of the arterio coronary venous pH difference which recovered towards baseline levels. This suggests that despite ongoing hypoperfusion, anaerobic metabolism could have been due to the loss of myocytes allowing the remaining myocytes to shift from anaerobic to aerobic metabolism. However, necrosis is unlikely to occur within 30 min, at which time significant recovery of the pH difference was noted. Furthermore, in the 30% FR animals no necrosis occurred, thus excluding myocyte drop-out as a mechanism for the metabolic adaptations in that group.

Discussion

This study has presented 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 30 or 90 min 70% FR prior to a 60 min TCO without the need for intervening reperfusion. The transmural distribution of this protection depended critically on the

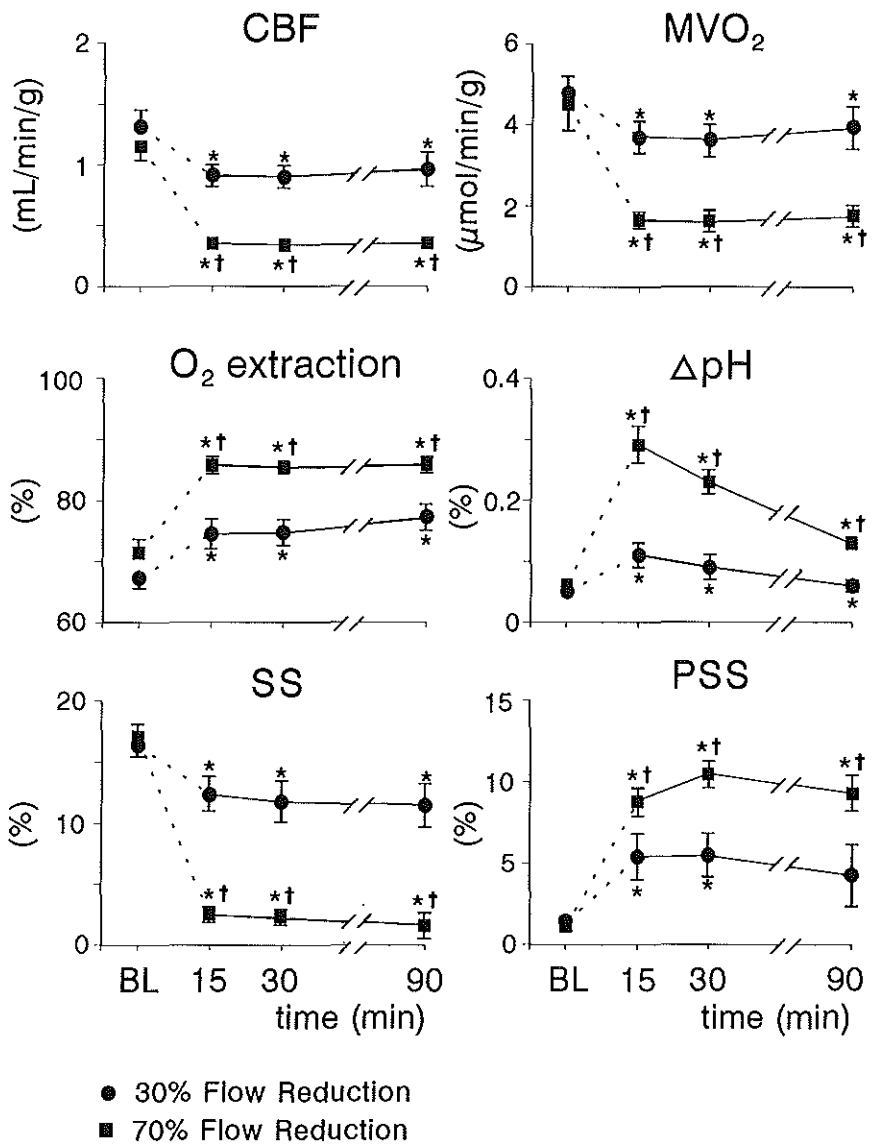

Figure 5. Alterations in perfusion, metabolism and contractile performance in the distribution area of the left anterior descending coronary artery produced by 30% (circles) and 70% (squares) coronary blood flow reductions (FR). Data have been pooled for all animals undergoing either 30% FR (n=19) or 70% FR (n=14). %Δ CBF = coronary blood flow, MVO_2 = myocardial oxygen consumption, O_2 = fractional myocardial oxygen extraction computed as the arterio-coronary venous oxygen content difference divided by the arterial oxygen content, ΔpH=difference in pH of the arterial and local coronary venous blood, SS = fractional segment shortening during left ventricular ejection, PSS = post-systolic segment shortening. *$p<0.05$ vs baseline, †$p<0.05$ 70% FR vs 30% FR.

duration of flow reduction. Thus, whereas a 30 min 70% FR produced similar decreases in infarct size in the inner- and outer half of the left ventricle, a 90 min 70% FR resulted in preferential epicardial infarct size limitation. 3) Protection against myocardial infarction or ventricular fibrillation was not afforded by either a 30 or 90 min 30% flow reduction. 4) The incidence of ventricular fibrillation during the 60 min total coronary artery occlusion was not altered by the 30% flow reduction but was significantly reduced when a 90 min 70% FR preceded the sustained ischaemia period. The implications of these findings will be discussed in detail.

Transmural Distribution of Infarct Size in Control Pigs and in Pigs subjected to a Brief Total Coronary Artery Occlusion.

In the present study we found a linear relation between IA and AR (both expressed as percent of left ventricular mass) with a positive intercept on the AR-axis, which was similar for the endocardial and epicardial half of the left ventricle in pigs subjected to a 60 min TCO. In anaesthetized and awake dogs, IA and AR are also linearly related with a positive intercept on the AR-axis,[26-28] but in contrast to swine, the AR-intercept in dogs is considerably higher in the epicardium than in the endocardium.[28] 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. But also in pigs, in which total coronary artery occlusions result in transmurally homogeneous blood flow reductions, infarction progresses from inner to outer layer,[29,30] 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 area produced by 60 min total coronary artery occlusion was larger although only slightly (but significant by ANCOVA) in the endocardial than in the epicardial half of the left ventricle.

In pigs preconditioned by a 10 min TCO + 15 min Rep the relation between infarct area and area at risk was linear, with a similar AR-intercept but with a lower slope than for the animals in the control group. The decrease in slope was similar in the endo- and epicardium indicating that in pigs 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 Degree and Duration of Flow Reduction for Infarct Size Limitation.

In the present study the myocardial protection was not different after 30 min or 90 min exposure to the coronary flow reductions, but critically depended on the degree of flow reduction. Thus, a 70% reduction of coronary flow preceding the 60 min TCO reduced infarct size, whereas a 30% reduction in coronary artery flow did not protect the myocardium. 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 intervening reperfusion was allowed. The present study suggests that a partial coronary

artery occlusion, immediately preceding a sustained coronary artery occlusion, can afford protection provided that the degree of flow reduction is sufficiently severe.

The protection produced by the 30 min 70% FR did not differ between endo- and epicardium. However, when the duration of 70% FR was extended from 30 min to 90 min, infarct size after the 60 min TCO was significantly larger in the endocardial than in the epicardial half of the left ventricle. A 90 min 70% FR *without* the 60 min TCO produced larger infarct size in the endocardium than in the epicardium, which likely results from the more severe flow reductions in the inner- than in the outer layers distal to a coronary artery stenosis. The additional necrosis produced by the 60 min TCO was not different for the endocardium and epicardium. Thus, the observation that a 90 min 70% FR 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. However, of greater importance is the observation that despite the production of significant endocardial necrosis by the 90 min 70% FR itself, endocardial infarct size after the subsequent 60 min TCO was still less than endocardial infarct size in the control group.

While ischaemia was not severe enough to produce myocardial necrosis after 90 min, a 30% reduction coronary blood flow *did* produce ischaemia as indicated by a 39±6% decrease in systolic segment shortening. It is well established that metabolism of an ischaemic 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 ischaemia-induced metabolic changes as the hypoperfusion is prolonged.[31-35] 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.[17,29,33] Thus, lack of necrosis after 90 min of 30% FR despite continuing myocardial hypoperfusion and hypofunction could be due to metabolic adaptations. This also supported by the observations regarding the 30% FR in the present study. Thus, the arterio-coronary venous pH difference increased during the early (15 min) phase of 30% FR, but partially recovered during the remainder of the 90 min period. 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.

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 both the endocardium and epicardium. In the present study we did not measure the transmural distribution of myocardial blood flow but previous studies, including from our own 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, 35] Thus, the observation that a 90 min 30% FR did not produce endocardial necrosis but a 90 min 70% FR produced epicardial necrosis is likely the result of the more severe epicardial flow deficit during 70% coronary artery flow reduction than the endocardial flow deficit associated with 30% flow reduction.

Ischaemic preconditioning and ventricular fibrillation during subsequent sustained ischaemia

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 ischaemia.[36, 37] 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.[37] In contrast, we failed to observe a decrease in the occurrence of ventricular fibrillation during the 60 min TCO when preceded by a 10 min TCO + 15 min Rep. An explanation for the different results in our study and that of Parratt and Vegh[35] is not readily found but could be due to a number of factors such as different durations of the preconditioning stimulus and the intervening reperfusion periods and the areas at risk. In the present study a partial coronary artery occlusion immediately preceding the sustained period of ischaemia (analogous to Harris' two-stage coronary artery occlusion)[38] 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 that partial flow reductions can decrease the incidence of ventricular fibrillation during a sustained coronary artery occlusion.[22]

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 [22, 23] these arrhythmias were rare in the present study. Consequently, the efficacy of preconditioning on fibrillation during reperfusion could not be assessed.

Methodological Considerations

In the present study experiments were performed in pigs. Pigs lack a significant innate coronary collateral circulation, which results in less variability in infarct size due to collateral blood flow during the sustained coronary artery occlusion, compared to species such as the dog.[3, 39] In agreement with this we observed a very tight relationship between AR and IA. Another advantage of the lack of collaterals is that the reductions as measured with a flow probe on a proximal coronary artery reflects the flow in the myocardium, thereby allowing more stringent control of the level of flow reduction.

In the present study infarct size produced by a 60 min coronary artery occlusion was determined in myocardium reperfused for two hours using para-nitrobluetetrazolium sodium. In viable myocytes para-nitrobluetetrazolium is reduced to form a dark purple diformazan precipitate by intracellular diaphorases that use NADH or NADPH as electron donors.[20] False positive staining is minimized by allowing myocardial reperfusion to facilitate washout of NADH and NADPH from necrotic myocardium. Schaper *et al*[39] reported that only 30 min of reperfusion is sufficient for accurate detection of infarct size. Horneffer *et al*[40] also evaluated para-nitrobluetetrazolium staining for the determination of myocardial infarct size in pigs and reported that infarct size produced by 15, 30 or 90 min coronary artery occlusion was not different when either 2 or 48 hours of reperfusion were allowed. Fujiwara *et al*[41] showed that determination of infarct size produced by a 60 min total coronary artery occlusion in porcine myocardium reperfused for 1 hour was identical to infarct size determined after 3 and 7 hours of reperfusion. In the latter study, histochemical analysis of infarct size correlated well with histological measurements. The available evidence clearly indicates that two hours of reperfusion following a 60 min coronary artery occlusion allows accurate histochemical determination of infarct size with para-nitrobluetetrazolium staining in pigs.

Clinical Relevance

Until recently, myocardial preconditioning 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 preconditioning 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. In this respect it is also of interest that Schulz *et al*[42] have shown that a complete coronary occlusion without intervening reperfusion preconditioned myocardium against infarction produced by a partial occlusion. 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 degree of flow reduction is sufficiently severe. Our data also suggest that mild flow reductions, even when sustained for 90 min, will not produce sufficient stimulation to precondition the myocardium. Our findings may have implications for interpretation of clinical 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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References

1. Murry CE, Richard VJ, Jennings RB, Reimer KA. Preconditioning with ischemia: A delay of lethal cell injury in ischemic myocardium. *Circulation* 1986;74:1124-1136.
2. Gross GJ, Auchampach JA, Blockade of ATP-sensitive potassium channels prevents myocardial preconditioning in dogs. *Circ Res* 1992;70:223-233.
3. Schott RJ, Rohmann S, Braun ER, Schaper W. Ischemic preconditioning reduces infarct size in swine myocardium. *Circ Res* 1990;66:1133-1142.
4. Sack S, Mohri M, Arras M, Schwarz ER, Schaper W. Ischaemic preconditioning - time course of renewal in the pig. *Cardiovasc Res* 1993;27:551-555.
5. Rohmann S, Weygandt H, Schelling P, Soei LK, Becker KH, Verdouw PD, Lues I, Haüsler G. Effect of Bimakalim (EMD 52692), an opener of ATP-sensitive potassium channels, on infarct size, coronary blood flow, regional wall function and oxygen consumption in swine. *Cardiovasc Res* 1994;28:858-863.
6. Iwamoto T, Miura T, Adachi T, Noto T, Ogawa T, Tsuchida A, Iimmura O. Myocardial infarct size-limiting effect of ischemic preconditioning was not attenuated by oxygen free-radical scavengers in the rabbit. *Circulation* 1991;83:1015-1022.
7. Cohen VC, Liu GS, Downey JM. Preconditioning causes improved wall motion as well as smaller infarcts after transient coronary occlusion in rabbits. *Circulation* 1991;84:341-349.
8. Yellon DM, Alkhulaifi AM, Browne EE, Pugsley WB. Ischaemic preconditioning limits infarct size in the rat heart. *Cardiovasc Res* 1992;26:983-987.
9. Liu Y, Downey JM. Ischemic preconditioning protects against infarction in rat heart. *Am J Physiol* (*Heart Circ Physiol* 32) 1992;263:H1107-H1112.
10. Ovize M, Przyklenk K, Kloner RA. Partial coronary stenosis is sufficient and complete reperfusion is mandatory for preconditioning the canine heart. *Circ Res* 1992;71:1165-1173.
11. Koning MMG, Simonis LAJ, De Zeeuw S, Nieukoop S, Post S, Verdouw PD. Ischaemic preconditioning by partial occlusion without intermittent reperfusion. *Cardiovasc Res* 1994;28:1146-1151.
12. Gewirtz H, Gross SL, Williams DO, Most AS. Contrasting effects of nifedipine and adenosine on regional myocardial flow disribution and metabolism distal to a severe coronary arterial stenosis: observations in sedated, closed-chest, domestic swine. *Circulation* 1984;69(5):1048-1057.
13. Most AS, Williams DO, Gewirtz H. Elevated coronary vascular resistance in the presence of reduced resting blood flow distal to a severe coronary artey stenosis. *Cardiovasc Res* 1985;19:599-605.
14. Pantely AJ, Bristow JD, Swenson LJ, Ladley HD, Johnson B, Anselone CG. Incomplete coronary vasodilatation during myocardial ischemia in swine. *Am J Physiol* (*Heart Circ Physiol* 18) 1985;249:H638-H647.
15. Sassen LMA, Soei LK, Heere TJM, van Woerkens LJ, Saxena PR, Verdouw PD. Nicorandil and cardiovascular performance in anethetized pigs with a concentric coronary artery stenosis. *Arch Pharmacol* 1989;340:733-739.
16. Pantely GA, Malone SA, Rhen WS, Anselone CG, Arai A, Bristow J, Bristow JD. Regeneration of myocardial phosphocreatine in pigs despite continued moderate ischemia. *Circ Res* 1990;67:1481-1493.
17. Arai AE, Pantely GA, Anselone CG, Bristow J, Bristow JD. Active downregulation of myocardial energy requirements during prolonged moderate ischemia in swine. *Circ Res* 1991;69:1458-1469.
18. Soei LK, Sassen LMA, Fan DS, van Veen T, Krams R, Verdouw PD. Myofibrillar Ca^{2+} sensitization predominantly enhances function and mechanical efficiency of stunned myocardium. *Circulation* 1994;90:959-969.
19. Schaper W, Hofmann M, Müller KD, Genth K, Carl M. Experimental occlusion of two small coronary arteries in the same heart. A new validation method for infarct size manipulation. *Basic Res Cardiol* 1979;74:224-229.
20. Klein HH, Puschmann S, Schaper J, Schaper W. The mechanism of the tetrazolium reaction in identifying experimental myocardial infarction. *Virchows Arch (Pathol Anat)* 1981;393:287-297.
21. Hochberg Y. A sharper Bonferroni procedure for mulitple tests of significance. *Biometrica* 1988;74:800-802.
22. Verdouw PD, Hartog JM. Provocation and suppression of ventricular arrhythmias in domestic swine. In: Stanton HC, Mersmann HJ, eds. *Swine in Cardiovascular Research* (volume II). Boca Raton, Florida: CRC Press Inc; 1986:121-156.
23. Balke CW, Kaplinsky E, Michelson EL, Naito M, Dreifus LS. Reperfusion ventricular tachyarrhythmias: Correlation with antecedent coronary artery occlusion tachyarrhythmias and duration of myocardial ischemia. *Am Heart J* 1981;101:449-456.
24. Takayama M, Norris RM, Brown MA, Armiger LC, Rivers JT, White HD. Postsystolic shortening of acutely ischemic canine myocardium predicts early and late recovery of function after coronary artery reperfusion. *Circulation* 1988;78:994-1007.
25. Schamhardt HC, Verdouw PD, Saxena PR. Effects of oxyfedrine on global haemodynamics and myocardial

lactate balance in acute ischaemia of the porcine heart preparation. *Arzn. Forsch. Drug Research* 1981;3:1091-1095.
26. Jugdutt BI, Grover CB, Hutchins GM, Bulkley BH, Becker LC. Myocardial infarction in the conscious dog: three-dimensional mapping of infarct, collateral flow and region at risk. *Circulation* 1979;60(5):1141-1150.
27. Miura T, Downey JM. Collateral perfusion of ischemic myocardium is inversely related to the size of the ischemic zone. *Basic Res Cardiol* 1988;83:128-136.
28. Koyanagi S, Eastham CL, Harrison DG, Marcus ML. Transmural variation in the relationship between myocardial infarct size and risk area. *Am J Physiol* 1982;242:H867-874.
29. Fujiwara H, Ashraf M, Sato S, Millard RW. Transmural cellular damage and blood flow distribution in early ischemia in pig hearts. *Circ Res.* 1982;51:683-693.
30. Klein HH, Schubothe M, Kreuzer H. Temporal and spatial development of infarcts in porcine hearts. *Basic Res Cardiol* 1984;79:440-447.
31. Verdouw PD, Remme WJ, Hugenholtz PG. Cardiovascular and antiarrhythmic effects of Aprindine (AC 1802) during partial occlusion of a coronary artery in the pig. *Cardiovasc Res* 1977;11:317-323.
32. De Jong JW, Verdouw PD, Remme WJ. Myocardial nucleoside and carbohydrate metabolism and hemodynamics during partial occlusion and reperfusion of pig coronary artery. *J Mol Cell Cardiol* 1977;9:297-312.
33. Bristow JD, Arai AE, Anselone CG, Pantely GA. Response to myocardial ischemia as a regulated process. *Circulation* 1991;84:2580-2587.
34. Schulz R, Guth BD, Pieper K, Martin C, Heusch G. Recruitment of an inotropic reserve in moderately ischemic myocardium at the expense of metabolic recovery: A model of short-term hibernation. *Circ Res* 1992;70:1282-1295.
35. Fedele FA, Gewirtz H, Capone RJ, Sharaf B, Most AS. Metabolic response to prolonged reduction of myocardial blood flow distal to a severe coronary artery stenosis. *Circulation* 1988;78:729-735.
36. Li Y, Whittaker P, Kloner RA. Cardioprotective effects of ischemic preconditioning are not mediated by prostanoids. *Cardiovasc Res* 1992;123:346-353.
37. Parratt J, Vegh A. Pronounced antiarrhythmic effects of ischemic preconditioning. *Cardioscience* 1994;5:9-18.
38. Harris AS. Delayed development of ventricular ectopic rhythms following experimental coronary occlusion. *Circulation* 1950;1:1318-1328.
39. Schaper W, Binz K, Sass S, Winkler B. Influence of collateral blood flow and of variations in MVO_2 on tissue-ATP content in ischemic and infarcted myocardium. *J Mol Cell Cardiol.* 1987;19:19-37.
40. Horneffer PJ, Healy B, Gott VL, Gardner TJ. The rapid evolution of a myocardial infarction in an end-artery coronary preparation. *Circulation.* 1987;76:V39-V42.
41. Fujiwara H, Onodera T, Tanaka M, Miyazaki S, Wu DJ, Matsuda M, Kawamura A, Ishida M, Takemura G, Fujiwara Y, Fujiwara T, Kawai C. Acceleration of cell necrosis following reperfusion after ischemia in the pig heart without collateral circulation. *Am J Cardiol.* 1989;63:14-18.
42. Schulz R, Post Heiner, Sakka S, Wallbridge DR, Heusch G. Intraischemic preconditioning: increased tolerance to sustained low-flow ischemia by a brief episode of no-flow ischemia without intermittent reperfusion.*Circulation* 1995;76:942-950.

Chapter 4

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

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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.

Keywords infarct size, myocardial blood flow, swine, tachycardia, transmural distribution of myocardial infarction.

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 (Figure 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 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.

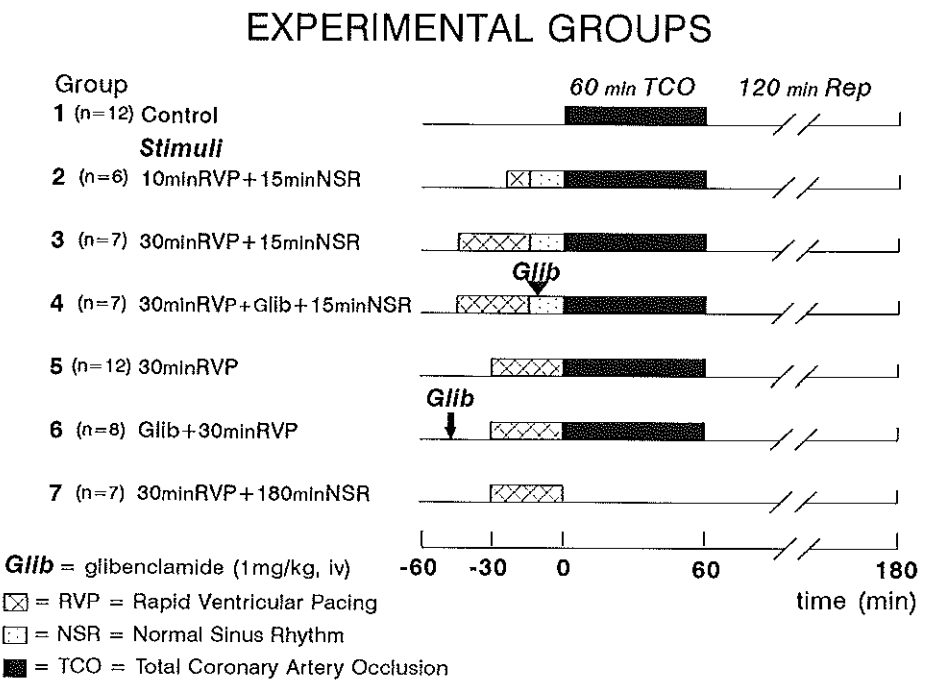

Figure 1. Experimental groups in which the effect of rapid ventricular pacing on infarct size after a 60 min total coronary artery occlusion was determined. Filled bar = 60 min total coronary artery occlusion (60min TCO), hatched bar = rapid ventricular pacing (RVP). NSR = normal sinus rhythm, Rep =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

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 (Figure 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 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.

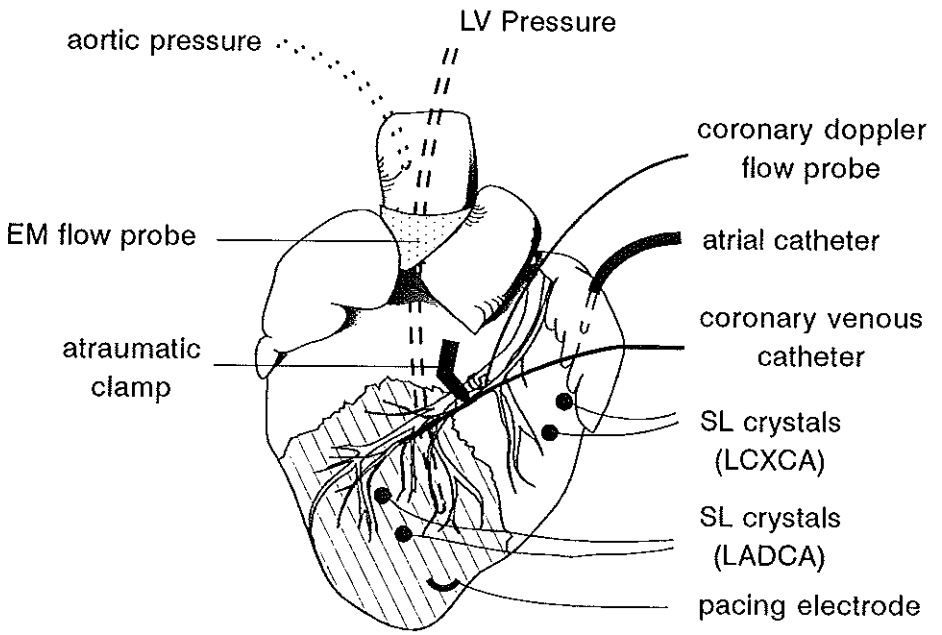

Figure 2. Schematic presentation of the experimental model. The pacing electrode was inserted into the superficial layers of the anteriolateral wall of the left ventricle. Pairs of ultrasonic crystals were inserted into the mesocardial layers of the left anterior descending coronary artery (LADCA) area 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.

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)(Figure 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 (Figure 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 $\cdot 10^6$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 perfomance 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 (Figure 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 by injection of 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) 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 in all pigs**

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 is indicated the total number of animals per group at the onset of each intervention. 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.

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; Figure 3). Separation of the left ventricular wall into two layers of equal

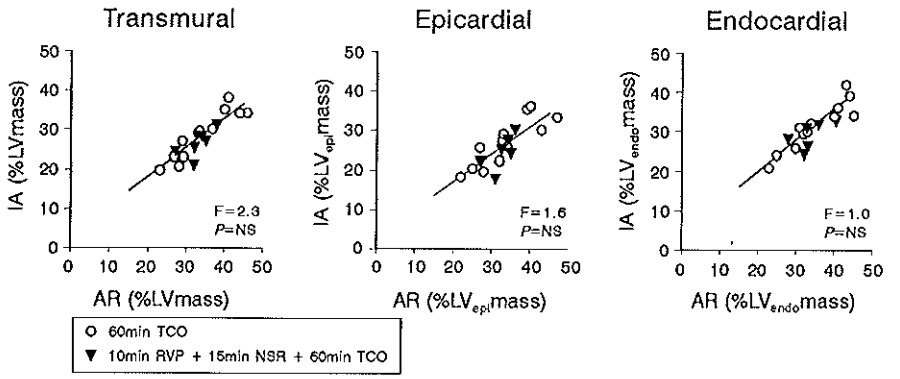

Figure 3. Effects of rapid ventricular pacing on the transmural, endocardial and epicardial relations between infarct area (IA) and area at risk (AR). Shown are the relations in the control group (60 min of total coronary artery occlusion, 60min TCO) and animals subjected to either 10 or 30 min of rapid 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.

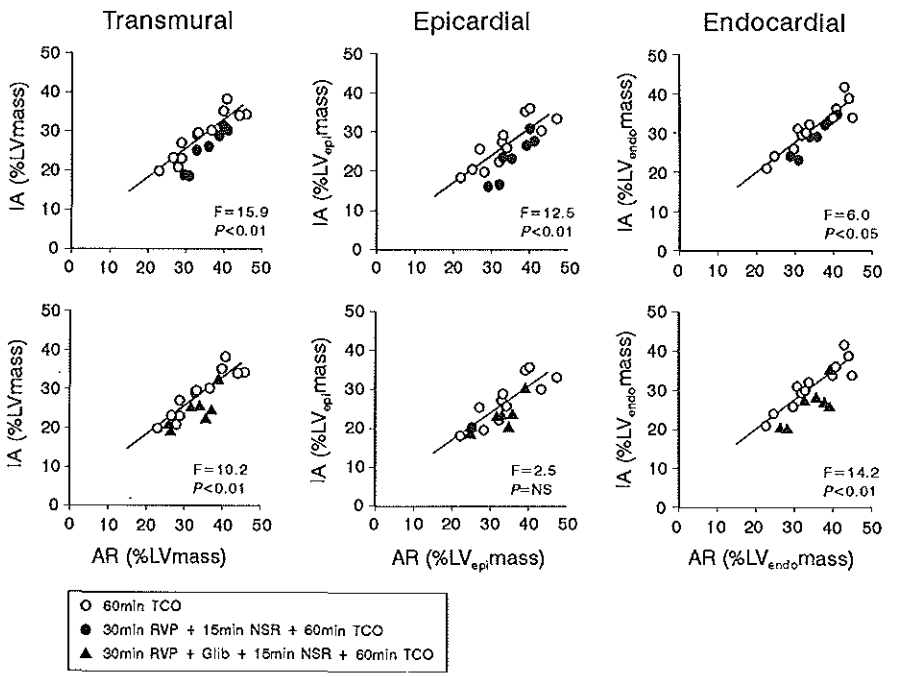

Figure 3 Effects of rapid ventricular pacing on the transmural, endocardial and epicardial relations between infarct area (IA) and area at risk (AR). Shown are the relations in the control group (60 min of total coronary artery occlusion, 60min TCO) and animals subjected to either 10 or 30 min of rapid 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.

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 (Figure 3). When the 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 (Figure 4). In this group of animals the transmural IA/AR ratio was also significantly lower than that in the control group (72±2% versus 85±2%, *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 (Figure 5). The IA/AR of this group of animals was 62±4% (*P*<0.01 versus control group). ANCOVA showed that 30min RVP immediately followed

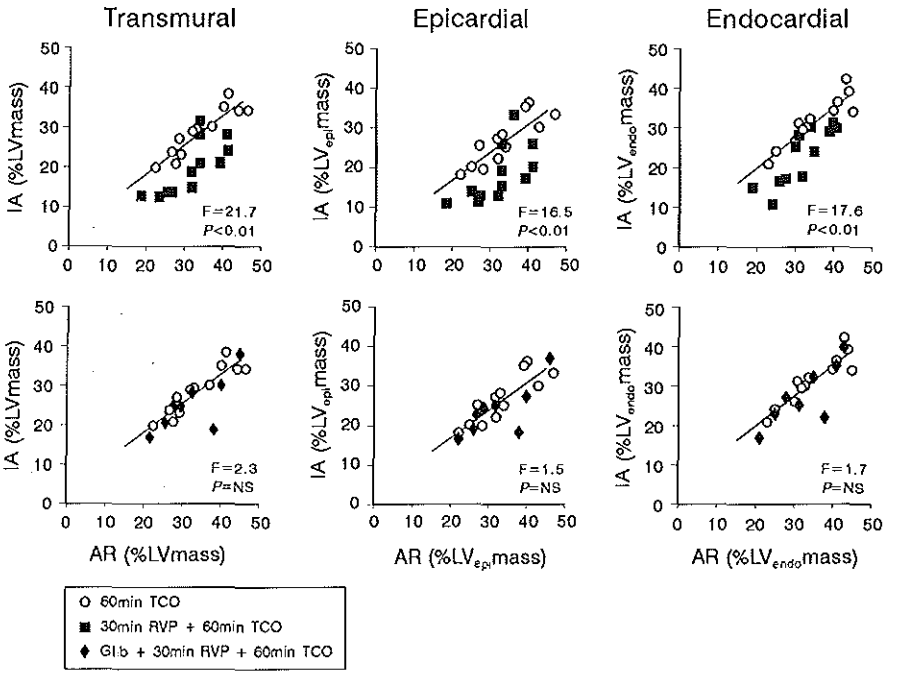

Figure 4. Effects of rapid ventricular pacing on the transmural, endocardial and epicardial relations between infarct area (IA) and area at risk (AR). In the top panels are shown the control animals (60 min of total coronary artery occlusion, 60min TCO) and the animals subjected to 30 min of rapid ventricular pacing (RVP) immediatley 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.

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 (Figure 5). Pretreatment with glibenclamide abolished the protective effect of 30min RVP in both the endocardial and epicardial halves (Figure 5). 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

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

	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.

(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 ($P<0.05$) and mean aortic pressure from 84±3 mmHg to 95±4 mmHg ($P=0.055$). The 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 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 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

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.**

	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 ($mmHg \cdot ml^{-1} \cdot min \cdot g$); cv$PO_2$ = 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.

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 min 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 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 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 we and others have shown that 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 aan 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 min 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 (chapter 3). 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 pulses per min 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 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 *et al*[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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References

1. Lawson CS, Downey JM. Preconditioning: state of art myocardial protection. *Cardiovasc Res* 1993;27:542-550.
2. Ovize M, Przyklenk K, Kloner RA. Partial coronary stenosis is sufficient and complete reperfusion is mandatory for preconditioning the canine heart. *Circ Res* 1992;71:1165-1173.
3. Koning MMG, Simonis LAJ, De Zeeuw S, Nieukoop S, Post S, Verdouw PD. Ischaemic preconditioning by partial occlusion without intermittent reperfusion. *Cardiovasc Res* 1994;28:1146-1151.
4. Przyklenk K, Bauer B, Ovize M, Kloner RA, Whittaker P. Regional ischemic "preconditioning" projects remote virgin myocardium from subsequent sustained coronary occlusion. *Circulation* 1993;87:893-899.
5. McClanahan TB, Nao BS, Wolke LJ, Martin BJ, Metz TE, Gallagher KP. Brief renal occlusion and reperfusion reduces myocardial size in rabbits. *The FASEB Journal* 1993;7:A118,682 (Abstract).
6. Gho BCG, Schoemaker RG, Van der Lee C, Sharma HS, Verdouw PD. Cardioprotection by transient renal ischemia, an in vivo study in rats. *Circulation* 1994;90:I-476 (Abstract).
7. Ovize M, Kloner RA, Przyklenk K. Stretch preconditions canine myocardium. *Am J Physiol (Heart Circ Physiol. 35)* 1994;266:H137-H146.
8. Vegh A, Szekeres L, Parrat JR. Transient ischaemia induced by rapid cardiac pacing results in myocardial preconditioning. *Cardiovasc Res* 1991;25:1051-1053.
9. Marber MS, Walker DM, Eveson DJ, Walker JM, Yellon DM. A single five minute period of rapid atrial pacing fails to limit infarct size in the in situ rabbit heart. *Cardiovasc Res* 1993; 27:597-601.
10. Szilvassy Z, Ferdinandy P, Bor P, Jakab I, Lonovics J, Koltai M. Ventricular overdrive pacing-induced anti-ischemic effect: a conscious rabbit model of preconditioning. *Am J Physiol (Heart Circ. Physiol. 35)* 1994;266:H2033-H2041.
11. del Balzo U, Rosen MR. T-wave changes persisting after ventricular pacing in canine heart are altered by 4-aminopyridine but not by lidocaine. Implications with respect to phenomenon of cardiac 'memory'. *Circulation* 1992;85:1464-72.
12. Geller JC, Rosen MR. Persistant T-wave changes after alteration of the ventricular activation sequence. New insights into mechanisms of 'cardiac memory'. *Circulation* 1993;88:1811-9.
13. Van Winkle DM, Chien GL, Wolff RA, Soifer BE, Kuzume K, Davis RF. Cardioprotection provided by adenosine receptor activation is abolished by blockade of the K_{ATP} channel. *Am J Physiol (Heart Circ Physiol 35)* 1994;266:H829-H839.
14. Rohmann S, Weygandt H, Schelling P, Soei LK, Becker KH, Verdouw PD, Lues I, Hausler G. Effect of Bimakalim (EMD 52692), an opener of ATP-sensitive potassium channels, on infarct size, coronary blood flow, regional wall function, and oxygen consumption in swine. *Cardiovasc Res* 1994;28:858-863.
15. Rohmann S, Weygandt H, Schelling P, Soei LK, Verdouw PD, Lues L. Involvement of ATP-sensitive potassium channels in ischemic preconditioning protection. *Basic Res Cardiol* 1994;89:563-576.
16. Sassen LMA, Bezstarosti K, Van der Giessen WJ, Lamers JMJ and Verdouw PD. L-propionylcarnitine increases post-ischemic blood flow but does not affect recovery of the energy charge. *Am J Physiol (Heart Circ Physiol 30)* 1991;261:H172-H180.
17. Atkinson DE, Walton GM. Adenosine triphosphate conservation in metabolic regulation. *J Biol Chem* 1967;242;3239-3241.
18. Chien GL, Wolff RA, DavisRF, Van Winkle DM. "Normothermic range" temperature affects myocardial infarct size. *Cardiovasc Res* 1994;28:1014-1017.
19. Duncker DJ, Herrlinger S, Pavek T, Crampton M, Lindstrom P and Bache RJ. Effects of temperature on myocardial infarct size in swine. *The FASEB Journal* 1994;8,5:A830,4809 (Abstract).
20. Schaper W, Hofmann M, Müller KD, Genth K, Carl M. Experimental occlusion of two small coronary arteries in the same heart. A new validation method for infarct size manipulation. *Basic Res Cardiol* 1979;74:224-9.
21. Verdouw PD, Hartog JM. Provocation and Suppression of ventricular arrhythmias in domestic swine. In: Swine in Cardiovascular Research. Eds: H.C. Stanton and H.J. Mersmann. CRC Press, Inc., Boca Raton, Florida, Vol II,121-156,1986.
22. Toombs CF, Moore TL, Shebuski RJ. Limitation of infarct size in the rabbit by ischaemic preconditioning is reversible with glibenclamide. *Cardiovasc Res* 1993;27:617-622.
23. Gross GJ, Auchampach JA. Blockade of ATP-sensitive potassium channels prevents myocardial preconditioning. *Circ Res* 1992;70:223-233.
24. Schultz R, Rose J, Heusch G. Involvement of ATP-dependent potassium channels in ischemic preconditioning in swine. *Am J Physiol (Heart Circ Physiol 36)* 1994;267: H1341-H1352.

25. Morris JJ, Pellom GL, Murphy CE, Salter DR, Goldstein JP, Wechsler AS. Quantification of the contractile response to injury: Assessment of the work-length relationship in the intact heart. *Circulation* 1987;76:717-727.
26. Schamhardt HC, Verdouw PD & Saxena PR. Effects of oxyfedrine on global haemodynamics and myocardial lactate balance in acute ischaemia of the porcine heart preparation. *Arzn. Forsch./Drug. Res.* 1981;31:,1091-1095.
27. Litovsky SH, Antzelevitch C. Differences in the electrophysiological response of canine ventricular subendocardium and subepicardium to acetylcholine and isoproterenol. A direct effect of acetylcholine in ventricular myocardium. *Circ Res* 1990;67:615-27.

Chapter 5

Myocardial protection by brief ischemia in noncardiac tissue

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Background Brief coronary artery occlusions (CAO) protect both the artery's own perfusion territory ("myocardial preconditioning") and adjacent "virgin" myocardium. Whether ischemia in remote organs protects myocardium is unknown. We examined whether brief occlusion of the anterior mesenteric artery (MAO) or left renal artery (RAO) protects against myocardial infarction.

Methods and Results Area at risk (AR) and infarcted area (IA) were determined in anesthetized rats after 180 minutes of reperfusion following a 60-minute CAO. At normothermia (36.5-37.5°C body temperature), IA/AR was 68±2% (mean ± SEM, n=11) in control rats and 50±3% (n=9, $P<.001$) in rats preconditioned by 15-minute CAO, 10 minutes prior to 60-minute CAO. A 15-minute MAO was equally protective (IA/AR=50±3%, n=10, $P<.001$), while 15-minute RAO failed to limit IA/AR (72±5%, n=8). Hypothermia (30-31°C body temperature) did not effect IA/AR (67±3%, n=11) in control animals, but enhanced protection by 15-minute CAO (IA/AR=22±3%, n=8), while protection by 15-minute MAO (IA/AR= 44±5%, n=11, $P<.001$) was minimally enhanced. Hypothermia unmasked protection by 15-minute RAO (IA/AR=46±6%, n=9, $P<.01$). Hexamethonium (20 mg/kg IV) did not alter protection by 15-minute CAO, but abolished protection by 15-minute MAO. When MAO was sustained throughout the study, cardioprotection was absent.

Conclusion Brief ischemia in "remote" organs protects myocardium against infarction as effectively as myocardial preconditioning. The mechanism of protection by MAO differs from that of CAO as ganglion blockade abolished protection by MAO but not by CAO. The neurogenic pathway is activated during reperfusion after 15-minute MAO as sustained MAO failed to produce cardioprotection.

Ischemic preconditioning has been described not only for the myocardium,[1] but also for the kidney,[2] skeletal muscle,[3] brain[4] and liver.[5] Furthermore, Przyklenk *et al*[6] reported that a brief coronary artery occlusion (CAO) preconditioned not only the myocardium within but also outside its perfusion territory ("remote" but intracardiac ischemic preconditioning). It is unknown, however, whether remote organ ischemia can protect the myocardium against infarction.

Therefore, we first examined whether brief remote organ ischemia prior to a 60-minute CAO limited myocardial infarct size. For this purpose, we produced transient ischemia in the small intestine or left kidney by occluding the anterior mesenteric artery (MAO) or the left renal artery (RAO) in rats and examined its effect on myocardial infarct size. Since body temperature may influence infarct size[7, 8] and cardioprotection by the adenosine deaminase inhibitor pentostatin was only observed in the presence of mild hypothermia[9] studies were performed at two temperatures. Because results indicated that brief MAO provided cardioprotection at both temperatures, MAO was selected to examine the mechanism of protection by remote organ ischemia. To investigate whether a neurogenic pathway was involved we repeated the studies after ganglion blockade with hexamethonium. To determine whether activation of the neurogenic pathway occurred during remote organ ischemia or the subsequent 10 minutes of Reperfusion, we also determined infarct size after 60-minute CAO in the presence of permanent MAO.

Methods

Experimental Design

Experiments in ad libitum fed male Wistar rats (± 300 g) 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.

Effect of 15-minute MAO or 15-minute RAO on Infarct Size Produced by 60-minute CAO (Protocol I).

Nine groups were studied in this protocol (Figure 1). Eight groups underwent a 60-minute occlusion of the left anterior descending coronary artery followed by 180 minutes of Reperfusion at normothermia (36.5-37.5°C body core temperature) (groups 1-4) or hypothermia (30-31°C body core temperature) (groups 6-9). Control groups 1 and 6 underwent a 25-minute sham period prior to the 60-minute CAO. Groups 2 and 7 underwent either a 15-minute CAO, groups 3 and 8 a 15-minute MAO and groups 4 and 9 a 15-minute RAO each followed by Reperfusion starting 10 minutes prior to 60-minute CAO. One group of normothermic rats (group 5) underwent only a 15-minute CAO to determine whether the classical ischemic preconditioning stimulus produced irreversible myocardial damage.

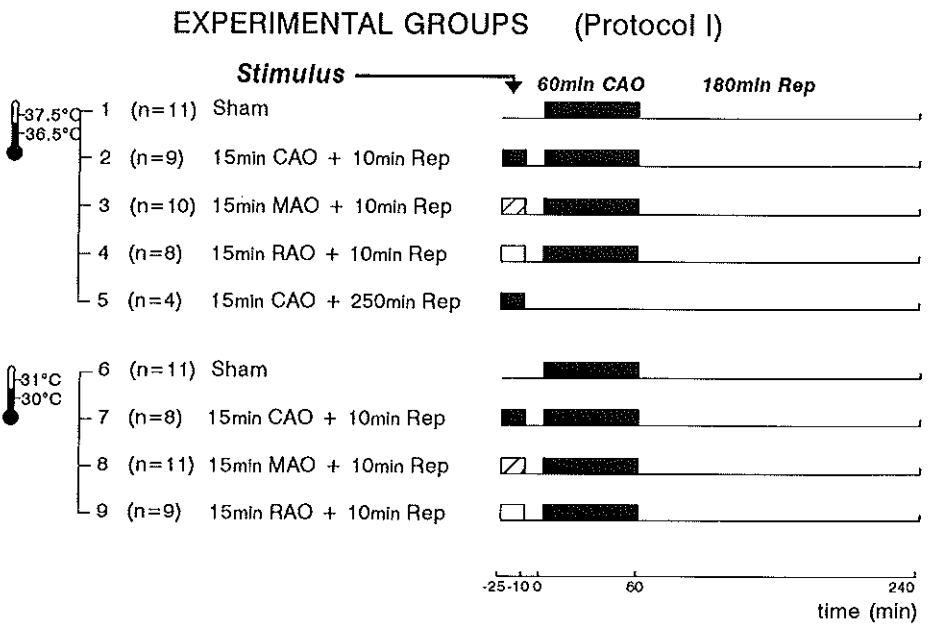

Figure 1. Schematic presentation of the 9 groups in which the protection by 15-minute renal artery and mesenteric artery occlusions against infarct size produced by 60-minute CAO was studied (Protocol I). CAO=left anterior descending coronary artery occlusion; MAO=anterior mesenteric artery occlusion; RAO=left renal artery occlusion; Rep=reperfusion.

Involvement of Neurogenic Pathway in Cardioprotection by 15-minute MAO (Protocol II).

Six groups of rats were studied after pretreatment with the ganglion blocker hexamethonium (20 mg/kg IV) 15 minutes before the ischemic stimulus was applied (Figure 2). Groups 10-12 were studied during normothermia and groups 13-15 during hypothermia. In groups 10 and 13 the effect of ganglion blockade on infarct size produced by 60-minute CAO was studied. The effect of ganglion blockade on cardioprotection by 15-minute CAO was studied in groups 11 and 14, and that by 15-minute MAO in groups 12 and 15.

Importance of Reperfusion for Cardioprotection by MAO (Protocol III).

To investigate whether activation of the neurogenic pathway occurred during occlusion or reperfusion of the anterior mesenteric artery we determined infarct size produced by 60-minute CAO in the presence of a permanent MAO (Figure 3).

Surgical and Experimental Procedures

Rats were anesthetized with pentobarbital (60 mg/kg IP) and intubated for positive pressure ventilation (Harvard) with room air. A PE-10 catheter was positioned in the thoracic aorta for

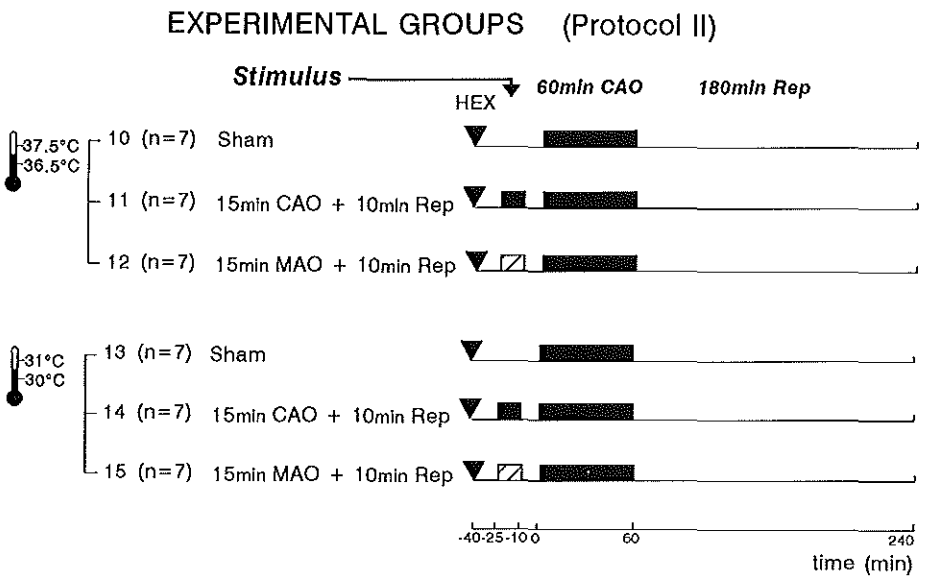

Figure 2. Schematic presentation of the 6 groups in which the effect of ganglion blockade by hexamethonium, (HEX, 20 mg/kg IV) on the protection by 15-minute MAO against myocardial infarct size produced by a 60-minute CAO was studied (Protocol II). For further details see Figure 1.

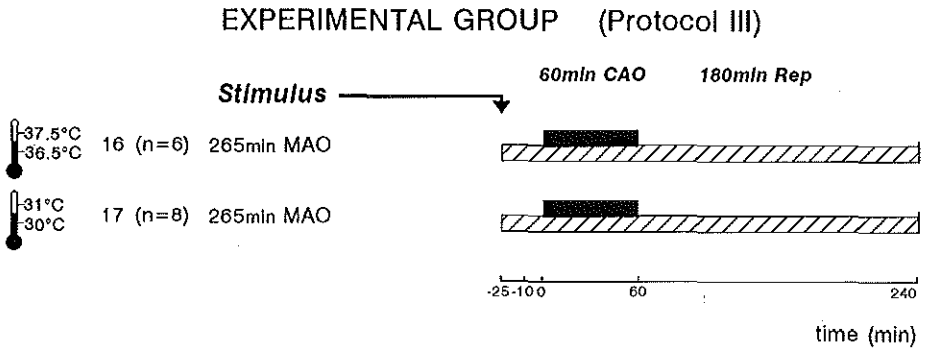

Figure 3. Schematic presentation of the 2 groups in which infarct size produced by 60-minute coronary artery occlusion (CAO) was studied in the presence of sustained anterior mesenteric artery occlusion (MAO) (Protocol III).

measurement of arterial blood pressure and heart rate (Baxter Diagnostic Inc.). A PE-50 catheter was positioned in the inferior caval vein for infusion of haemaccel. After intercostal thoracotomy the pericardium was opened and a silk (6-0) suture was looped under the coronary artery for later production of CAO.[10, 11] Following laparotomy, a catheter was positioned in the abdominal cavity to allow intraperitoneal infusions of pentobarbital for maintenance of anesthesia. Then, the anterior mesenteric artery or the left renal artery was dissected free and a suture was placed around the artery to facilitate later MAO and RAO with an atraumatic clamp. After applying the ischemic stimulus, the abdomen was closed. The control and the classical ischemic

preconditioning groups underwent the same procedure, but without dissection of the renal or mesenteric artery.

Body core temperatures were continuously measured rectally using an electronic thermometer (Electromedics Inc.) and were maintained in the designated range using either heating pads or ice-filled packages. Except during application of the coronary artery occlusions and reperfusions the thoracotomy site was covered with aluminium foil to prevent heat loss from the thoracic cavity. The adequacy of this procedure was verified in five rats in which simultaneous measurements of rectal and intrathoracic temperature showed no differences at baseline (37.2±0.2°C and 36.9±0.02°C, respectively) or at the end of 60-minute CAO (36.9±0.2°C and 36.8±0.2°C, respectively). Rats that fibrillated during ischemia or reperfusion were allowed to complete the protocol when conversion to normal sinus rhythm occurred spontaneously within 1 minute or when resuscitation, by gentle thumping on the thorax, was successful within 2 minutes after onset of fibrillation. Occlusion as well as reperfusion were visually verified by appearance and disappearance of myocardial, small intestinal or renal cyanosis.

Measurement of Area at Risk and Infarcted Area

At the end of the experiment the heart was quickly excised and cooled in ice-cold saline, before it was mounted on a modified Langendorff apparatus and perfused retrogradely via the aorta with 10 ml ice-cold saline to wash out blood.[10, 11] After the coronary ligature was retied the heart was perfused with 3 ml Trypan Blue (0.4%, Sigma Chemical Co.) to stain the normally perfused myocardium dark blue and delineate the non-stained area at risk (AR). The heart was then frozen at -80°C for 10 minutes and cut into slices of 1 mm from apex to base. From each slice, the right ventricle was removed and the left ventricle (LV) was divided into the AR and the remaining left ventricle. The AR was then incubated for 10 minutes in 37°C Nitro-Blue-Tetrazolium (Sigma Chemical Co.; 1 mg per 1 ml Sörensen buffer, pH=7.4), which stains vital tissue purple but leaves infarcted tissue unstained. After the infarcted area (IA) was isolated from the non-infarcted area the different area's of the left ventricle were dried and weighed separately.

Data Analysis and Presentation

Infarct area (% total LV_{mass}) was analysed using ANCOVA with IA as dependent variable, experimental groups as independent factor and AR (% total LV_{mass}), the heart rate- systolic arterial blood pressure product and temperature as covariates. Infarct size (IA/AR in %) was analysed using one way ANOVA followed by unpaired t-test and modified Bonferroni correction.[12] Hemodynamic variables were compared using two way ANOVA for repeated measures followed by the paired or unpaired t-test and modified Bonferroni correction. Data are presented as mean ± SEM.

Results

Mortality

Eight out of the 89 rats that entered protocol I (one rat in each of the groups 1, 2, 3, 8 and 9 and three rats in group 4) and seven out of the 49 rats that entered protocol II (1 rat in each of the groups 12 and 15, 2 rats in group 13, and 3 rats in group 14) were excluded because of sustained ventricular fibrillation. In group 16 (protocol III) 1 of the 9 rats had to be excluded.

Areas at Risk in the Three Study Protocols

There were no significant differences between AR of the experimental groups (Table 1).

Effect of 15-minute MAO or 15-minute RAO on Infarct Size Produced by 60-minute CAO (Protocol I).

Normothermia (Figure 4, Table 1). There was a strong linear relationship between IA and AR of control rats which underwent the 60-minute CAO (IA=0.76(±0.04)AR-1.93(±1.20); r^2=0.98, P<.001). A single 15-minute CAO limited IA/AR produced by 60-minute CAO to 50±3% against 68±2% in the control groups (P<.001). The 15-minute CAO itself resulted in negligible necrosis (IA/AR=3±1%, n=4). A 15-minute MAO was equally protective (IA/AR=50±3%, P<.001) as 15-minute CAO, while 15-minute RAO failed to protect the myocardium (IA/AR=72±5%).

Hypothermia (Figure 4, Table 1). IA/AR of the hypothermic control groups (67±3%) was similar to IA/AR of the normothermic control group (IA=0.74 AR-1.80, r^2=0.90, P<.001) . In contrast, protection by 15-minute CAO was greater during hypothermia (IA/AR=22±3%, P<.001) than during normothermia (P<.01). The limitation of IA/AR to 44±5% (P<.005) by 15-minute MAO was not different from that produced by 15-minute MAO during normothermia. The 15-minute RAO, ineffective during normothermia, limited IA/AR to 46±6% during hypothermia (P<.01 versus hypothermic control).

Involvement of Neurogenic Pathway in Cardioprotection by 15-minute MAO (Protocol II).

Figure 5 and Table 1 illustrate that during normothermia as well as hypothermia ganglion blockade had no effect on infarct size produced by 60-minute CAO (IA/AR=68±3% and IA/AR=67±3%, respectively) and infarct size limitation by 15-minute CAO (IA/AR=54±3%, P<.001 and IA/AR=18±4%, P<.001 respectively). In contrast, cardioprotection by 15-minute MAO was completely abolished by ganglion blockade during both normothermia (IA/AR=74±2%) and hypothermia (IA/AR=69±3%).

Importance of Mesenteric Artery Reperfusion for Cardioprotection by MAO (Protocol III).

Figure 6 and Table 1 show that when MAO was sustained throughout the experimental protocol, myocardial infarct size was not different from that of the control animals (IA/AR=70±3% and 63±3% at normothermia and hypothermia, respectively).

Table 1. **Effect of Remote Organ Ischemia on Infarct Size produced by 60-minute Coronary Artery Occlusion in Rats**

	AR (%LV_{mass})		IA/AR(%)	
	36.5-37.5°C	30-31°C	36.5-37.5°C	30-31°C
Protocol I				
(Ganglion intact)				
Sham + 60min CAO	31±4 (n=11)	36±4 (n=11)	68±2	67±3
15min CAO+10min Rep+60min CAO	47±4 (n=9)	40±5 (n=8)	50±3*	22±3*†
15min MAO+10min Rep+60min CAO	42±4 (n=10)	41±3 (n=11)	50±3*	44±5*
15min RAO+10min Rep+60min CAO	35±8 (n=8)	37±2 (n=9)	72±5	46±6*†
Protocol II				
(After ganglion blockade)				
Sham+60min CAO	37±5 (n=7)	35±3 (n=7)	68±3	67±3
15min CAO+10min Rep+60min CAO	45±3 (n=7)	35±2 (n=7)	54±3*	18±4*†
15min MAO+10min Rep+60min CAO	40±3 (n=7)	37±3 (n=7)	74±2	69±3
Protocol III				
Permanent MAO+60min CAO	36±4 (n=6)	34±2 (n=8)	70±3	63±3

Sham=control group undergoing 60 min CAO *without* ischemic stimulus. CAO=coronary artery occlusion; MAO=mesenteric artery occlusion; RAO=renal artery occlusion; Rep=reperfusion; Hex=hexamethonium (20 mg/kg IV). Permanent MAO started 25 min before the onset of 60 min CAO and was maintained until the end of the three hour reperfusion period. Date are mean ± SEM. *P<.05 vs Control; †P<.05 vs corresponding 36.5-37.5°C group.

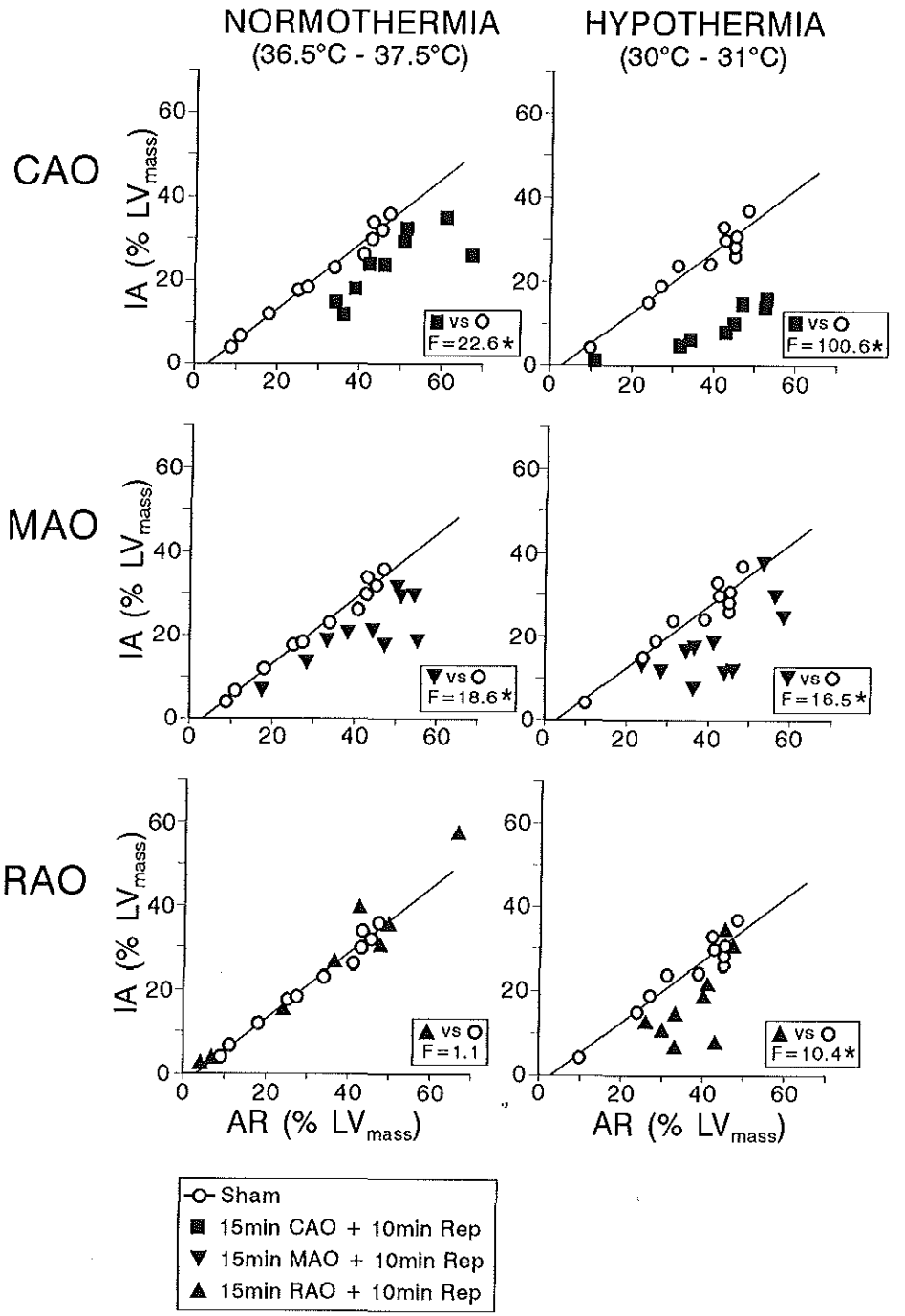

Figure 4. Scatterplots show effects of 15-minute CAO, 15-minute MAO, or 15-minute RAO on the relation between IA and AR produced by 60-minute CAO during normothermia and hypothermia. The regression lines are for the 60-minute CAO control groups. *$P<0.05$. For further details see Figure 1.

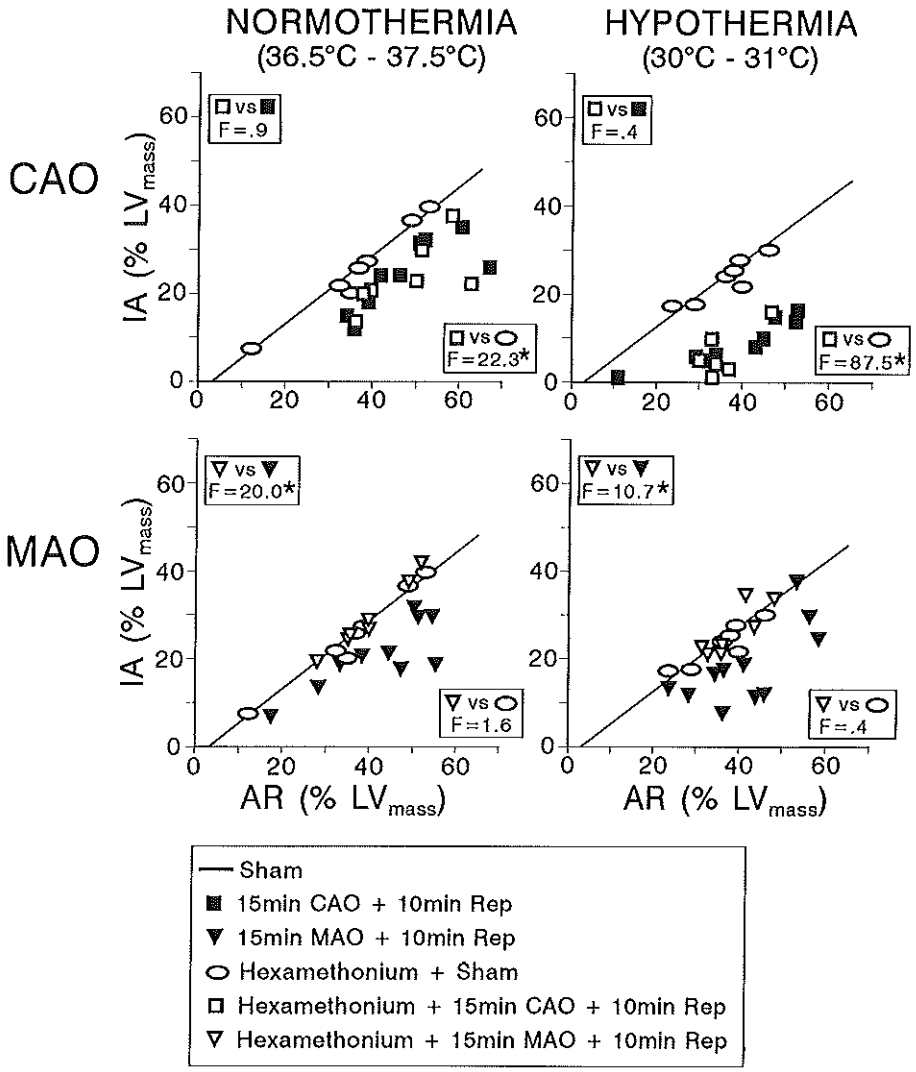

Figure 5. Scatterplots show that ganglion blockade with hexamethonium inhibits protection by 15-minute MAO but has no effect on the protection by 15-minute CAO during normothermia and hypothermia. The plots also show that hexamethonium per se had no effect on myocardial infarct size. The regression lines are for the control (60-minute CAO) animals that did not receive hexamethonium (groups 1 and 5). *$P<0.05$. For further details see Fig. 1.

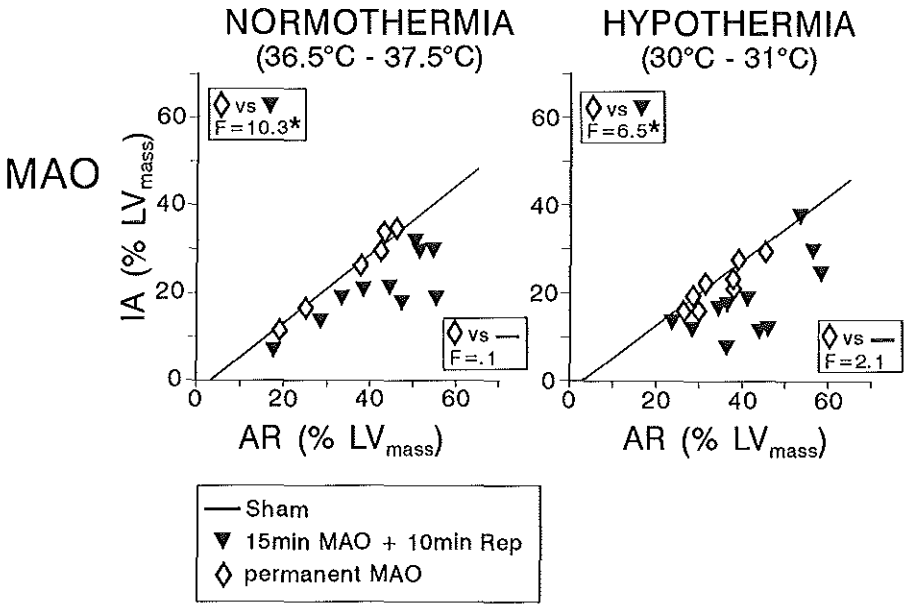

Figure 6. Scatterplots show the lack of effect of sustained MAO on the relation between IA and AR produced by 60-minute CAO. *$P<0.05$. For further details see Figure 1.

Table 2. Systemic Hemodynamics in Rats of Protocol I

	BL	Organ Ischemia (15 min)	Organ Rep (10 min)	CAO (60 min)	Rep 180 min
Normothermia					
Sham (n=10)					
HR	340±8	350±12	344±9	355±9	332±6
MAP	77±5	87±6*	86±5*	70±5	66±5*
15min CAO + 10min Rep (n=9)					
HR	366±11	357±16	360±11	359±7	351±8
MAP	89±5	78±6*	77±4	75±3*	69±3*
15min MAO + 10 min Rep (n=10)					
HR	358±7	368±6	356±6	356±8	351±7
MAP	98±6	110±4*	84±6*	73±4*	68±4*
15min RAO + 10min Rep (n=4)					
HR	350±17	348±19	354±15	330±12	333±10
MAP	92±7	95±7	99±4	69±6	70±6
Hypothermia					
Sham (n=7)					
HR	284±12‡	266±15*‡	266±14*‡	288±8‡	249±7*‡
MAP	86±4	84±6	86±5	95±4	74±3
15min CAO + 10min Rep (n=8)					
HR	320±12‡	303±8‡	288±12‡	276±14*‡	245±14*‡
MAP	94±4	80±4*	83±4*	75±5*	61±3*
15min MAO + 10min Rep (n=5)					
HR	267±14‡	260±12‡	254±13‡	252±13‡	217±8‡
MAP	90±4	99±6	83±3	85±7	71±4
15min RAO + 10min Rep (n=7)					
HR	252±11‡	252±16‡	263±16‡	251±27‡	222±11*‡
MAP	67±5	76±6	79±5	72±6	59±5

BL=baseline; Rep=reperfusion; CAO=coronary artery occlusion; Sham=control group undergoing 60 min CAO without organ ischemia stimulus; HR=heart rate (beats/min); MAP=mean arterial blood pressure (mmHg); MAO=mesentric artery occlusion; RAO=renal artery occlusion; Rep=reperfusion. *$P<.05$ vs Baseline. ‡$P<.05$ vs corresponding normothermia group. There were no significant differences between the preconditioning groups and the corresponding sham group for both heart rate and mean aortic pressure. All data are mean ± SEM.

Hemodynamics

Heart Rate and Mean Arterial Blood Pressure

Protocol I. Under normothermic baseline conditions heart rate (351±5 bpm), mean arterial blood pressure (86±3 mmHg), or the product of heart rate and systolic arterial blood pressure (34500±1300 bpm•mmHg) were not different in groups 1-4 (Table 2). Hypothermia did not affect mean arterial blood pressure (84±3 mmHg) but decreased heart rate (285±8 bpm, $P<.001$) and the rate pressure product (28410±1400bpm•mmHg, $P<.001$). There were also no differences in baseline values of heart rate and arterial blood pressure between groups 6-9.

None of the ischemic stimuli had an effect on heart rate during either normothermia or hypothermia. A 15-minute CAO resulted in a 12±3 mmHg ($P<.05$) decrease in mean arterial blood pressure, which did not recover during the 10 minutes of Reperfusion. In contrast, 15-minute MAO and 15-minute RAO produced increases in mean arterial blood pressure of 11±3 mmHg ($P<.05$) and 6±3 mmHg ($P<.05$), respectively. In the MAO-groups arterial blood pressure decreased to below baseline values during the 10 minutes of Reperfusion but was maintained at 11±3 mmHg ($P<.05$) above baseline in the RAO-groups.

Protocol II. Administration of hexamethonium caused decreases in mean arterial blood pressure of 24±3 mmHg ($P<.05$) and 20±6 mmHg ($P<.05$) during normothermia and hypothermia, respectively, and decreases in heart rate of 34±5 bpm ($P<.05$) and 58±6 bpm ($P<.05$ vs normothermia), respectively. 15-minute CAO decreased mean arterial blood pressure slightly further (7±3 mmHg, $P<.05$), whereas MAO caused an increase in mean arterial blood pressure (18±2 mmHg, $P<.05$). These responses were not different from those observed in the absence of hexamethonium in protocol I (Table 3).

Protocol III. Heart rate did not change in either group during the experimental protocol. After onset of permanent MAO mean arterial blood pressure increased by 14±3 mmHg ($P<.05$) during normothermia and by 16±5 mmHg ($P<.05$) during hypothermia, but returned to baseline levels during the 60-minute CAO (Table 4).

Lack of Effect of Systemic Hemodynamics on Infarct Size

Multivariate regression analysis of the groups in protocol I demonstrated that in the two sham- and two MAO-groups AR explained 99% ($r^2=0.99$) and 93% ($r^2=0.93$), respectively, of the variability in IA with no contributions of temperature or rate-pressure product measured at the onset of 60-minute CAO. In both CAO- and RAO-groups AR explained 88% and 90% of IA variability, respectively, whereas AR together with temperature explained 94% and 94% of the variability of IA with no contribution of the rate-pressure product. Similarly, ANCOVA (with temperature as independent factor and AR and the rate-pressure product as co-variants) demonstrated that temperature but not different hemodynamic conditions explained the enhanced protection in the hypothermic RAO- and CAO-groups. There was no correlation between the mean arterial blood pressure response to the preconditioning stimuli and IA/AR in the CAO-,

Table 3. **Systemic Hemodynamics in Rats of Protocol II**

	BL	Hex (15 min)	Organ Ischemia (15 min)	Organ Rep (10 min)	CAO (60 min)	Rep (180 min)
Normothermia						
Hex control (n=7)						
HR	354±9	323±5*	325±6	320±5	333±5	329±7
MAP	89±9	64±4*	64±5	64±5	66±4	66±3
Hex15min CAO+10 min Rep (n=7)						
HR	341±10	308±4*	313±7	309±5	314±9	315±8
MAP	92±6	65±2*	62±5	61±4	62±3	66±3
Hex 15min MAO+10 min Rep (n=7)						
HR	358±7	320±8*	334±6	319±11	337±14	341±6
MAP	85±6	64±4*	82±5†	61±4	66±7	67±4
Hypothermia						
Hex control (n=7)						
HR	251±10‡	197±5*‡	213±9†‡	206±8	225±8†‡	223±6†‡
MAP	91±3	71±3*	81±3	76±4	74±4	74±2
Hex 15min CAO+10min Rep (n=7)						
HR	260±12‡	195±7*‡	202±9‡	204±8‡	211±8‡	203±10‡
MAP	87±6	69±4*	58±2†	66±4	69±7	62±5
Hex 15min MAO+10 min Rep (n=7)						
HR	268±18‡	213±8*‡	221±9‡	215±10‡	231±14‡	219±9‡
MAP	82±6	62±2*	79±4†	62±3	67±3	59±5

BL=baseline (pre hexamethonium); Hex=hexamethonium (15 min post-administration); Rep=reperfusion; CAO=coronary artery occlusion; Sham=control group undergoing 60 min CAO without organ ischemia stimulus; HR=heart rate (beats/min); MAP=mean arterial blood pressure (mmHg); MAO=mesentric artery occlusion; RAO=renal artery occlusion; *$P<0.05$ HEX vs Baseline; †$P<.05$ vs Hex; ‡$P<.05$ vs corresponding normothermia group. All data are mean ± SEM.

MAO- and RAO-groups, indicating that the pressure responses to 15-minute CAO, MAO or RAO were not responsible for the decrease in IA/AR. This is supported by the observation that despite a pressor response to the permanent MAO, this stimulus failed to limit infarct size.

In both the hexamethonium treated sham- and MAO-groups, AR explained 99% of the variability in IA with no contribution of either temperature or rate-pressure product measured at the onset of 60-minute CAO. In the CAO-groups, AR explained 82% of the IA variability, whereas AR together with temperature explained 97% of IA variability, again with no contribution of the rate-pressure product. Taken together with the observation that

Table 4. **Systemic Hemodynamics in Rats of Protocol III**

	BL	Organ Ischemia (15 min)	Organ Ischemia (25 min)	CAO (60 min)	Rep 180 min
Normothermia					
Permanent MAO (n=6)					
HR	353±8	360±14	355±15	362±6	340±23
MAP	95±7	109±7*	104±8	90±8	54±3*
Hypothermia					
Permanent MAO (n=8)					
HR	247±6‡	243±5‡	232±5‡	234±6‡	248±9‡
MAP	97±6	113±4*	101±3	93±3	77±4*

BL=baseline; Rep=reperfusion; CAO=coronary artery occlusion; HR=heart rate (beats/min); MAP=mean arterial blood pressure (mmHg); MAO=mesentric artery occlusion; Rep=reperfusion. *$P<.05$ vs Baseline. ‡$P<.05$ vs corresponding normothermia group. All data are mean ± SEM.

hexamethonium had no effect on the relation between IA and AR or IA/AR despite the decreases in heart rate and arterial blood pressure, the data clearly indicate that AR, temperature and brief remote organ ischemia, but not hemodynamic conditions were determinants of IA.

Discussion

Until now the protective effects of ischemic preconditioning have only been investigated in models in which the preconditioning stimulus was applied to the organ that was also subjected to the prolonged period of ischemia.[1-5] The present study investigated whether ischemia in an organ other than the heart could limit infarct size produced by a sustained coronary artery occlusion and examined the mechanism(s) leading to cardioprotection. For this purpose we first investigated if protection by remote organ ischemia could be organ specific, and evaluated the effects of ischemia in two different organs, the kidney and small intestine. Secondly, we performed our studies at two different body temperatures (36.5-37.5°C and 30-31°C). This approach was chosen because earlier studies have shown that infarct size development depends on temperature[7, 8] but also because the ability of the adenosine deaminase inhibitor pentostatin to limit myocardial infarct size was only observed at lower body temperature.[9] The major results of the first part of the present study were that (i) ischemia in remote organs can limit myocardial infarct size as effectively as ischemic myocardial preconditioning with 15-minute CAO since 15-minute MAO limited infarct size to the same extent as ischemic myocardial preconditioning during normothermia, (ii) the degree of protection depends on body temperature as 15-minute RAO failed to protect the myocardium during normothermia but was protective during hypothermia, and (iii) the protection by ischemic myocardial preconditioning was more pronounced during hypothermia than during normothermia though infarct size produced by 60-minute CAO per se was not different during normothermia and hypothermia. Because of the

results of the first part of the study we selected the 15-minute MAO stimulus to examine its mechanism of protection. To investigate the involvement of a neurogenic pathway, we examined the effect of ganglion blockade on the protection by 15-minute MAO as well as 15-minute CAO. Ganglion blockade abolished the protection by 15-minute MAO during both normo- and hypothermia, but had no effect on infarct size produced by 60-minute CAO and protection by ischemic myocardial preconditioning at either temperature. These results demonstrate the involvement of a neurogenic pathway in the protection by 15-minute MAO, indicating that protection by remote organ ischemia may be different from that by ischemic myocardial preconditioning. However, our data do not exclude a common intramyocardial endpoint for the mechanism of protection by remote organ ischemia and ischemic myocardial preconditioning, e.g. activation of protein kinase C.[13-15]

The final question we addressed was whether activation of the neurogenic pathway occurred during MAO or in the ensuing 10 minutes of Reperfusion. The observation that permanent MAO failed to limit myocardial infarct size produced by 60-minute CAO indicates that reperfusion of the small intestine was mandatory to activate the neurogenic pathway. These data could be interpreted to suggest that upon reperfusion substances released in the mesenteric bed (e.g. oxygen derived free radicals[16], cytokines[17]) stimulate afferent neurofibers. From the present study it cannot be determined whether these neurofibers are activated within or outside the mesenteric bed. Future studies should therefore be directed at examining the factors involved in activating the neurogenic pathway upon release of the MAO and how this activation results in limitation of myocardial infarct size.

The finding that 15-minute MAO protected the myocardium during both normothermia and hypothermia, whereas the 15-minute RAO was only protective during hypothermia might suggest that 15-minute RAO resulted in a subthreshold stimulus during normothermia. The renal and mesenteric artery are of similar size in terms of amount of total blood flow, but whereas the anterior mesenteric flow is considered almost completely nutrient flow, less than 10% of renal blood flow is nutrient flow.[18] Consequently, the amount of tissue that became ischemic during renal artery occlusion was less than during mesenteric artery occlusion so that the stimulus by RAO may have been below threshold. We can therefore not exclude that multiple or a single longer renal occlusion or bilateral renal artery occlusion could have protected the myocardium. In this respect it should be kept in mind that in ischemic myocardial preconditioning the severity of ischemia appears to be more important than its duration in order to elicit cardioprotection.[19] Nevertheless, brief interruption of renal artery blood flow protected the heart during hypothermia. There have been preliminary reports that a renal artery occlusion may reduce infarct size produced by a coronary artery occlusion.[20, 21] In our earlier study[21] we did not control temperature as rigorously as in the present study. In subsequent experiments we observed that without appropriate measures, temperature can decrease easily by as much as 3-4 °C during the course of surgical instrumentation. We can therefore not exclude that in our earlier study the

effects by the 15-minute RAO occlusions were due to the presence of hypothermia.

An intriguing finding in the present study was that hypothermia per se had no effect on infarct size in the control rats, but unmasked a protective effect by 15-minute RAO. In contrast, the protection by 15-minute MAO tended to be enhanced but this was not statistically significant. The lack of effect of hypothermia on infarct size in the control rats seems at variance with previous observations in rabbits.[7] However, in that study a different temperature range (35-42°C) was employed while the sustained coronary artery occlusion lasted only 30 minutes. At 30 minutes of occlusion infarction progresses rapidly in the rabbit so that a small delay of infarction by a decrease in body temperature may have a greater effect on infarct size. It is possible that in rats infarcts produced by 60-minute CAO are less susceptible to the delay in infarction produced by a decrease in body temperature. This hypothesis is supported by two recent studies in swine. Whereas Duncker *et al*[8] observed that a decrease in body temperature from 39°C to 35°C reduced infarct size produced by 45-minute CAO by more than 80%, McClanahan *et al*[9] reported that a decrease in temperature from 37°C to 35°C had no effect on infarct size following 60-minute CAO. These findings indicate that the effect of temperature on infarct size may depend critically on the experimental model, including the duration of the sustained CAO.

In contrast to the lack of effect of temperature on infarct size in the control rats, hypothermia markedly modified the efficacy of the preconditioning stimuli. With the exception of the mesenteric preconditioning stimulus, which was only slightly but not significantly enhanced by the presence of hypothermia, protection by intramyocardial ischemic preconditioning was enhanced and a cardioprotective effect of brief renal ischemia now emerged. The mechanism of this synergistic (intramyocardial ischemia) or unmasking (renal ischemia) action of hypothermia is not readily explained. The present study excludes a contribution of activation of a neural pathway, as hexamethonium did not affect the enhanced protection of ischemic myocardial preconditioning by hypothermia. McClanahan *et al*[9] reported that either mild hypothermia or adenosine deaminase inhibition alone had no effect on infarct size. In contrast, when these stimuli were combined a significant reduction in myocardial infarct size was observed. Their findings are in agreement with the present study and suggest that body temperature even when it does not alter infarct size by itself can significantly modify the cardioprotective effects of other physiological or pharmacological interventions. The protection by 15-minute MAO was not significantly increased when experiments were performed at hypothermia, which might suggest that the stimulus was already maximally effective at normothermia.

Methodological Considerations

In the present study body core temperature was measured rectally. That this temperature reflects intrathoracic temperature was demonstrated in five rats in which simultaneous measurements of rectal and thoracic cavity temperatures were not different (see Method section).

The question could be raised whether these rectal measurements reflect intramyocardial temperature. In a previous study in swine,[8] rectal temperature exceeded moycardial temperature on average by only 0.3 °C at baseline, while at the end of 45-minute CAO, intramyocardial temperature was 0.3 °C lower than rectal temperature. Those findings suggests that although subtle differences in temperature of myocardium and rectum may have been present in this study, these were small compared to the 6 °C rectal temperature difference in the normothermic and hypothermic groups.

An increase in myocardial stretch produced by rapid volume loading can limit infarct size produced by a 60-minute CAO in dogs.[22] We did not measure left ventricular diastolic volume or pressure, and can therefore not exclude that MAO- or RAO-induced pressor responses produced stretch mediated cardioprotection. However, such a mechanical pathway of protection appears unlikely as ganglion blockade abolished the MAO-induced protection, even though the pressor response persisted.

Clinical Implications

The present study may have important clinical implications as it suggests that ischemia in remote organs could result in cardioprotection when preceding a coronary thrombotic event. Thus, patients suffering from abdominal angina or perhaps even intermittent claudication might conceivably have a longer time-window for thrombolytic therapy to salvage ischemic myocardium. The present study also supports earlier reports that myocardium can be protected by stimuli which do not produce myocardial ischemia such as myocardial stretch[22] or ventricular pacing.[23] The ability of such diverse stimuli, e.g. non-ischemic myocardial stimuli and non-myocardial ischemic stimuli, to protect the myocardium may hamper the unequivocal demonstration of ischemic myocardial preconditioning in man.[24-26]

Conclusion

The present study is the first to demonstrate that not only brief ischemia in an adjacent myocardial region, but also a brief period of ischemia followed by reperfusion in a remote organ, such as small intestine or kidney, can protect the myocardium against irreversible damage produced by a prolonged coronary artery occlusion. Mesenteric artery occlusion and reperfusion resulted in protection both during hypo- and normothermia, whereas the protection by renal ischemia was only apparent under hypothermic conditions. The mechanism of protection by brief mesenteric artery occlusion involved a neurogenic pathway that required mesenteric artery reperfusion for its activation.

References

1 Murry CE, Jennings RB, Reimber KA. Preconditioning with ischemia: a delay of lethal cell injury in ischemic myocardium. *Circulation* 1986;74:1124-1136.

2 Zager RA, Baltes LA, Sharma HM, Jurkowitz MS. Responses of the ischemic acute renal failure kidney to additional ischemic events. *Kidney Int* 1984;26:689-700.

3 Mounsey RA, Pang CY, Forrest C. Preconditioning: a new technique for improved muscle flap survival. *Otolaryngology-Head and Neck Surg* 1992;107:549-552.

4 Kitagawa K, Matsumoto M, Tagaya M, Hata R, Ueda H, Niinobe M, Handa N, Fukunaga R, Kimura K, Mikoshiba K, Kamada T. 'Ischemic tolerance' phenomenon found in the brain. *Brain Res* 1990;528:21-24.

5 Lloris-Carsi JM, Cejalvo D, Toledo-Pereyra LH, Calvo MA, Suzuki S. Preconditioning: effect upon lesion modulation in warm liver ischemia. *Transplant Proc* 1993;25:3303-3304.

6 Przyklenk K, Bauer B, Ovize M, Kloner RA, Whittaker P. Regional ischemic 'preconditioning' protects remote virgin myocardium from subsequent sustained coronary occlusion. *Circulation* 1993;87:893-899.

7 Chien GL, Wolff RA, Davis RF, Van Winkle DM. "Normothermic range" temperature affects myocardial infarct size. *Cardiovasc Res* 1994;28:1014-1017.

8 Duncker DJ, Klassen CL, Herrlinger SH, Pavek TJ, Ishibashi Y, Bache RJ. Effect of temperature on myocardial infarction in swine. *Am J Physiol* 1996 (in press).

9 McClanahan TB, Mertz TE, Martin BJ, Gallagher KP. Pentostatin reduces infarct size in pigs only when combined with mild hypothermia. *Circulation* 1994;90:I478(Abstract)

10 Fishbein MC, MacLean D, Maroko PR. Experimental myocardial infarction in the rat. *Am J Pathol* 1978;90:57-70.

11 Schoemaker RG, Urquhart J, Debets JJM, Struyker-Boudier HAJ, Smits JFM. Acute haemodynamic effects of coronary artery ligation in conscious rats. *Basic Res Cardiol* 1990;85:9-20.

12 Hochberg Y. A sharper Bonferroni procedure for multiple tests of significance. *Biometrica* 1988;74:800-802.

13 Downey JM, Cohen MV, Ytrehus K, Liu Y. Cellular mechanisms in ischemic preconditioning: the role of adenosine and protein kinase C. Das DK (ed). In: Cellular, biochemical, and molecular aspects of reperfusion injury. *Ann New York Acad Sc* 1994;723:82-98.

14 Mitchell MB, Meng X, Ao L, Brown JM, Harken AH, Benerjee A. Preconditioning of isolated rat heart is mediated by protein kinase C. *Circ Res* 1995;76:73-83.

15 Bugge E, Ytrehus K. Ischaemic preconditioning is protein kinase C dependent but not through stimulation of alpha adrenergic or adenosine receptors in isolated rat heart. Cardiovasc Res 1995;29:401-406.

16 Iwamoto T, Miura T, Adachi T, Noto T, Ogawa T, Tsuchida A, Iimura O. Myocardial infarct size-limiting effect of ischemic preconditioning was attenuated by oxygen free-radical scavengers in the rabbit. Circulation 1991;83;1015-1022.

17 Janssen BJ, Van Essen H, Struyker Boudier HA, Smits JF. Hemodynamic effects of activation of renal and mesenteric sensory nerves in rats. *Am J Physiol* 1989;257:R29-36.

18 Drexler H, Depenbusch JW, Truog AG, Zelis R, Flaim SF. Effects of diltiazem on cardiac function and regional blood flow at rest and during exercise in a conscious rat preparation of chronic heart failure (myocardial infarction). *Circulation* 1985;71:1262-1270.

19 Koning MMG, Gho BCG, Van Klaarwater E, Duncker DJ, Verdouw PD. Endocardial and epicardial infarct size after preconditioning by a partial coronary artery occlusion without intervening reperfusion. Importance of the degree and duration of flow reduction. *Cardiovasc Res* 1995;30:1017-1027.

20 McClanahan TB, Nao BS, Wolke LJ, Martin BJ, Metz TE, Gallagher KP. Brief renal occlusion and reperfusion reduces myocardial infarct size in rabbits. *FASEB* 1993;7:A118,682 (Abstract).

21 Gho BCG, Schoemaker RG, Van der Lee C, Sharma HS, Verdouw PD. Cardioprotection by transient renal ischemia, an in vivo study in rats. *Circulation* 1994;90:I1476(Abstract).

22 Ovize M, Kloner RA, Przyklenk K. Stretch preconditions canine myocardium. *Am J Physiol* 1994;266:H137-H146.

23 Koning MMG, Gho BCG, Van Klaarwater E, Opstal RLJ, Duncker DJ, Verdouw PD. Rapid ventricular pacing produces myocardial protection by non-ischemic activation of K^+_{ATP} channels. *Circulation* 1996;93:178-186.

24 Lawson CS. Does ischemic preconditioning occur in the human heart ? *Cardiovasc Res* 1994;28:1461-1466.

25 Kloner RA, Yellon D. Does ischemic preconditioning occur in patients? *J Am Coll Cardiol* 1994;24:1133-1142.

26 Verdouw PD, Gho BCG, Duncker DJ. Ischaemic preconditioning: is it clinically relevant? *Eur Heart J* 1995;16:1169-1176.

Chapter 6

The myocardial infarct size limiting effect of low body temperature in rats depends on the duration of the coronary artery occlusion.

The myocardial infarct size limiting effect of low body temperature in rats depends on the duration of the coronary artery occlusion

Background A reduction in bodytemperature has been reported to result in smaller myocardial infarct size produced by a 30- and 45-minute coronary artery occlusion (CAO) in rabbits and swine respectively. However, in rats we previously found that infarct size produced by a 60-minute CAO at normothermia (36.5°C-37.5°C) or at hypothermia (30°C-31°C) did not differ. Consequently, in the present study we examined whether the protective effect of low body temperature depends on the duration of the CAO.

Methods and Results Area at risk (AR) and infarcted area (IA) were determined in anesthetized rats after 180 minutes of reperfusion following CAO's varying between 15 and 120 minutes. During normothermia, IA/AR was 3±1% (mean ± SEM, n=4), 48±9% (n=6), 62±6% (n=11), 68±2% (n=11) and 75±3% (n=4) after 15-, 30-, 45-, 60- and 120-minute CAO, respectively. During hypothermia, IA/AR was 14±3% (n=5 , P<0,01), 54±2% (n=7, P=NS), 69±2% (n=10, P=NS) and 72±1% (n=2, P=NS) after 30-, 45-, 60- and 120-minute CAO, respectively (P vs corresponding normothermia groups). Although, hypothermia was accompanied by a lower heart rate (275±8 bpm, n=24, vs 355±5 bpm during normothermia, n=28), elimination of hypothermia-induced bradycardia by atrial pacing at 360 bpm did not increase the IA/AR produced by 30-minute CAO (12±2%, n=5, P=NS vs 30-minute CAO during spontaneous sinus rhythm).

Conclusion The myocardial infarct size limiting effect of low body temperature depended critically on the duration of the coronary artery occlusion, but was independent of its effects on heart rate and systolic arterial blood pressure.

Key Words: infarct size, temperature

Introduction

Recently, we showed that myocardial infarct size produced by a 60-minute coronary artery occlusion (CAO) in rats was not different when the experiments were performed at normothermia (36.5°C-37.5°C) or hypothermia (30°C-31°C) [1]. This finding is at variance with previous studies in rabbits [2] and swine [3] in which infarct size produced by 30- [2] and 45-minute [3] CAO was smaller at lower body temperatures. Reasons for the apparent discrepancy could be differences in species or the duration of the CAO. Consequently, the present study investigated the importance of the duration of CAO on the infarct size limiting effect of hypothermia. Since hypothermia was associated with bradycardia, which could possibly limit infarct size per se [4], we studied an additional group of hypothermic rats in which heart rates were increased to heart rates at baseline of the normothermic animals.

Methods

Experimental Design

Experiments in ad libitum fed male Wistar rats (± 300 g) 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.

Ten groups of rats were studied (Figure 1). Five groups (groups 1-5) were subjected to left anterior descending coronary artery occlusions of different durations followed by 180 minutes of reperfusion during normothermia (36.5°C-37.5°C body core temperature), while five groups (groups 6-10) were studied during hypothermia (30°C-31°C body core temperature). Groups 1, 2, 3, 4 and 5 underwent CAO's of 15, 30, 45, 60 or 120 minutes, respectively, while groups 6, 7, 8 and 9 were subjected to CAO's of 30, 45, 60 or 120 minutes, respectively. Group 10 was subjected to 30-minute CAO followed by 180 minutes of reperfusion, while heart rate was raised to 360 bpm to determine the contribution of bradycardia to the temperature-induced infarct size limitation. Infarct size data of rats of groups 1, 4 and 8 have been reported earlier [1].

Surgical and Experimental Procedures

Rats were anesthetized with pentobarbital (60 mg/kg IP) and intubated for positive pressure ventilation (Harvard) with room air. A PE-10 catheter was positioned in the thoracic aorta for measurement of arterial blood gases, arterial blood pressure and heart rate (Baxter Diagnostic Inc.). A PE-50 catheter was positioned in the inferior caval vein for infusion of polygeline (35mg/ml). After intercostal thoracotomy the pericardium was opened and a silk (6-0) suture was looped under the coronary artery for later production of CAO (1, 5, 6). In rats of group 10, a pacing electrode (negative pole) was implanted in the wall of the left atrial auricle while the zero

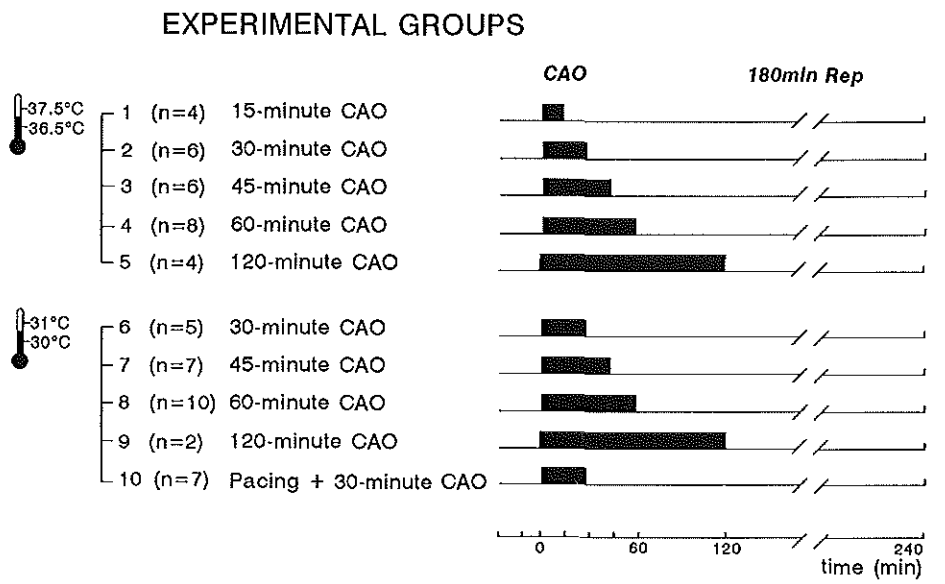

Figure 1. Schematic presentation of the 10 groups in which the effect of temperature on the relation between infarct size and duration of the coronary artery occlusion was studied. CAO=left anterior descending coronary artery occlusion; Rep=reperfusion.

electrode was positioned in the major pectoral muscle. Following laparotomy, a catheter was positioned in the abdominal cavity to allow intraperitoneal infusions of pentobarbital for maintenance of anesthesia.

Body core temperatures were continuously measured rectally using an electronic thermometer (Electromedics Inc.) and were maintained in the designated range using either heating pads or ice-filled packages. Except during application of the coronary artery occlusions and reperfusions the thoracotomy site was covered with aluminium foil to prevent heat loss from the thoracic cavity. The adequacy of this procedure has been verified earlier (chapter 5)[1]. Rats that fibrillated during occlusion or reperfusion were allowed to complete the protocol when conversion to normal sinus rhythm occurred spontaneously within 1 minute or when resuscitation, by gentle thumping on the thorax, was successful within 2 minutes after onset of fibrillation. Occlusion as well as reperfusion were visually verified by appearance and disappearance of myocardial cyanosis.

Measurement of Area at Risk and Infarcted Area

At the end of the experiment the heart was quickly excised and cooled in ice-cold saline before it was mounted on a modified Langendorff apparatus and perfused retrogradely via the aorta with 10 ml ice-cold saline to wash out blood [5,6]. After the coronary ligature was retied the heart was perfused with 3 ml Trypan Blue (0.4%, Sigma Chemical Co.) to stain the normally

perfused myocardium dark blue and delineate the non-stained area at risk (AR). The heart was then frozen at -80°C for 10 minutes and cut into slices of 1 mm from apex to base. From each slice, the right ventricle was removed and the left ventricle (LV) was divided into the AR and the remaining left ventricle. The AR was then incubated for 10 minutes in a 37°C Nitro-Blue-Tetrazolium solution (Sigma Chemical Co.; 1 mg per 1 ml Sörensen buffer, pH=7.4), which stains vital tissue purple but leaves infarcted tissue unstained. After the infarcted area (IA) was isolated from the non-infarcted area, the different area's of the left ventricle were dried and weighted separately. In addition, the isolation technique show an increased variability in measuring infarct size at smaller area at risks, we therefore excluded animals in which the area at risk was below 20% of left ventricular dry weight.

Data Analysis and Presentation

The effects of temperature on infarct size (IA/AR * 100%) were analysed using Two way ANOVA with IA/AR as dependent variable, and temperature and duration of CAO as independent factors. When a significant effect was observed, post-hoc testing was performed using the unpaired t-test with a modified Bonferroni correction [7]. Heart rate, mean arterial blood pressure and the rate-pressure product (heart rate * systolic arterial bloodpressure) were analyzed using three way (CAO duration, temperature and time) ANOVA for repeated measures followed by the paired or unpaired t-test and modified Bonferroni correction. Data are presented as mean ± SEM.

Results

Mortality.

One of the rats (group 4) that entered the study was excluded because of irreversible ventricular fibrillation, while one animal had to be excluded because of technical failure.

Areas at Risk (Table 1).

There were no significant differences between AR of the 10 experimental groups.

Time course of infarct size development (Figure 2, Table 1).

Normothermia. IA/AR of groups 1, 2, 3, 4 and 5 was 3±1%, 48±9%, 62±6%, 68±2% and 75±3% after 15-, 30-, 45-, 60- and 120-minute CAO, respectively, demonstrating that the suspectibility of the myocardium to the development of irreversible damage increased sharply between 15 and 30 minutes (3% of the AR per min).

Hypothermia IA/AR of goups 6, 7 and 8 was 14±3% (n=5, $P<0.01$ vs corresponding normothermia group), 54±2% (n=7, P=NS), 69±2% (n=10, P=NS) and 72±1% (n=2, P=NS) after 30-, 45-, 60-, 120-minute CAO, respectively. These data show thatduring hypothermia the most sensitive period for the development of irreversible damage was delayed, but that the sensitivity

Table 1. **Effect of Temperature on Infarct Size produced by Coronary Artery Occlusion of Different Durations in Rats**

	AR (%LV_{mass})		IA/AR(%)	
	36.5°C-37.5°C	30°C-31°C	36.5°C-37.5°C	30°C-31°C
Control				
15-minute CAO	44±4 (n=4)	-	3±1	-
30-minute CAO	39±4 (n=6)	40±3 (n=5)	48±9	14±3*
45-minute CAO	44±3 (n=6)	38±3 (n=7)	62±6	54±2
60-minute CAO	38±3 (n=8)	39±3 (n=10)	71±2	69±2
120-min CAO	43±3 (n=4)	34±2 (n=2)	75±3	72±1
Pacing +30-minute CAO	-	42±3 (n=7)	-	12±2†

CAO=coronary artery occlusion; Pacing=atrial pacing at 360 bpm started 25 minutes before the onset CAO and was maintained until the end of the three hour reperfusion period. Date are mean ± SEM. *P<.05 vs normothermic group (36.5°C-37.5°C); †P<.05 vs corresponding group with the same temperature and occlusion duration. Table 1

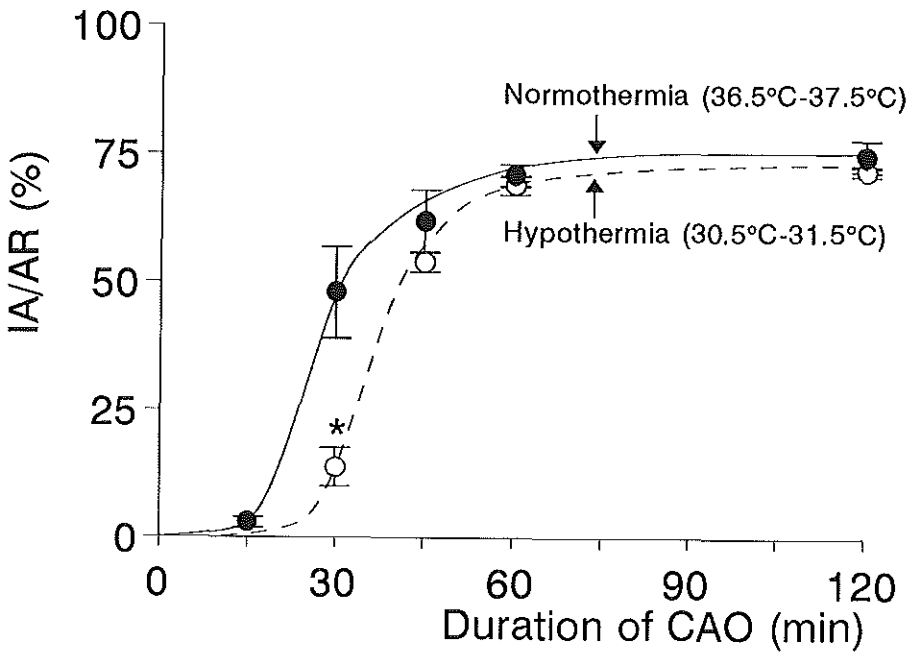

Figure 2. Effect of temperature on the infarct size after CAO's of different duration. Experiments were performed at normothermia (36.5°C-37.5°C, ●) and hypothermia (30°C-31°C, ○). *P<.05 vs normothermic group. IA and AR are infarcted area and area at risk respectively. Data are mean±SEM.

per se was unaltered (2.7% of AR per min).When the occlusions were maintained for longer than 45 minutes, IA/AR was not different for the normothermic and hypothermic rats.

When the 30-minute CAO experiments were performed during atrial pacing at 360 bpm IA/AR was 12±2% (group 10, P<0.05 vs group 2), which was not different from the IA/AR of 14±3% in group 6.

Hemodynamics

Heart Rate, Mean Arterial Blood Pressure and Rate-Pressure Product (Figure 3).

Under normothermic baseline conditions heart rate (355±5 bpm, n=28), mean arterial blood pressure (89±4 mmHg, n=28), or the rate-pressure product (36600±1500 bpm•mmHg, n=28) were not different in groups 1-5. Hypothermia did not affect mean arterial blood pressure (92±3 mmHg, n=24) but decreased heart rate (275±8 bpm, P<.001) and the rate-pressure product (28700±1500 bpm•mmHg, P<.001, n=24). There were no differences in baseline values of heart rate, mean arterial blood pressure and the rate-pressure product between groups 6-9. In group 10 heart rate, mean arterial blood pressure and the rate-pressure product during atrial pacing were not significantly different from the respective baseline levels in the normothermia group 2.

Table 2. **Heart Rate and Mean Arterial Blood Pressure in Rats undergoing Coronary Artery Occlusion under Normo- and Hypothermia.**

	BL	Pre CAO	CAO (15 min)	CAO (30 min)	CAO (45 min)	CAO (60 min)	Rep (180 min)
Normothermia (36.5°C-37.5°C)							
15-minute CAO (n=4)							
HR	328±5	328±5	323±15	-	-	-	330±13
MAP	66±3	66±3	63±5	-	-	-	56±4
30-minute CAO (n=6)							
HR	363±8	355±8	357±8	348±9	-	-	357±5
MAP	87±4	97±3	78±5	84±6	-	-	79±9
45-minute CAO (n=6)							
HR	368±5	347±13	355±7	343±10	360±9	-	360±17
MAP	107±6	90±9	79±6	83±5	93±5	-	66±7
60-minute CAO (n=7)							
HR	351±9	351±9	356±7	360±8	364±10	361±11	336±7
MAP	78±7	82±8	75±10	75±9	74±7	67±6	62±6
Hypothermia (30°C-31°C)							
30-minute CAO (n=5)							
HR	258±15	240±16	260±16	252±20	-	-	254±13
MAP	93±5	83±8	86±7	92±8	-	-	83±2
45-minute CAO (n=7)							
HR	266±12	257±5	277±13	282±15	287±14	-	272±14
MAP	92±2	90±4	84±6	99±5	101±4	-	77±3
60-minute CAO (n=7)							
HR	286±14	269±17	285±15	290±14	286±12	289±9	251±8
MAP	84±6	84±6	83±8	94±7	97±8	98±5	75±4
***Pacing** + 30-minute CAO (n=7)*							
HR	284±5	360±1	348±12	360±1	-	-	360±1
MAP	79±8	78±5	78±6	86±7	-	-	72±4

BL=baseline; CAO=coronary artery occlusion; Rep=Reperfusion; HR=heart rate (bpm); MAP=mean arterial blood pressure (mmHg); Pacing=atrial pacing at 360 bpm. $^{*}P<.05$ vs Baseline. $^{\ddagger}P<.05$ vs corresponding normothermia group. [$P<.05$ vs corresponding time point. There were no significant differences between the groups for both heart rate and mean aortic pressure. All data are mean ± SEM.

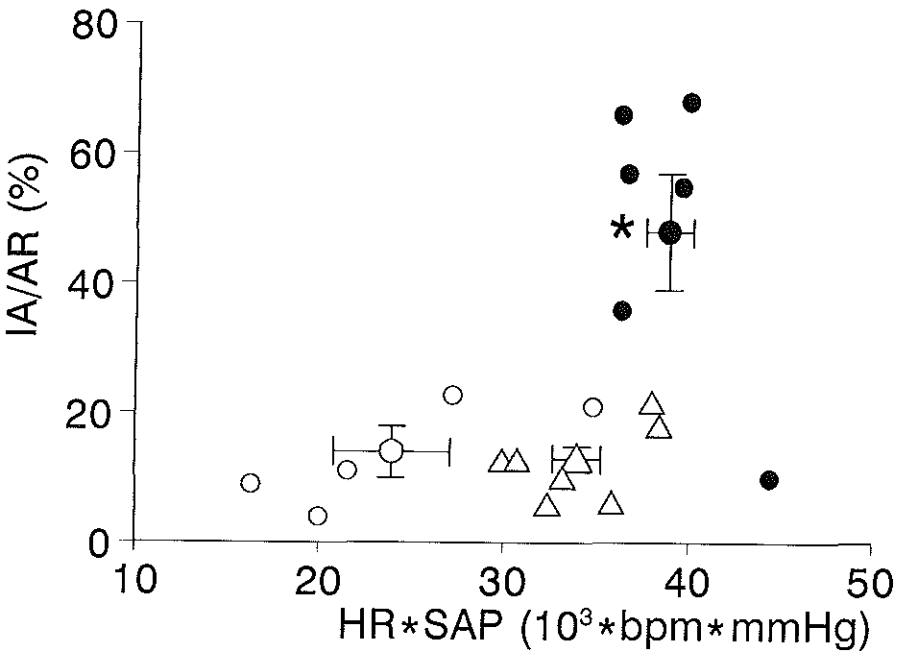

Figure 3. Relation between the rate-pressure product (HR*SAP) and infarct size (IA/AR) for rats that underwent a 30-minute CAO. ● normothermia, O hypothermia, Δ hypothermia and atrial pacing at 360 bpm. IA and AR are infarcted area and area at risk respectively. Data are mean±SEM.

Figure 3 shows that the bradycardia-induced decrease in the rate-pressure-product did not contribute to the limitation in infarct size in the hypothermia group.

Discussion

Some ten years ago Voorhees *et al* [8] reported that in dogs at temperatures below the normal range (~26°C) infarct size produced by a 5-hour coronary artery occlusion was less than in normothermic animals (~39°C). The results of that study must be interpreted with caution because infarct size was determined immediately at the end of the 5 hour occlusion period using nitroblue tetrazolium staining. To minimize false positive staining myocardial reperfusion must be allowed to facilitate washout of nicotinamide-adeninedinucleotide from necrotic myocardium[9]. Voorhees *et al* [8] did not allow reperfusion of the ischemic myocardium and infarct size may therefore have been underestimated. Furthermore, the authors reported collateral blood flow data which were much higher than usually measured during coronary artery occlusion in dogs [8]. Finally, infarct size was expressed as a percent of the left ventricle without taking into acount the size of the area at risk.

Recently, Chien *et al* (2) reported a steep relation between body core temperature in the

"normothermic" range (35°-42°C) and myocardial infarct size in rabbits subjected to a 30-minute CAO and 3 hours of reperfusion, so that an increase of 1°C resulted in 12% infarction of the area at risk with no infarction occuring at a body core temperature of 34.5°C. Duncker *et al* [3] showed an even steeper relation between body core temperature and infarct size produced by a 45 minute coronary artery occlusion and four hours of reperfusion in swine as as 20% of the area at risk became infarcted with a 1°C increase in temperature in the range of 35°C to 39°C.

In contrast to these studies in an earlier study in rats we could not show a protective effect of hypothermia on infarct size determined 3 hours after a 60-minute CAO [1]. We hypothesized that the discrepancy with the earlier studies could be caused by either a difference in the duration of the CAO or a difference in species. The present study demonstrates that the infarct size limiting effect of hypothermia depends on the duration of the coronary artery occlusion. Thus, when in rats the coronary artery was occluded for 30 minutes, hypothermia was protective (5.2% of the area at risk per 1°C) , but when the duration of the CAO was extended to 60 minutes the protective effect of hypothermia could not be detected (Figure 4).

Rabbits and swine like rats display a steep relation between occlusion duration and infarct size. It is likely that in species like the dog and baboon in which infarction progresses more slowly [10, 11], the effect of temperature will be less than in species such as swine, rabbits and rats.

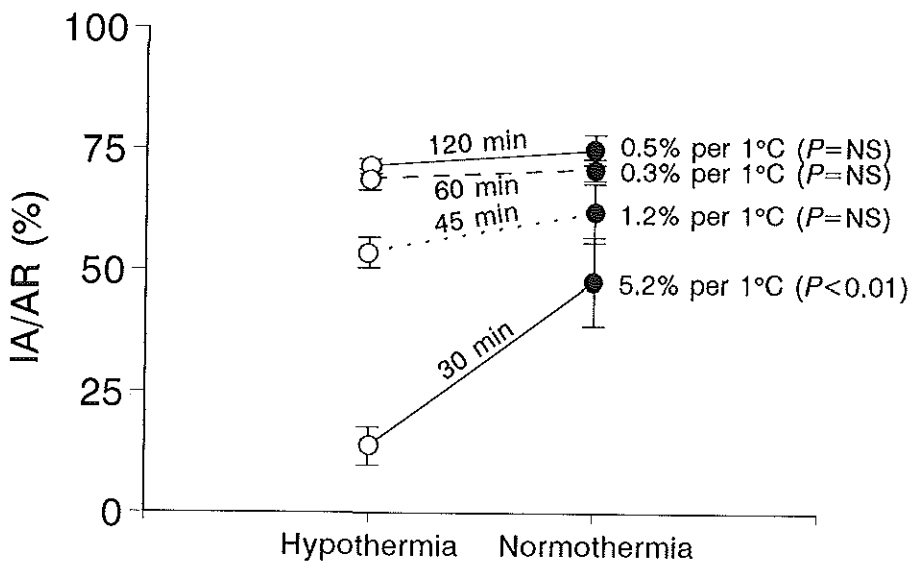

Figure 4. Sensitivity (% infarction of AR per 1°C) for development of irreversible damage during a 30-minute CAO in rats. Experiments were performed at normothermia (●) and hypothermia (O). **P*<.05 vs normothermic group, † *P*<.05 vs normothermic group. IA and AR are infarcted area and area at risk respectively. Data are mean±SEM.

Mechanisms by which hypothermia could exert protection

The role of myocardial oxygen demand at the onset of the coronary artery occlusion as a determinant of infarct size is controversial. A positive correlation [12, 13] as well as no relation [13-17] between the rate-pressure product and infarct size have been reported. Nienaber *et al* [4] produced bradycardia in dogs with a synthetic opiate to lower the metabolic demand at the onset of a 24 hour coronary artery occlusion thereby producing a smaller infarction compared to a group of animals with a high metabolic demand at the onset of coronary artery occlusion. It cannot be excluded that the obtained protection by bradycardia was actually a direct result of μ-opoid receptor stimulation [18]. In collateral deficient species such as rabbit and swine infarct size does not appear to be correlated with the rate-pressure product [14, 17]. In the study by Duncker *et al* [3] univariate or stepwise multivariate regression analysis did not reveal a significant correlation between temperature and systemic hemodynamic variables at baseline or myocardial blood flow under baseline conditions, suggesting that temperature did not exert its effect by altering myocardial oxygen demand at the onset of occlusion. Similarly, in rabbit hearts [2] and rat hearts (present study) the infarct size limiting effect of hypothermia was unmitigated when hypothermia-induced bradycardia was prevented. It is likely that during coronary artery occlusion when contraction ceases, energy utilization is no longer reflected by the rate-pressure product.

Cellular determinants of myocyte viability distal to a coronary artery occlusion are incompletely understood, but may include depletion of high energy phosphate pools below a critical level (ATP<10% of normal), or damage to mitochondrial membranes and sarcolemma with altered ion homeostasis. Since many enzyme systems in mammalian membranes (including ATPases) are temperature sensitive, a decrease in temperature might decrease infarct size through reduction of energy utilization with consequent slowing of high energy phosphate depletion. In agreement with this hypothesis, Jones *et al* [20] reported that a 3°C decrease in temperature markedly slowed the rate of ATP depletion and lactate production in the globally ischemic isolated dog heart. There is evidence that a decrease in temperature can result in decreased fluidity and, consequently, decreased ion permeability of membranes [21, 22]. The latter mechanism has been implicated in the protective effect of hypothermia against calcium overload associated with the calcium paradox [21] and during reoxygenation following hypoxia in isolated perfused rodent hearts [23]. Thus, potential mechanisms through which a lower temperature decreases infarct size likely include a decrease in energy utilization and/or maintained ion homeostasis during ischemia and reperfusion.

Methodological Considerations

Measurement of temperature. In the present study body core temperature was measured rectally. That this temperature reflects intrathoracic temperature was demonstrated in five rats in which simultaneous measurements of rectal and thoracic cavity temperatures were not different [1]. The question could be raised whether these rectal measurements reflect

intramyocardial temperature. In a previous study in swine [3], rectal temperature exceeded moycardial temperature on average by only 0.3°C at baseline, while at the end of 45-minute CAO, intramyocardial temperature was 0.3°C lower than rectal temperature. Those findings suggest that although subtle differences in temperature of myocardium and rectum may have been present in the present study, these were likely to be small compared to the 6°C rectal temperature difference in the normothermic and hypothermic groups.

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 [19]. 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. Recently, it was shown that even in collateral deficient species like rabbit [24] and swine [14] the relation between the area at risk and the infarcted area has a positive intercept through the area at risk axis. As a result the ratio of infarcted area divided by the area at risk depends on the size of the area at risk. However, in rats the intercept through the area at risk axis is not different from zero, implying that infart size expressed as a percentage of the area at risk is independent of the area at risk [1], which justifies the use of infarct size expressed as a percent of the area at risk in the present study.

Collateral blood flow to the Area at Risk. In species with substantial and variable degrees of native collateral circulation such as the dog, collateral blood flow to the ischemic region exerts a protective effect on infarct size [10, 13, 25, 26]. The rat does not possess an extensive collateral circulation and collateral blood can therefore be excluded as a confounding factor.

Conclusions.

The myocardial infarct size limiting effect of low body temperature depended critically on the duration of the coronary artery occlusion, but was independent of its effects on heart rate and systolic arterial blood pressure.

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References

1. Gho BCG, Schoemaker RG, van den Doel MA, Duncker DJ, Verdouw PD. Myocardial protection by brief ischemia in noncardiac tissue. *Circulation* 1996;94:2193-2200.
2. Chien GL, Wolff RA, Davis RF, Van Winkle DM. "Normothermic range" temperature affects myocardial infarct size. *Cardiovasc Res* 1994;28:1014-1017.
3. Duncker DJ, Klassen CL, Herrlinger SH, Pavek TJ, Ishibashi Y, Bache RJ. Effect of temperature on myocardial infarction in swine. *Am J Physiol (Heart Circ. Physiol. 39)* 1996;270:H1189-H1199.
4. Nienaber Ch, Gottwik M, Winkler B, Schaper W. The relationship between the perfusion deficit, infarct size and time after experimental coronary artery occlusion. *Basic Res Cardiol* 1983;78:210-225.
5. Fishbein MC, MacLean D, Maroko PR. Experimental myocardial infarction in the rat. *Am J Pathol* 1978;90:57-70.
6. Schoemaker RG, Urquhart J, Debets JJM, Struyker-Boudier HAJ, Smits JFM. Acute haemodynamic effects of coronary artery ligation in conscious rats. *Basic Res Cardiol* 1990;85:9-20.
7. Hochberg Y. A sharper Bonferroni procedure for multiple tests of significance. *Biometrica* 1988;74:800-802.
8. Voorhees WD, Abendschein DR, Tacker WA. Effect of whole-body hypothermia on myocardial blood flow and infarct salvage during coronary artery occlusion in dogs. *Am Heart J* 1984;107:945-949.
9. Schaper W, Experimental infarcts and the microcirculation. In: Therapeutic Approaches to Myocardial Infarct Size Limitation, Hearse DJ and Yellon DM (eds). Raven Press, New York, 1984; p. 79.
10. Schaper W, Frenzel H, Hort W, Winkler B. Experimental coronary artery occlusion. II. Spatial and temporal evolution of infarcts in the dog heart. *Basic Res Cardiol* 1979;74:233-239.
11. Vatner SF, Patrick TA, Knight DR, Manders WM, Fallon JT. Effects of calcium channel blocker on responses of blood flow, function, arrhythmias, and extent of infarction following reperfusion in conscious baboons. *Circ Res* 1988;62:105-115.
12. Garcia-Dorado D, Theroux P, Elizaga J, Aviles FF, Alonso J, Solares J. Influence of tachycardia and arterial hypertension on infarct size in the pig. *Cardiovasc Res* 1988;22:620-626.
13. Reimer KA, Jennings RB, Cobb FR, Murdock RH, Greenfield Jr. JC, Becker LC, Bulkley BH, Hutchins GM, Schwartz RP, Bailey KR, Passamani ER. Animal models for protecting ischemic myocardium: results of the NHLBI Cooperative Study. Comparison of unconscious and conscious dog models. *Circ Res* 1985;56:651-665.
14. Koning MMG, Simonis LAJ, de Zeeuw S, Nieukoop S, Post S, Verdouw PD. Ischaemic preconditioning by partial occlusion without intermittent reperfusion. *Cardiovasc Res* 1994; 28:1146-1151.
15. Lowe JE, Reimer KA, Jennings RB. Experimental infarct size as a function of the amount of myocardium at risk. *Am J Pathology* 1978;90:363-380.
16. Miura, T., D.M. Yellon, D.J. Hearse, M. Downey. Determinants of infarct size during permanent occlusion of a coronary artery in the closed chest dog. *J Am Coll Cardiol* 1987;9:647-654.
17. Miura T, Ogawa T, Iwamoto T, Shimamoto K, Iimura O. Dipyridamole potentiates the myocardial infarct size-limiting effect of ischemic preconditioning. *Circulation* 1992;86:979-985.
18. Schultz JEJ, Hsu A, Gross GJ. Morphine-induced cardioprotection is mediated via the ATP-sensitive potassium (K_{ATP}) channel in the rat heart. *The FASEB J* 1996;10:A12(abstract).
19. Jennings RB, Sommers MM, Smyth GA, Flack HA, Linn H. Myocardial necrosis induced by temporary occlusion of a coronary artery in the dog. *Arch Pathol* 1960;70:68-78.
20. Jones RN, Reimer KA, Hill ML, Jennings RB. Effect of hypothermia on changes in high-energy phosphate production and utilization in total ischemia. *J Molecular and Cellular Cardiol* 1982;14,*Suppl*3:123-130.
21. Holland Jr CE, Olson RE. Prevention by hypothermia of paradoxical calcium necrosis in cardiac muscle. *J Molecular and Cellular Cardiol* 1975;7:917-928.
22. McMurchie EJ, Raison JK, Cairncross KD. Temperature-induced phase changes in membranes of heart: A contrast between the thermal response of poikilotherms and homeotherms. *Comp Biochem Physiol* 1973;44B:1017-1026.
23. Hearse DJ, Humphrey SM, Bullock GR. The oxygen paradox and the calcium paradox: two facets of the same problem? *J Molecular and Cellular Cardiol* 1978;10:641-668.
24. Ytrehus, K., M.V. Cohen, J.M. Downey. Volume of risk zone influences infarct size in rabbits and may account for unexplained variability in infarction studies. *Circulation* 88:I-136,1993.
25. Judgutt BI, Hutchins GM, Bulkley BH, Becker LC. Myocardial infarction in the conscious dog: three-dimensional mapping of infarct, collateral flow and region at risk. *Circulation* 1979;60:1141-1150.

26. Rivas F, Cobb FR, Bache RJ, Greenfield Jr JC. Relationship between blood flow to ischemic regions and extent of myocardial infarction. Serial measurement of blood flow to ischemic regions in dogs. *Circ Res* 1976;38:439-447.
27. Marber MS, Walker DM, Eveson DJ, Walker JM, Yellon DM. A single five minute period of rapid atrial pacing fails to limit infarct size in the in situ rabbit heart. *Cardiovasc Res* 1993; 27:597-601.

Chapter 7

Does protein kinase C play a pivotal role in the mechanisms of ischemic preconditioning?

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Does Protein Kinase C Play a Pivotal Role in the Mechanisms of Ischemic Preconditioning?

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Jos M.J. Lamers, PhD; Pieter D. Verdouw, PhD.

Summary. This communication reviews the evidence for the pivotal role of protein kinase C in ischemic myocardial preconditioning. It is believed that several intracellular signalling pathways via receptor-coupled phospholipase C and its "cross-talk" with phospholipase D converge to activation of protein kinase C isozymes which is followed by phosphorylation of until now (a number of) unknown target proteins which produce ischemic preconditioning.

After briefly introducing the general biochemical properties of protein kinase C, its isozymes and the limitations of the methodology used to investigate the role of protein kinase C, studies are discussed in which pharmacological inhibition and activation and (immunore)activity and/or isozymes measurements of protein kinase C isozymes were applied to access the role of activation of protein kinase C in ischemic myocardial preconditioning.

Conclusion. It is concluded that definitive proof for the involvement of protein kinase C in preconditioning requires future studies which must focus on the isozyme(s) of protein kinase C that are activated, the duration of action, cellular translocation sites and the identity and stability of (covalently bound phosphate) of phosphorylated substrate proteins.

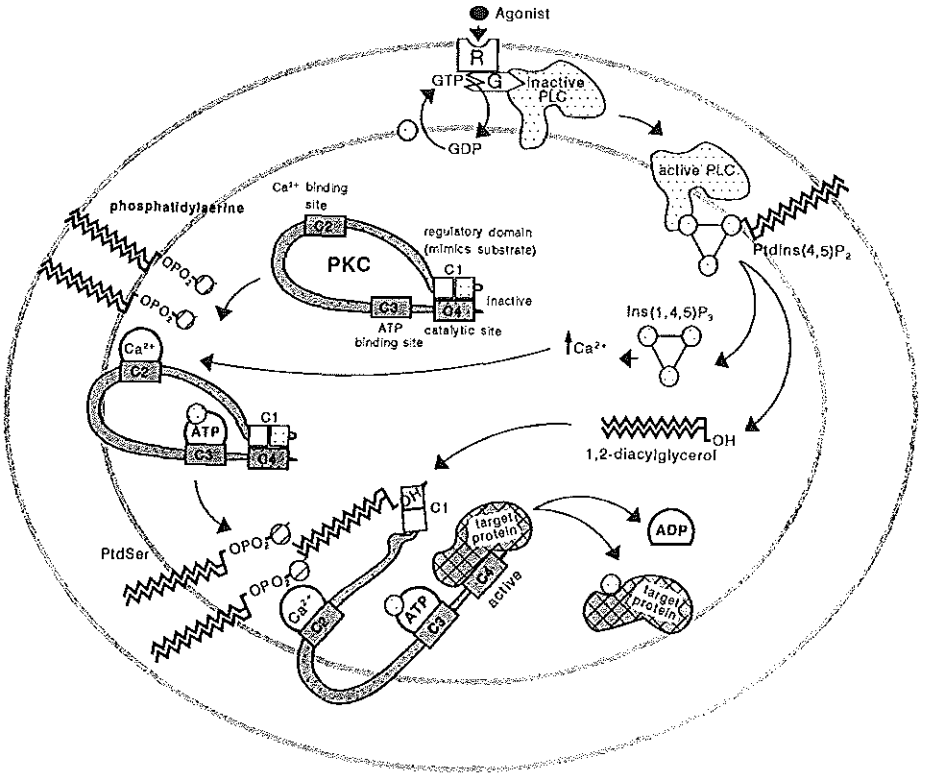
Key words. myocardial ischemia; infarct size; myocardial protection; ischemic preconditioning; protein kinase C; phospholipase C; phospholipase D

Introduction

Ischemic preconditioning is not an organ specific phenomenon, as it does not only occur in the heart [1, 2], but also in kidney [3], liver [4] and skeletal muscle [5], while the brain is protected against the consequence of a new ischemic event at 24 hours after the preconditioning stimulus was applied [6]. Furthermore, brief ischemic in organs other than the heart may also limit irreversible damage produced be a subsequent coronary artery occlusion. Thus, in rats a 15-minute occlusion of the mesenteric artery 10 minutes prior to a 60-minute coronary artery occlusion limited myocardial infarct size by 40% [7]. Since ganglion blockade abolished myocardial protection by mesenteric artery occlusion-reperfusion but not by brief coronary artery occlusion-reperfusion, the mechanism of protection by brief ischemia-reperfusion in other organs appears to differ from that by brief myocardial ischemia-reperfusion [7].

Initially, ischemic preconditioning studies focussed on time characteristics and the search for extracellular endogenous and exogenous factors that either mimicked or inhibited the phenomenon. It proved that protection occurred during two distinct episodes: a first window of protection (FWOP) that lasted 2 to 3 hours after the preconditioning stimulus was applied [1, 2], and a second window of protection (SWOP) between 24 and 72 hours [8-10]. The mechanisms of protection for these two windows are most likely not the same. Endogenous rapidly factors produced agonists may activate intracellular pathways during FWOP, while the slower process of induction of heat-shock proteins may be involved during the SWOP. Initially, attention focussed on activation of adenosine A_1-receptors [11, 12] or K^+_{ATP} channel opening [13, 14], as the mechanisms for protection during FWOP. More recently activation of protein kinase C has received wide attention [15]. Prior to reviewing the role of protein kinase C, we first review the current state of knowledge of the molecular mechanism(s) of ischemic preconditioning and introduce the generally known biochemical properties of protein kinase C and before discussing the limitations of the techniques used to investigate the potential role of protein kinase C. Finally, the evidence that activation of protein kinase C and the intracellular signalling pathways leading to its activation play a pivotal role in the mechanism of ischemic preconditioning is summarized. However, not all studies support a role for protein kinase C in preconditioning and this issue remains therefore controversial at the present time.

Most studies on ischemic preconditioning used infarct size as endpoint, but other endpoints such as recovery of contractile function, and protection against reperfusion-induced ventricular arrhythmias have also been used. Because these other endpoints require different experimental conditions (i.e. shorter duration of the sustained occlusions) we have limited ourselves to studies which used infarct size as endpoint. For this same reason we have excluded studies on ischemic preconditioning in other organs.



PKC Isotype	Domain structure
$\alpha, \beta_1, \beta_2, \gamma$	C1 C2 C3 C4
$\delta, \epsilon, \eta, \theta, \mu$	C1 C3 C4
ζ, ι, λ	C1 C3 C4

Figure 1. Isozymes of protein kinase C, the functional domains in their primary structure, and receptor-coupled phospholipase C-mediated signal transduction leading to protein kinase C activation. The various protein kinase C isozymes share some sequence homology and have all a common ATP-binding site(C3) and catalytic site (C4). Only protein kinase C-α, -β_1, -β_2 and -γ have a Ca^{2+} binding site (C2) and 1,2-diacylglycerol (1,2-DAG) - binding site (C1). In the inactivated state the isozymes of protein kinase C are folded so that an endogenous "pseudo substrate" region on the N-terminal part of the protein occupies the catalytic site (C-terminal part). When agonists (see text) bind to their specific receptors linked to phospholipase C in the cardiac sarcolemma, receptor activation is followed by phospholipase C catalyzed hydrolysis of $PtdIns(4,5)P_2$ to form inositol-1,4,5-trisphosphate ($Ins(1,4,5)P_3$). $Ins(1,4,5)P_3$ releases Ca^{2+} from the $Ins(1,4,5)P_3$ receptor-sensitive Ca^{2+} storage sites in the cardiac sarcoplasmic reticulum. $PtdIns(4,5)P_2$ hydrolysis also forms 1,2-diacylglycerol (1,2-DAG), which increases the affinity of some isozymes for Ca^{2+}. When the intracellular free Ca^{2+} concentration increases, some isozymes become more tightly associated with membranes containing the negatively charged head groups of PtdSer, allowing 1,2-DAG to reach its binding site (C1) on the protein kinase C. The 1,2-DAG-protein kinase C complex approaches

the membrane by placing the kinase in a pocket of negatively-charged head groups of PtdSer in which Ca^{2+} remains attracted. When this occurs, the conformation of protein kinase C changes, exposing the unoccupied catalytic site, thereby allowing the kinase to phosphorylate cellular proteins. There is also evidence that specific binding proteins (e.g. MARCKS and RACK) determine the cellular translocation process of protein kinase C isozymes. The membrane-bound protein kinase C-DAG-(Ca^{2+})-membrane complex only slowly dissociates and this property is the basis for the commonly used "translocation assay" for assessment of PKC activation. Figure adapted from Zeisel *et al* [34], with permission of the FASEB Journal.

Current state of knowledge of the molecular mechanism(s) of ischemic preconditioning

Activation of receptors by exogenously administered stimuli such as adenosine [11, 12], bradykinin [16, 17], noradrenaline [18, 19], acetylcholine [20, 21], endothelin-1 [22] or opiates [23] mimic myocardial protection by ischemic preconditioning. Intracellular signalling by these stimuli, via GTP-binding-protein-linked receptors and phospholipase C and possibly phospholipase D (see later) [24, 25], leads to activation of one or more isozymes of the protein kinase C family which then phosphorylate putative target proteins [15, 26]. Possible target proteins are those that regulate opening of K^+_{ATP} channels [13, 14, 27], activate ecto-5'-nucleotidase [28] (during FWOP) or modulate transcriptional regulation of the expression of heat shock proteins [29, 30] (during SWOP). For instance, K^+_{ATP} channels are opened when an ischemic preconditioning stimulus is applied, while blockade of K^+_{ATP} channels prevents ischemic preconditioning [13, 14]. It is likely that modulation of K^+_{ATP} channels in the mitochondria, sarcoplasmic reticulum or the nucleus are involved in the mechanism of protection as blockade of the action potential shortening by dofetilide does not abolish protection by ischemic preconditioning [31]. Since protein kinase C can be activated via various receptors linked to phospholipase C- and possibly phospholipase D-mediated signalling pathways, these receptors may act synergistically [15]. Opening of K^+_{ATP} channels by pharmacological substances lowers the threshold for ischemic preconditioning [32], which is consistent with the hypothesis that K^+_{ATP} channels are target proteins for protein kinase C. Kitakaze *et al* [28] reported that ischemic preconditioning increased ecto-5'-nucleotidase activity and that activation of protein kinase C increases ecto-5'-nucleotidase activity in isolated rat cardiomyocytes, supporting the candidacy of ecto-5'-nucleotidase as another target protein of protein kinase C.

General biochemical properties of protein kinase C

In general, the conformation of protein kinases consists of two regions which are connected by a region functioning as a hinge. The protein substrate fits into the groove between the two regions and interacts with a catalytic domain and cofactors interacting with the regulatory domain [33]. The specificity of protein kinases such as cyclic AMP dependent protein kinase, Ca^{2+}-calmodulin dependent protein kinase (CaM-PK) and protein kinase C for their substrate proteins is determined by both the primary sequence of these proteins around their phosphorylation site and the capacity of these sites to interact with the catalytic domain of the protein kinase. Generally, protein kinases are inactivated by the interaction between a pseudo substrate region

in the protein kinase's primary sequence and the active site [33]. This restraint is removed during activation by changes in protein kinase conformation due to interaction of second messengers (e.g. cyclic AMP, 1,2-diacylglycerol (1,2-DAG) and Ca^{2+}) with the protein kinase's regulatory sites and by competition between the protein substrates and the pseudo substrate site, all present at the N-terminal region of the primary structure of the protein kinases (Figure 1). Indeed, activation of most protein Serine/Threonine (Ser/Thr) kinases is preceded by receptor activation followed by synthesis or release of low-molecular-weight protein kinase effectors or second messengers.

Protein kinases can be divided in several types. Protein kinase A is a cyclic AMP dependent protein-Ser/Thr kinase, while protein kinase C is a group of protein-Ser/Thr kinases which are phosphatidylserine (PtdSer-), 1,2-DAG- and/or Ca^{2+}-dependent. Recently, protein kinase D was discovered in COS cells to be dependent on 1,2-DAG and phorbol esters, but information on this enzyme in myocardium is not yet available [35]. In myocardial cells protein kinase C regulates various processes, including myocardial contraction, ion transport, energy metabolism, gene expression and hypertrophic growth [25, 36, 37]. The role of protein kinase C in growth and proliferation has been implied by its identification as a high-affinity intracellular receptor for tumor-promoting phorbol esters which directly activate most protein kinase C isozymes in a relatively unspecific manner. Phosphorylation of target proteins by protein kinase C isozymes depends on their intracellular location at the time of action. This compartmentalization may be caused by the architecture and intracellular localization of anchor proteins e.g. the so-called receptors for activated C kinase (RACK) [38, 39]. Therefore, after protein kinase C is activated it translocates to other cellular compartments such as the sarcolemma where it exerts its principal action (Figure 1). However, several other compartments such as mitochondria, myofibrils, sarcoplasmic reticulum and the perinuclear zone also possess specific receptor sites for protein kinase C isozymes [40].

Protein kinase C isozymes.

The protein kinase C family can be divided into three distinct subfamilies on basis of their structure and catalytic and regulatory properties (Figure 1). Classical protein kinase C isozymes (cPKC's) such as protein kinase C-α, -β_1, -β_2 and -γ are activated by Ca^{2+}, PtdSer and 1,2-DAG or phorbol esters such as phorbol-12-myristate-13-acetate (PMA). Novel protein kinase C isozymes (nPKC's) such as protein kinase C-δ, -ϵ, -η, -θ and -μ are Ca^{2+} independent and only need PtdSer and 1,2-DAG (or PMA) to become activated. Atypical protein kinase C isozymes (aPKC's) are protein kinase C-ζ, -ι and -λ which are also Ca^{2+}independent and only require PtdSer, to become activated (Figure 1). At present, the still growing number of isozymes can be discriminated by immunoblot or immunohistofluorescence analysis. Most investigators use histone III-S as substrate and γ-^{32}P-labelled ATP as phosphate donor to measure protein kinase C activity, which reflects the activity of some of the isozymes present in the cellular fraction.

Histone III-S, is a poor substrate for some nPKC's (δ, ϵ and η) compared to the other isozymes (α, β_1, β_2, γ) [41]. Moreover, techniques such as hydroxylapatite high-pressure-liquid-chromatography can be used to determine the activity of protein kinase C isozymes after separation [42]. Measurement of the mRNA concentration using specific cDNA probes on Northern blotting can also be used for the detection of protein kinase C isozymes [43], but mRNA levels offer only limited information because these do not always reflect the functional activities of the isozymes.

In a preliminary study it was found that α, ß, ϵ and ζ are the most prominent isozymes in the rat heart [44]. Similar observations have been made in cultured neonatal rat cardiomyocytes [45] and adult rat cardiomyocytes [46]. However, in a more recent study in adult rat ventricular myocytes, protein kinase C-ϵ was abundantly present and protein kinase C-α could not be detected [47, 48], while in another study protein kinase C- α, -δ, -ϵ, -η, -θ, -ζ, -λ and -ι were detected in adult rat heart [49]. In the canine heart the presence of protein kinase C-α, -β_1, -β_2, -γ, -δ, -ϵ, -ι, -θ and -ζ has been described [50]. Thus, the species and the type of assays determine the pattern of protein kinase C isozymes. Furthermore, the affinity and the specificity of the antibodies used to detect the protein kinase C isozymes determine the abundancy of detection but not the absolute concentration of the isozyme in vivo. In the pig, using rabbit polyclonal antibodies, we have demonstrated the presence of protein kinase C-α and -ϵ, while the isozymes -δ and -ζ were undetectable. Other isozymes were not studied [51]. The studies carried out on myocardial homogenates do not only include myocytes but also fibroblasts, smooth muscle cells and endothelial cells. Consequently, the protein kinase C isozymes of these cells in these homogenates all together are measured. So far, in only one study immunohistochemistry was used to detect the isozymes in situ [48]. In that study it was concluded that protein kinase C-δ is probably the most important isoform involved in preconditioning in the rat heart [48].

Protein kinase C in coupling phospholipase C- to phospholipase D-activation

Endogenous ligands such as adenosine A_1 -, α_1-adrenergic- and muscarinic agonists, bradykinin, angiotensin II, endothelin-1 or opiates stimulate, via the receptor-G-protein coupled to phospholipase, the intracellular signalling pathway. [25, 48, 52-56]. Phospholipase C catalyzes hydrolysis of phosphatidylinositol-4,5-biphosphate (PtdIns (4,5)P_2) which leads to formation of the second messengers inositol-1,4,5-trisphosphate (Ins(1,4,5)P_3) and 1,2-DAG (Figure 1) [25]. These messengers, directly or indirectly, activate Ca^{2+}-independent and/or Ca^{2+}-dependent protein kinase C isozymes and calmodulin-dependent protein kinase (CaM-PK). The activated protein kinase C isozymes and/or CaM-PK phosphorylate specific target proteins which may be responsible for FWOP. Phospholipase D, another 1,2-DAG forming enzyme, can be strongly stimulated by phorbol esters [36, 45, 51, 57, 58]. It uses phosphatidylcholine (PtdCho) as substrate and its activation leads initially to formation of phosphatidic acid (PtdOH) and choline [59]. PtdOH is rapidly hydrolysed to 1,2-DAG and inorganic phosphate (P_i) by PtdOH hydrolase. The 1,2-

Table 1. **Protein kinase C inhibitors and protection by ischemic preconditioning.**

Model	Species	CP-Stimulus x(I+R) (min)	Prolonged-Stimulus I(R) (min)	Protein kinase C inhibitor	Evaluation (ISL/FR)	Result	Reference
Isolated heart	Rat	3x(5I+5R)	30I(120R)	Polymyxin B, before PC-stim	ISL	Abolishes	[66]
		3x(5I+5R)	30I(120R)	Chelerythrine, before PC-stim	ISL	Abolishes	
		1x(2I+10R)	20I(40R)	Staurosporine, before PC-stim	FR	Abolishes	[48]
		Phenylephrine+10R	20I(40R)	Staurosporine, before PC-stim	FR	Abolishes	
		1x(2I+10R)	20I(40R)	Chelerythrine, before PC-stim	FR	Abolishes	
		Phenylephrine+10R	20I(40R)	Chelerythrine, before PC-stim	FR	Abolishes	
	Rabbit	1x(5I+10R)	30I(120R)	Staurosporine, before and after PC-stim	ISL	Failed to abolish	[17]
		1x(5I+10R)	30I(120R)	Staurosporine, after PC-stim	ISL	Abolishes	
		Bradykinin	30I(120R)	Staurosporine, after PC-stim	ISL	Abolishes	
		Bradykinin	30I(120R)	Polymyxin B, 50 min starting 5 min before PC-stim	ISL	Abolishes	
		1x(5I+10R)	30I(180R)	Polymyxin B, 5 min after PC-stim	ISL	Abolishes	[52]
		Phenylephrine	30I(120R)	Polymyxin B, before and after PC-stim	ISL	Abolishes	[55]
		Angiotensin II	30I(120R)	Polymyxin B, 50 min starting 5min before PC-stim	ISL	Abolishes	[56]
In Vivo	Rat	1x(5I+10R)	45I(150R)	Chelerythrine, after PC-stim	ISL	Abolishes	[53]
		3x(3I+5R)	90I(240R)	Calphostin C, before and after PC-stim	ISL	Abolishes	[68]
	Rabbit	1x(5I+10R)	30I(180R)	Staurosporine, 5 min after PC-stim	ISL	Abolishes	[52]
		1x(5I+10R)	30I(180R)	Polymyxin B, 5 min after PC-stim	ISL	Abolishes	
		1x(5I+10R)	30I(180R)	Chelerythrine, 8 min after PC-stim	ISL	Abolishes	[67]
		1x(5I+10R)	30I(180R)	Staurosporine, before PC-stim	ISL	Partially abolishes	[63]
		1x(5I+10R)	30I(180R)	Colchicine, 30 min before PC-stim	ISL	Abolishes	
	Dog	4x(5I+10R)	60I(240R)	H-7 (IV), before, during and after PC-stim	ISL	Failed to abolish	[64]
		4x(5I+10R)	60I(240R)	H-7 (IC), before, during and after PC-stim	ISL	Failed to abolish	
		4x(5I+10R)	60I(240R)	Polymyxin B, before, during and after PC-stim	ISL	Failed to abolish	
		4x(5I+5R)	90I(360R)	Polymyxin B, 5 min before and during PC-stim	ISL	Abolishes	[28]
		4x(5I+5R)	90I(360R)	GF109203X, 5 min before and during PC-stim	ISL	Abolishes	
		Methoxamine	90I(360R)	Polymyxin B, 5 min before and during PC-stim	ISL	Abolishes	
		Methoxamine	90I(360R)	GF109203X, 5 min before and during PC-stim	ISL	Abolishes	
	Pig	2x(10I+30R)	60I(120R)	Staurosporine	ISL	Failed to abolish	[69]
		BIS	60I(120R)	Bisindolylmaleimide	ISL	Mimics	

I=ischemia; R=reperfusion; CP=cardioprotective; BIS=bisindolylmaleimide; H-7=1-(5-isoquinolinesulfonyl)-2-methylpiperazine; ISL=infarct size limitation; FR=functional recovery.

DAG formed by phospholipase D potentially contributes to activation of protein kinase C isozymes [51]. Stimuli such as noradrenaline, angiotensin II and endothelin-1 stimulate both phospholipase C and phospholipase D [45, 58, 60]. In fact, protein kinase C has been proposed to function as a switch which reduces the rate of PtdIns(4,5)P_2 hydrolysis catalyzed by phospholipase C and stimulates the rate of PtdCho hydrolysis catalyzed by phospholipase D [57, 58, 59]. Through this "cross talk" mechanism between phospholipase C and D, the cardiomyocytes may be continuously supplied with 1,2-DAG after receptor stimulation, because the cellular concentration of PtdCho is about 100 times higher than that of PtdIns(4,5)P_2.The continuous production of 1,2-DAG could be of importance for maintenance of activation of the protein kinase C isozymes involved in ischemic preconditioning.

Evidence for a role of protein kinase C in ischemic preconditioning

Inhibition of protein kinase C (Table 1)

Selective inhibition of protein kinase C activation by administration of inhibitors prior to or after applying the cardioprotective stimulus is one approach to investigate the role of protein kinase C in ischemic preconditioning. Depending on the inhibitor, protein kinase C function can be blocked at its catalytic or regulatory sites (Figure 1). It is also possible to downregulate protein kinase C activity by prolonged (1-2 days) stimulation with phorbol ester [61]. This last approach has not been used in ischemic preconditioning, but is of interest in view of the development of tolerance to ischemic preconditioning when a very large number of brief occlusion-reperfusion sequences are applied[62].

The most prominent drugs used to inhibit protein kinase activation or translocation are staurosporine [17, 48, 52, 63], 1-(5-isoquinolinesulfonyl)-2-methylpiperazine (H-7) [27, 64], chelerythrine [53, 65-67], calphostin-C [68], polymyxin B [17, 52, 55, 56, 64, 66], bisindolylmaleimide [28, 69] and colchicine [63]. Staurosporine, H-7, and polymyxin B are non-specific inhibitors of protein kinase C compared to cyclic AMP-dependent protein kinase, CaM-PK or protein-Tyr kinases [70]. All non-selective inhibitors act on the catalytic domain of protein kinase C, which contains a high degree of sequence homology with other protein kinases. The more specific inhibitor calphostin-C [71] acts on the regulatory domain (Figure 1). Chelerythrine, another specific inhibitor [67] interacts with the catalytic domain but also competes with the classically used protein substrates of protein kinase C [57]. Furthermore, polymyxin B directly blocks K^+_{ATP} channels, one of the possible target proteins of protein kinase C and is therefore not well suitable to investigate the role of protein kinase C in ischemic preconditioning [72, 73]. Moreover, it is unknown whether protein kinase C inhibitors are equipotent for all enzyme isozymes. It is quite feasible that the degree of inhibition depends on both the isozyme [60, 74-76] and species. Table 1 shows that polymyxin B [66], staurosporine [48] and chelerythrine [48] abolished the protective effect in isolated rat hearts and calphostin-C [68] and chelerythrine [53] in the *in vivo* rat model. Polymyxin B [17, 52, 55, 56] and

Table 2. **Protein kinase C activators and myocardial infarct size.**

Model	Species	CP-Stimulus x(I+R) (min)	Prolonged-Stimulus I(R) (min)	Evaluation (ISL/FR)	Result	Reference
Isolated heart	Rat	SAG	20I(40R)	FR	Mimics	[48]
	Rabbit	PMA	30I(180R)	ISL	Mimics	[52]
		OAG	30I(180R)	ISL	Mimics	
		PMA	30I(120R)	ISL	Mimics	[63]
In Vivo	Rat	DOG	45I(150R)	ISL	Mimics	[53]
	Rabbit	PMA	30I(180R)	ISL	Mimics	[63]
	Pig	PMA	None	ISL	Failed to mimic	[69]

I=ischemia; R=reperfusion; CP=cardioprotective; SAG=1-stearoyl-2-arachidonoyl glycerol; PMA=phorbol-12-myristate-13-acetate; OAG=oleyl acetyl-glycerol; DOG=1,2-dioctanoyl *sn*-glycerol; ISL=infarct size limitation; FR=functional recovery.

staurosporine [17] abolished protection in *in vitro* and polymyxin B [52], staurosporine [52, 63], chelerythrine [67] and colchicine [63] in *in vivo* rabbit model. In dogs, the data are different as Przyklenk *et al* [64] observed that polymyxin B and H-7 did not abolish cardioprotection by ischemic preconditioning. In a preliminary study in pigs, staurosporine and bisindolylmaleimide limited infarct size [69]. These studies suggest a different role for protein kinase C in rats and rabbits than for dogs and pigs. The different results in rat, rabbit and pig could be related to species-dependent differences in myocardial expression, in the task performed and the site of translocation of the various isozymes [38, 39, 48, 64, 77]. Moreover, the various isozymes might be activated differently by Ca^{2+}, 1,2-DAG and free fatty acids [24, 25, 36, 37].

Reviewing the studies using pharmacological blockade one is tempted to conclude that the protein kinase C family is involved in the mechanism of ischemic preconditioning in rat and rabbit. However, none of the studies investigated whether blockade of protein kinase C actually occurred in the *in vivo* model. *In vitro* assays of enzyme activity will not provide conclusive answers either because these have to be performed in subcellular fractions in the absence of the inhibitor due to the isolation procedure and in the presence of optimal amounts of 1,2-DAG and/or Ca^{2+}. The ideal experimental design would be to study the enzyme activity *in vivo* by measuring phosphorylation degrees of one or more of the unknown specific target proteins.

Activation of protein kinase C (Table 2)

Activation of protein kinase C by administration of phorbol esters, such as 12-O-tetradecanoyl phorbol-13-acetate (TPA) and PMA [27, 66] or 1,2-DAG analogues such as 1-stearoyl-2-arachidonoyl glycerol (SAG) [48], 1,2-dioctanoyl *sn*-glycerol (DOG) [65] and oleyl acetyl-glycerol (OAG) [64] prior to a sustained coronary artery occlusion has been a second approach to investigate the role of protein kinase C in ischemic preconditioning. The advantage of using phorbol esters over 1,2-DAG as activating substances is that they are not metabolized and produce prolonged protein kinase C activation. Protein kinase C translocation takes place after phorbol ester (or the 1,2-DAG analog) is bound to the enzyme's regulatory domain whereby it obtains not only an increased affinity for acidic membrane phospholipids (PtdSer) but also an increased activity (Figure 1).

PMA and several 1,2-DAG analogs mimic preconditioning in the rat [53] and rabbit [52,63], but PMA failed to limit infarct size in pigs [69]. Przyklenk *et al* [64] measured protein kinase C translocation after administration of PMA in dogs. Although these studies lack information regarding activation (translocation) of the isozyme(s) in relation to the protective effect, the results with activators suggest a role for protein kinase C in ischemic preconditioning in rat and rabbit. Furthermore, the route of administration and the dose used may be different for the large animal studies and the in vitro and vivo studies of small animals. For instance, Vogt *et al* [69] used intramyocardial administration of PMA (1μM) to activate protein kinase C but failed to mimic the protective effect of ischemic preconditioning. However, the dose could have been too high

Table 3. **Protein kinase C translocation/activation and protection by ischemic preconditioning.**

Model	Species	CP-Stimulus x(I+R) (min)	Prolonged-Stimulus I(R) (min)	PKC assay	Result	Reference
Isolated heart	Rat	1x(2I)	none	Immunohisto fluorescence	PKC-δ to sarcolemma, ϵ to nucleus, no ζ, α, β_I	[48]
		Phenylephrine	none	Immunohisto fluorescence	PKC-δ to sarcolemma, ζ to nucleus, no ϵ, α, β_I, η	
In Vivo	Dog	4x(5I+10R)	None	Fluorescent to binding by confocal microscopy	No PKC translocation	[64]
		4x(5I+10R)	None	Activity by protein phosphorylation	No PKC activation	
		None	10I	Activity by protein phosphorylation	PKC activation vs Conl	
		4x(5I+10R)	10I	Activity by protein phosphorylation	PKC activation vs Conl	
		PMA	None	Activity by protein phosphorylation	PKC activation	
	Dog	4x(5I+5R)	None	Activity by protein phosphorylation	PKC activation	[28]
	Pig	1x(10I+7.5R)	None	Immunoreactivity on Western blot	PKC-ϵ translocation	[58]
		1x(10I+7.5R)	None	Activity by protein phosphorylation	PKC-ϵ translocation	
	Pig	2x(10+30)	None	Activity by protein phosphorylation	PKC activation	[69]
		PMA	None	Activity by protein phosphorylation	PKC activation	

I=ischemia; R=reperfusion; CP=cardioprotective; PMA=phorbol-12-myristate-13-acetate; PKC= protein kinase C.

because Cohen *et al* [78] found that 0.2 nM PMA was protective while 2 nM PMA was damaging in the isolated rabbit heart. It is feasible that in that study the low dose of PMA activated an isozyme that is protective, while at the higher dose an isozyme was activated that was damaging [78].

Measurements of protein kinase C-translocation (activation) (Table 3)

Translocation of protein kinase C from the cytosol to the membrane has been investigated employing: *(1)* immunoblot analysis using protein kinase C isozyme-specific antibodies of SDS-electrophoretically separated subcellular fractions isolated from myocardial homogenates [44, 51, 69]; *(2)* immunohistofluorescence detection (with confocal microscopy) of protein kinase C isozymes in sections of myocardial tissue [48, 64]; *(3)* assay of total protein kinase C activity in subcellular fractions isolated from myocardial homogenates by measurement of Ca^{2+}- and/or 1,2-DAG-dependent ^{32}P incorporation from γ-^{32}P-labelled ATP into histone III-S or a protein kinase C isoenzyme-specific substrate protein such as peptide-ϵ [51, 54, 64, 79] or other peptides [28, 64]. All three methods have their limitations. For instance, in protein phosphorylation or immuno-reactivity assays, cardiac biopsies are usually rapidly frozen in liquid N_2, followed by preparation of particulate fractions from the homogenates. It can not be excluded, however, that relocalization or (in)activation occurs during isolation of the subcellular fractions. In the subfractions both the basal rate and the maximum rate of histone III-S (or other substrate protein) phosphorylation are measured in the presence of Ca^{2+}, PtdSer and 1,2-DAG. The results of these measurements only reflect the total protein kinase C activities in the subcellular fractions. Moreover, histone III-S is, a relatively poor substrate for some nPKC's (δ, ϵ and η) compared to the cPKC's [41]. Therefore, measurements of the rate of ^{32}P incorporation into the synthetic protein kinase C-ϵ-specific substrate peptide-ϵ may provide the required information about the ϵ isozyme [51]. It should also be noted that mixed micelles of Ca^{2+}-1,2-DAG-PtdSer embedded in Triton-X-100 micelles, used to activate protein kinase C in the ^{32}P incorporation assays, only mimic the cellular membrane environment of protein kinase C in the intact cell. It is unknown whether myocardium is preconditioned homogenously or heterogeneously. In the latter case, the sampling site of the biopsy may pose a restriction. Because, protein kinase C assayed in subcellular fractions isolated from homogenates of whole myocardial tissue represents a mixture of activities of myocytes, fibroblasts, smooth muscle cells and endothelial cells. Immunohistofluorescence measurements must therefore be performed to provide information on the cell type involved in protein kinase C translocation/activation. Measurements of protein kinase C isozyme activity by immunohistofluorescence must, however, be interpreted with caution, because the specific antibodies are not always capable to distinguish active from inactive protein kinase C isozymes.

Weinbrenner *et al*, using Western blotting, showed in rats a rapid translocation of the Ca^{2+}-dependent protein kinase C isozyme α and the Ca^{2+}-independent isozymes (δ, ϵ and ζ) to the

sarcolemma after brief ischemia and increased expression of the Ca^{2+}-independent of protein kinases C-δ and -ϵ in the cytosol after prolonged ischemia [24]. Mitchell *et al*, using immunohistofluorescence, showed in rat hearts that protein kinase C-δ translocated from the cytosol to the sarcolemma after both brief ischemia and α_1-adrenergic stimulation [48]. Brief ischemia also caused translocation of protein kinase C-ϵ from the cytosol to the nuclear region. Measurements of other protein kinase C-isozymes (α, β_1, ζ and η) did not provide evidence for occurrence of translocation after brief ischemia or α_1-adrenergic stimulation [48]. These results provide the first evidence that (at least in the rat) specific protein kinase C isozymes are involved in ischemic preconditioning.

Przyklenk *et al* used a probe consisting of the protein kinase C inhibitor bisindolylmaleimide conjugated to fluorescein that selectively binds to active protein kinase C and observed no difference in the total amount and the cellular distribution of protein kinase C fluorescence with preconditioning in dogs [64]. The advantage of this method over immunofluorescence is that it distinguishes between active and inactive protein kinase C. In their study Przyklenk *et al* also obtained quantitative information on the changes in the amount and subcellular distribution of protein kinase C by measuring the rate of ^{32}P incorporation into the threonine group of a protein kinase C-specific peptide, which was not further specified [64]. A small rise in protein kinase C activity was found in the membrane fraction isolated from biopsies obtained after 10 min of ischemia compared to those isolated after four sequences of 5 min occlusion-reperfusion or no intervention [64]. However, no difference in protein kinase C activity between matched groups of controls and 'ischemic preconditioned' dogs could be measured at time points comparable to the onset of the long occlusion or at 10 min into sustained ischemia [64]. Using the same protein kinase C analysis, Vogt *et al* found a modest (10 to 20%) redistribution of protein kinase C from the cytosol to the membrane fraction in pig hearts subjected to 10 min of ischemia [69]. In contrast to the studies by Przyklenk *et al* [64], Kitakaze *et al* [28] observed in the same canine model a marked translocation of Ca^{2+}- and lipid-dependent protein kinase C activity in cytosol and membrane fractions isolated from preconditioned epi- as well as endomyocardium. These authors ascribed their positive findings to the time of measurements (5 min after the preconditioning stimulus against 10 min by Przyklenk *et al* [64]).

We studied translocation of protein kinase C enzyme activity by ^{32}P incorporation into histone III-S and ϵ-peptide and immunoreactivity of a number of protein kinase C isoforms (α, ϵ, δ and ζ) of cytosolic and membrane fractions isolated from biopsies of porcine myocardium preconditioned by a 10-minute coronary artery occlusion and 7.5-minutes of reperfusion [51, 80]. Ca^{2+} and 1,2-DAG-stimulated protein kinase C activity with histone III-S as substrate was higher in the cytosolic and particulate fractions isolated from the preconditioned myocardium than from the control region. Significant Ca^{2+}-independent, 1,2-DAG-stimulated phosphorylation of ϵ-peptide was found in the cytosolic fractions, but not in the particulate fractions. However, no significant increase of 1,2-DAG-stimulated phosphorylation of ϵ-peptide in the cytosolic fraction

from the preconditioned myocardium was observed. These were rather unexpected findings in view of our protein kinase C-ϵ immunoreactivity measurements (see below). The cytosolic and particulate fractions were also examined by immunoblot analysis using rabbit polyclonal antibodies specific for protein kinase C-α, -δ, -ϵ and -ζ isozymes [51]. This analysis revealed significant levels of expression of the Ca^{2+}-independent isozyme protein kinase C-ϵ, the abundant presence of protein kinase C-α, while protein kinase C-δ and -ζ were virtually undetectable. The immunoreactivity data also indicate that neither the (Ca^{2+} and 1,2-DAG)-stimulated histone III-S and peptide-ϵ kinase activities of the cytosolic nor those of the particulate fractions reflect the relative immunoreactivities in the corresponding fractions. In contrast to the ^{32}P-incorporation data, the immunofluorescence data suggested that the total amount and subcellular distribution of protein kinase C-α and -ϵ was not altered in the preconditioned region compared to the non-ischemic region of the left circumflex coronary artery. Therefore, by using immunofluorescence we were unable to detect the occurrence of ischemia-induced expression of protein kinase C or ischemia-induced translocation of protein kinase C from the cytosolic to the particulate fraction of the protein kinase C isozyme -α, -δ, -ϵ or ζ. On the other hand, binding proteins, such as myristoylated-alanine-rich-C-kinase-substrate (MARCKS) and RACK may determine whether activated protein kinase C isozymes translocate and are providing another mechanism for functional specificity to specific intracellular locations. Thus, in the in vitro phosphorylation assays of the subcellular fractions, different amounts of MARCKS- or RACK-bound protein kinase C isozymes can alter the protein kinase C activity measured [81]. This could cause the discrepancy between the activity assays and Western blotting. Nevertheless, our results on ^{32}P incorporation demonstrate an increase in cytosolic and membrane-bound protein kinase C activities due to brief ischemia and supports a role for protein kinase C in ischemic preconditioning in pigs [51].

Concluding remarks

At present MARCKS is the only endogenous target protein for protein kinase C, that has been shown to be phosphorylated in preconditioned rabbit myocardium. However, the former is believed to be an intracellular location site rather than a protein factor intimately involved in the protective response [82]. Irrespective of the target protein(s) we are dealing with, its (their) covalently bound phosphates must be relatively stable during the 2 to 3 hours in which the cardioprotection is present (FWOP). Furthermore, the precise time point that protein kinase C is maximally translocated (activated) during ischemia or reperfusion (preconditioning stimulus) is unknown and consequently also the time point at which the enzyme reaches the target proteins for catalyzing their phosphorylation. It is quite feasible that protein kinase C is removed from its translocation site or proteolytically degraded after it has performed its action and thereafter it is not longer detectable by immunoreactivity or activity measurements. It is therefore mandatory to determine the time course of translocation/activation and subsequent

relocalization/inactivation or proteolytic degradation of the protein kinase C isozyme and the time course(s) of phosphorylation and dephosphorylation or proteolytic degradation of the target protein(s). Because the time course of weaning of the protective effect of the FWOP is roughly known, the time course of dephosphorylation/inactivation of the target protein could be correlated to the time course of weaning of protection. Candidate target proteins of protein kinase C involved in the FWOP are e.g. the K^+_{ATP} channel [13, 14] and/or the ecto-5'-nucleotidase [28], but experimental evidence for phosphate incorporation into these proteins or regulating proteins is lacking. If the K^+_{ATP} channel or the ecto-5'-nucleotides are target proteins the most likely translocation site for the protein kinase C isozyme(s) involved in the FWOP is the sarcolemma.

Protein kinase C is involved in the agonist-receptor interaction induced changes in gene expression of many cells [25, 36, 37, 74, 75, 41, 83]. Taking into account the time required for inducing heat shock/stress proteins [29, 30] the former may only play a role in SWOP. Therefore, a transcription factor involved in the regulation of expression of heat shock/stress proteins could be another potential target protein of protein kinase C. If true, the nucleus may be the translocation site for the protein kinase C isozyme(s).

References

1. Murry CE, Jennings RB, Reimer KA. Preconditioning with ischemia: a delay of lethal cell injury in ischemic myocardium. *Circulation* 1986;75:1124-1136.
2. Lawson CS, Downey JM. Preconditioning : state of the art myocardial protection. *Cardiovascular Res* 1993;27:542-550.
3. Zager RA, Baltes LA, Sharma HM, Jurkowitz MS. Responses of the ischemic acute renal failure kidney to additional ischemic events. *Kidney Int* 1984;26:689-700.
4. Lloris-Carsi JM, Cejalvo D, Toledo-Pereyra LH, Calvo MA, Suzuki S. Preconditioning: effect upon lesion modulation in warm liver ischemia. *Transplant Proc* 1993;25:3303-3304.
5. Mounsey RA, Pang CY, Forrest C. Preconditioning: a new technique for improved muscle flap survival. *Otolaryngology-Head and Neck Surg* 1992;107:549-552.
6. Kitagawa K, Matsumoto M, Tagaya M, Hata R, Ueda H, Niinobe M, Handa N, Fukunaga R, Kimura K, Mikoshiba K, Kamada T. 'Ischemic tolerance' phenomenon found in the brain. *Brain Res* 1990;528:21-24.
7. Gho BC, Schoemaker RG, Van den Doel MA, Duncker DJ, Verdouw PD. Myocardial protection by brief ischemia in non-cardiac tissue. *Circulation* 1996;94:2193-2200.
8. Kuzuya T, Hoshida S, Yamashita N, Fuji H, Oe H, Hori M, Kamada T, Tada. Delayed effects of sublethal ischemia on the acquisition of tolerance to ischemia. *Circ Res* 1993;72:1293-1299.
9. Marber MS, Latchman DS, Walker M, Yellon DM. Cardiac stress protein elevation 24 hours after brief ischemia or heat stress is associated with resistance to myocardial infarction. *Circulation* 1993;88:1264-1272.
10. Baxter GF, Goma FM, Yellon DM. Duration of the "second window of protection" following ischaemic preconditioning in the rabbit. *J Mol Cell Cardiol* 1995;27:A162 (Abstract).
11. Thornton J, Liu GS, Olsson RA, Downey JM. Intravenous pretreatment with A1-selective adenosine analogues protects the heart against infarction. *Circulation* 1992;85:659-665.
12. Toombs CF, McGee DS, Johnston WE, Vinten-Johanson J. Myocardial protective effects of adenosine: infarct size reduction with pretreatment and continued receptor stimulation during ischemia. *Circulation* 1992;86:986-994.
13. Gross GJ, Auchampach JA. Blockade of ATP-sensitive potassium channels prevents myocardial preconditioning in dogs. *Circ Res* 1992;70:223-233.
14. Rohmann S, Weygandt H, Schelling P, Soei LK, Verdouw PD, Lues I. Involvement of ATP-sensitive potassium channels in preconditioning protection. *Basic Res Cardiol* 1994;89:563-576.
15. Downey JM, Cohen MV, Ytrehus K, Liu Y. Cellular mechanisms in ischemic preconditioning: The role of adenosine and protein kinase C. In: Cellular, Biochemical, and Molecular Aspects of Reperfusion Injury. Ed: Das DK; *Ann NY Acad Sci* 1994;723:882-898.
16. Wall TM, Sheehy R, Hartman JC. Role of bradykinin in myocardial preconditioning. *J Pharmacol Exp Ther* 1994;270:681-689.
17. Goto M, Liu Y, Yang X-M, Ardell JL, Cohen MV, Downey JM. Role of bradykinin in protection of ischemic preconditioning in rabbit heart. *Circ Res* 1995;77:611-621.
18. Hu K, Nattel S. Mechanisms of ischemic preconditioning in rat hearts. Involvement of alpha 1B-adrenoceptors, pertussis toxin-sensitive G proteins, and protein kinase C. *Circulation* 1995;92(8):2259-2265.
19. Strasser RH, Marquetant R. Sensitization of the beta-adrenergic system in acute myocardial ischaemia by a protein kinase C-dependent mechanism. *Eur Heart J* 1991;12 Suppl F:48-53.
20. Yao Z, Gross GJ. Acetylcholine mimics ischemic preconditioning via a glibenclamide-sensitive mechanism in dogs. *Am J Physiol.* 264 (*Heart Circ Physiol* 33) 1993;H2221-H2225.
21. Yao Z, Gross GJ. Role of nitric oxide, muscarinic receptors, and the ATP-sensitive K^+ channel in mediating the effects of acetylcholine to mimic preconditioning in dogs. *Circ Res* 1993;73:1193-1201.
22. Wang P, Gallagher KP, Downey JP, Cohen MV. Pretreatment with endothelin-1 mimics ischemic preconditioning against infarction in isolated rabbit heart. *J Mol Cell Cardiol* 1996;28:579-588.
23. Schultz JEJ, Hsu A, Gross GJ. Morphine-induced cardioprotection is mediated via the ATP-sensitive potassium channel in the rat heart (Abstract). *FASEB J* 1996;10:A12, 68.
24. Sugden PH, Bogoyevitch MA. Intracellular signalling through protein kinases in the heart. *Cardiovasc Res* 1995;30:478-492.
25. De Jonge HW, Van Heugten HAA, Lamers JMJ. Review. Signaltransduction by the phosphatidylinositol cycle in myocardium. *J Mol Cell Cardiol* 1995;27:93-106.
26. Cohen MV, Downey JM. Preconditioning during ischemia: basic mechanism and potential clinical applications. *Cardiol Rev* 1995;3:137-149.

27. Hu K, Duan D, Nattel S. Protein kinase C-mediated activation of ATP-sensitive potassium current - The missing link in ischemic preconditioning. *Circulation* 1995;92:2259-2265.
28. Kitakaze M, Node K, Minamino T, Komamura K, Funaya H, Shinozaki Y, Chujo M, Mori H, Inoue M, Hori M, Kamada T. Role of activation of protein kinase C in the infarct size-limiting effect of ischemic preconditioning through activation of ecto-5'-nucleotidase. *Circulation* 1996;93:781-791.
29. Donnely TJ, Sievers RE, Visseren FLJ, Welch WJ, Wolfe CL. Heat shock protein induction in rats. A role for improved myocardial salvage after ischemia reperfusion. *Circulation* 1992;85:769-778.
30. Yellon DM, Pasini E, Cargnoni A, Marber MS, Latchman DS, Ferrari R. The protective role of heat stress in the ischaemic and reperfused rabbit myocardium. *J Mol Cell Cardiol* 1992;24:895-907.
31. Sleph P, D'Alonzo A, Dzwonczyk S, Parham C, Darbenzio R, Grover GJ. Preconditioning is not abolished by the delayed rectifier K^+(IKR) blocker dofetilide. *FASEB J* 1996;10:A319-1841.
32. Yao Z, Gross GJ. Activation of ATP-sensitive potassium channels lowers threshold for ischemic preconditioning in dogs. *Am J Physiol* 1994;267:H1888-H1894.
33. Kemp BE, Parker MW, Hu S, Tiganis T, House C. Substrate and pseudosubstrate interactions with protein kinases: determinants of specificity. *Trends Biochem Sci* 1994;19:440-444.
34. Zeisel SH. Choline phospholipids: signal transduction and carcinogenesis. *FASEB J* 1993;7:551-557.
35. Rozengurt E, Sinnet-Smith J, Van Sint J, Valverde AM. Protein kinase D (PKD): A novel target for diacylglycerol and phorbol esters. *Mutation Res* 1995;333:153-160.
36. Nishizuka Y. Intracellular signalling by hydrolysis of phospholipids and activation of protein kinase C. *Science* 1992;258:607-614.
37. Pucéat M, Brown JH. Protein kinase C in the heart. In: Kuo JF, ed. Protein kinase C. London: Oxford University Press 1994;249-268.
38. Mochly-Rosen D, Khaner H, Lopez J. Identification of intracellular receptor proteins for activated protein kinase C. *Proc Natl Acad Sci* 1991;88:3997-4000.
39. Ron D, Mochly-Rosen D. An autoregulatory region in protein kinase C: the pseudoanchoring site. *Proc Natl Acad Sci* 1995;92:492-496.
40. Pears C, Stabel S, Cauzabon S, Parker P. Studies on the phosphorylation of protein kinase C-α. *Biochem J* 1992;283:515-518.
41. Hug H, Sarre TF. Protein kinase C isozymes: divergence in signal transduction. *Biochem J* 1993;291:329-343.
42. Kikkawa U, Go M, Koumoto J, Nishizuka Y. Rapid purification of protein kinase C by high performance liquid chromatography. *Biochem Biophys Res Commun* 1986;135:636-643.
43. Ruzycky A. Protein kinase C and cardiovascular contractility. In: Gwathmey JK, Briggs GM, Allan PD Ed., Heart failure, basic science and clinical aspects. New York: Marcel Dekker, Inc. 1993.
44. Weinbrenner CE, Simonis G, Marquetant R, Strasser RH. Selective regulation of calcium-dependent and calcium-independent subtypes of protein kinase C in acute and prolonged myocardial ischemia (Abstract). *Circulation* 1993;88(Suppl 1):I-101.
45. Lamers JMJ, Eskildsen-Helmond YEG, Resink AM, De Jonge HW, Bezstarosti K, Sharma HS, Van Heugten HAA. Endothelin-1-induced phospholipase C-ß and D and protein kinase C isoenzyme signalling leading to hypertrophy in rat cardiomyocytes. *J Cardiovasc Pharmacol* 1995;26(suppl. 3):S100-S103.
46. Bogoyevitch MA, Parker PJ, Sugden PH. Characterization of protein kinase C isotype expression in adult rat heart. *Circ Res* 1993;72:757-767.
47. Rybin VO, Steinberg SF. Protein kinase C isoform expression and regulation in the developing heart. *Circ Res* 1994;74:299-309.
48. Mitchell MB, Meng X, Ao L, Brown JM, Harken AH, Banerjee A. Preconditioning of isolated rat heart is mediated by protein kinase C. *Circ Res* 1995;76:73-81.
49. Taguchi K, Erdbrügger W, Phillip T, Michel CM. Identification of protein kinase C (PKC) isoforms in the rat cardiovascular tissues (Abstract). *FASEB J* 1996;10:A683.
50. Gerardo JA, Lust RM. Expression of PKC isoforms in adult canine cardiac myocytes (Abstract). *FASEB J* 1996;10:A314.
51. Eskildsen-Helmond YEG, Gho BCG, Bezstarosti K, Dekkers DHW, Soei LK, Van Heugten HAA, Verdouw PD, Lamers JMJ. Exploration of the possible roles of phospholipase D and protein kinase C in the mechanism of preconditioning in the myocardium. In: Myocardial Preservation, Preconditioning, and Adaptation. Eds Das DK, Engelman RM, Cherian KM. *Ann NY Acad Sci*, 1996;793:210-225.
52. Ytrehus K, Liu Y, Downey JM. Preconditioning protect the ischemic rabbit heart by protein kinase C activation. *Am J Physiol* 1994;266:H1145-H1152.

53. Speechly-Dick ME, Mocanu MM, Yellon DM. Protein kinase C. Its role in ischemic preconditioning in the rat. *Circ Res* 1994;75:586-590.
54. Kloner RA, Przyklenk K, Whittaker P, Hale S. Editorial. Preconditioning stimuli and inadvertent preconditioning. *J Mol Cell Cardiol* 1995;27:743-747.
55. Tsuchida A, Liu Y, Liu GS, Cohen MV, Downey JM. α_1-Adrenergic agonists precondition rabbit ischemic myocardium independent of adenosine by direct activation of protein kinase C. *Circ Res* 1994;75:576-585.
56. Liu Y, Tsuchida A, Cohen MV, Downey JM. Pretreatment with angiotensin II activates protein kinase C and limits myocardial infarction in isolated rabbit hearts. *J Mol Cell Cardiol* 1995;27:883-892.
57. Guillemain I, Rossignol B. Protein kinase C and phospholipase D activation in rat parotid glands. *FEBS Letters* 1995;363:13-16.
58. Eskildsen-Helmond YEG, van Heugten HAA, Lamers JMJ. Regulation and functional significance of phospholipase D in myocardium. *Mol Cell Biochem* 1996;157:39-48.
59. Exton JH. Phosphatidylcholine breakdown and signal transduction. *Biochem Biophys Acta* 1994;1212:26-42.
60. Nakamura S, Nishizuka Y. Lipid mediators and PKC activation for the intracellular signalling network. *J Biochem* 1994;115:1029-1034.
61. Johnson JA, Adak S, Mochly-Rosen D. Prolonged phorbol ester treatment down-regulates protein kinase C isozymes and increases contraction rate in neonatal cardiac myocytes. *Life Sciences* 1995;57:1027-1038.
62. Cohen MV, Yang XM, Downey JM. Conscious rabbits become tolerant to multiple episodes of ischemic preconditioning. *Circ Res* 1994;74:998-1004.
63. Liu Y, Ytrehus K, Downey LM. Evidence that translocation of protein kinase C is a key event during ischemic preconditioning of rabbit myocardium. *J Mol Cell Cardiol* 1994;26:661-668.
64. Przyklenk K, Sussman MA, Simkhovich BZ, Kloner RA. Does ischemic preconditioning trigger translocation of protein kinase C in the canine model. *Circulation* 1995;92:1546-1557.
65. Speechly-Dick ME, Grover GJ, Yellon DM. Does ischemic preconditioning in the human involve protein kinase C and the ATP-dependent K^+ channel. Studies of the contractile function after simulated ischemia in an atrial in vitro model. *Circ Res* 1995;77:1030-1035.
66. Bugge E, Ytrehus K. Ischemic preconditioning is protein kinase C dependent but not through stimulation of α-adrenergic or adenosine receptors in the isolated rat heart. *Cardiovasc Res* 1995;29:401-406.
67. Liu Y, Cohen MV, Downey JM. Chelerythrine, a highly selective protein kinase C inhibitor, blocks the anti-infarct effect of ischemic preconditioning in rabbit hearts. *Cardiovasc Drugs Ther* 1994;8:881-882.
68. Li Y, Kloner RA. Does protein kinase C play a role in ischemic preconditioning in rat hearts? *Am J Physiol* 1995;268:H426-H431.
69. Vogt A, Barancik M, Weihrauch D, Arras M, Podzuweit T, Schaper W. Protein kinase C inhibitors reduce infarct size in pig hearts in vivo (Abstract). *Circulation* 1994;90(suppl. I):I-647.
70. Tamaoki T. Use and the specificity of staurosporine, UCN-01, and calphostin C as protein kinase inhibitors. *Methods Enzymol* 1991;201:340-347.
71. Kobayashi E, Nakano H, Morimoto M, Tamaoki T. Calphostin C (UCN-1028C), a novel microbial compound, is a highly potent and specific inhibitor of protein kinase C. *Biochem Biophys Res Commun* 1989;159:548-553.
72. Harding AE, Jaggar JH, Squires PE, Dunne MJ. Protein kinase C and the modulation of ATP-sensitive potassium channels in insulin-secreting cells. *Biochem Soc Trans* 1993;21:388S.
73. Harding EA, Jaggar JH, Squires PE, Dunne MJ. Polymyxin B has multiple blocking actions on the ATP-sensitive potassium channel in insulin-secreting cells. *Pflügers Arch (Eur J Phys)* 1994;426:31-39.
74. Steinberg SF, Goldberg M, Rybin VO. Protein kinase C isoform diversity in the heart. *J Mol Cell Cardiol* 1995;27:141-153.
75. Harrington EO, Ware JA. Diversity of the protein kinase C gene family. Implications for cardiovascular disease. *Trends Cardiovasc Med* 1995;5:193-198.
76. Hug H, Sarre TF. Protein kinase C isoenzymes: divergence in signal transduction? *Biochem J* 1990;291:329-343.
77. Mochly-Rosen D, Heinrich CJ, Cheever L, Khaner H, Simpson PC. A protein kinase C isoenzyme is translocated to cytoskeletal elements on activation. *Cell Regul* 1990;1:693-706.
78. Cohen MV, Liu Y, Downey JM. Activation of protein kinase C is critical to the protection of preconditioning. Wainwright CL and Parrat JR. In: Myocardial preconditioning. New York: Chapman & Hall; Texas (USA): R.G. Landes Company. 1996:185-206.
79. Schaap D, Parker PJ. Expression, purification, and characterization of protein kinase C-ϵ. *J Biol Chem* 1990;265:7301-7307.

80. Koning MMG, Simonis LAJ, De Zeeuw S, Nieukoop S, Post S, Verdouw PD. Ischaemic preconditioning by partial occlusion without intermittent reperfusion. *Cardiovasc Res* 1994;28:1146-1151.
81. Mochly-Rosen D, Khaner H, Lopez J, Smith BL. Intracellular receptors for activated protein kinase C, identification of a binding site for the enzyme. *J Biol Chem* 1991;266:14866-14868.
82. Brooks G, Walsh R, Downey J. Phosphorylation of 80K/MARCKS, a specific substrate of protein kinase C occurs after 5' ischemia but only in preconditioned hearts (Abstract). *Circulation* 1993;88(SupplI):101.
83. Dekker LV, Parker PJ. Protein kinase C - a question of specificity. *Trends Biochem Sci* 1994;19:73-77.

Chapter 8

Myocardial protein kinase C isozymes-α, -δ, -ϵ and -ζ in classic ischemic preconditioning and cardioprotection by remote organ ischemia

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Myocardial protein kinase C isozymes-α, -δ, -ϵ and -ζ in classic ischemic preconditioning and cardioprotection by remote organ ischemia

Background. We have shown in rats that not only brief coronary artery occlusion (CAO) (ischemic preconditioning) but also brief anterior mesenteric artery occlusion (MAO) limits myocardial infarct size produced by a sustained coronary artery occlusion. It has been postulated that intracellular translocation of protein kinase C following activation of receptors is involved in ischemic preconditioning. In the present study we investigated the cellular distribution of myocardial protein kinase C isozymes -α, -δ, -ϵ and -ζ in rats after brief MAO and CAO in order to investigate the possible involvement of activation of protein kinase C in the cardioprotection by brief MAO and CAO.

Methods and Results. In 3 groups of anesthetized rats left ventricular free wall was isolated after a 25-minute sham period or after 15 minutes CAO and 10 minutes reperfusion or after 15 minutes MAO and 10 minutes reperfusion. Tissue samples were processed to isolate cytosolic and membrane fractions wherein the immunoreactivities of protein kinase C-α, -δ, -ϵ and -ζ were measured by Western blotting. Total protein kinase C activity was also measured in the subcellular fractions using (γ^{32}P-)ATP and histon III-S as a substrate. Protein kinase C-α, -δ, -ϵ and -ζ immunoreactivities expressed as percentage of the chemiluminescence counts per μg of the total protein content of the cytosolic and membrane fraction of the matched control experiment tended to decrease in the cytosolic fractions of the CAO. Only the decrease in cytosolic protein kinase C-δ was significant (P=0.01). No changes of the protein kinase C-α, -δ, -ϵ and -ζ immunoreactivities in the membrane fractions were observed. In the MAO group no changes of the protein kinase C-α, -δ, -ϵ and -ζ immunoreactivities in the cytosolic as well as in the membrane fractions were observed. By histon III-S phosphorylation no changes of protein kinase C isozymes in the cytosolic fractions of the CAO and MAO were found. Moreover, Ca^{2+} and (1,2)diacylglycerol dependent activity was virtually absent in membranes.

Conclusion. On basis of the immunoreactivity measurements we conclude that protein kinase C-δ may translocate after CAO but not MAO but disappearance of its immunoreactivity from the cytosol is not accompanied by an increase of immunoreactivity in the membrane. The observed change in cytosolic protein kinase C-δ immunoreactivity in CAO did also not lead to changes in protein kinase C activity measured by histon III-S phosphorylation.

Key Words. myocardial infarct size, signal transduction, protein kinase C, ischemic preconditioning, intestinal ischemia, remote cardioprotection

Introduction

Exposure to brief periods of acute myocardial ischemia protects the heart against infarction during a subsequent longer episode of ischemia [1, 2]. This phenomenon (ischemic preconditioning) is neither a tissue specific phenomenon as it has been described not only for the myocardium, but also for the kidney [3], brain [4], liver [5], skeletal muscle [6], lung [7] and intestine [8] nor a species specific phenomenon as it has been demonstrated in i.e. the rat [9], rabbit [10], dog [1] and pig [11]. Furthermore there are indications that ischemic preconditioning also can be elicited in the human heart during angina pectoris preceding an acute myocardial infarction and percutaneous transluminal coronary angioplasty [12, 13]. In our previous study we have shown that a brief period of mesenteric artery occlusion and reperfusion (MAO) also protects the myocardium [14]. Thus, ischemic preconditioning may be an intrinsic protective property occurring within each cell type but the mechanism leading to this phenomenon does not necessarily have to be the same in each cell type. For instance, we observed that MAO-induced cardioprotection can be but the CAO-induced cardioprotection cannot be blocked at a neurogenic level[14]. However, MAO- and CAO-induced cardioprotection may still have a common endpoint, e.g. activation of protein kinase C isozyme(s). Despite the numerous efforts to elucidate the molecular mechanism(s) by which transient ischemia protect(s) the myocardium the mechanism of ischemic preconditioning remains incompletely understood. Advanced hypotheses point toward a role for activation of adenosine [15, 16] or activation of K^+_{ATP} receptor channels [17, 18]. More recent studies suggest that endogenous ligands such as adenosine-A_1, α_1-adrenergic and muscarinic agonists, bradykinin, angiotensin II, endothelin-1 [19, 20] and opioids [21] initiate an intracellular signalling pathway by acting via G-protein-coupled receptors, which leads to activation of phospholipase C-β [19, 22-27]. Currently there is some evidence suggesting the involvement of phospholipase D in addition to phospholipase C in the production of (1,2)diacylglycerol ((1,2)DAG) [28]. Activation of phospholipase C-β (and D) increases the levels of the second messenger inositol-1,4,5-triphosphate (Ins(1,4,5)P_3) which mobilizes intracellular Ca^{2+} and 1,2-diacylglycerol ((1,2)DAG) which activates protein kinase C isozymes [19]. These messengers can act synergistically by activation of the Ca^{2+}-dependent, (1,2)DAG stimulated protein kinase C isozymes and Ca^{2+}-calmodulin dependent protein kinase (CaM-PK). Activated protein kinase C isozymes and/or CaM-PK then phosphorylate specific proteins that ultimately lead to cardioprotection. In line with this hypothesis, the rate of dephosphorylation by phosphoprotein phosphatases of the specific phosphoproteins involved in preconditioning, should determine the period in which the protective state is maintained. The protective state of the myocardium appears to occur in two stages. The first window of protection (FWOP) occurs immediately after the preconditioning stimulus has been applied and lasts about 2 hours [1, 29]. A second window of protection (SWOP) appears approximately 24 hours after applying the stimulus [30, 31] and has weaned off after 3 days [32]. It is most likely that FWOP is based upon

changes in protein kinase C induced phosphorylation of specific proteins. The protective state expresses itself by a delay in the development of infarction[1]. Thus early reperfusion after a prolonged coronary artery occlusion remains a necessity to effectively limit infarct size after ischemic preconditioning. Consistent with the protein kinase C hypothesis is the assumption that all known stimuli which mimic the protective state of preconditioning converge into the activation of specific isozymes of the protein kinase C family via receptor-mediated activation of phospholipase C-β or -D [33, 34]. K^+_{ATP} -channels [17,18] and ecto-5'-nucleotidase [35] has already been proposed as target proteins of protein kinase C. Long-term protein kinase C stimulation is known to down regulate the enzyme providing a possible mechanism of tolerance development to continuous stimulation [36].

Unfortunately, the pattern of translocation or activation of protein kinase C and the protein kinase C isozymes has only been investigated by immunohistochemistry in the rat after α_1-adrenergic stimulation [25]. In this study we measured the (immunore)activities of protein kinase C isozymes of subcellular fractions in the cardioprotection in rats obtained by brief anterior mesenteric artery occlusion and classic ischemic preconditioning in order to elucidate the underlying (different) mechanisms, or speaking in terms of Brooks and Hearse [37] in order to resolve the question whether protein kinase C is a player or spectator in ischemic preconditioning?

Materials and 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. Ad libitum fed male Wistar rats (± 300 g, TNO Zeist, The Netherlands) were used in all experiments.

Surgical procedures

The in situ rat heart model for ischemia and reperfusion and remote cardioprotection has been extensively described in our previous study [14]. Briefly, rats were anesthetized with pentobarbital (60 mg/kg intra peritoneal) and intubated for positive pressure ventilation (Harvard) with room air. A PE-10 catheter was positioned in the thoracic aorta via the right carotid artery for measurement of arterial blood pressure and heart rate (Baxter Diagnostic Inc.). A PE-50 catheter was positioned in the inferior caval vein via the left femoral vein for infusion of Haemaccell (Behring Pharma). After intercostal thoracotomy the pericardium was opened and a silk (6-0) suture was looped under the left anterior descending coronary artery (LADCA) for sham or later production of a coronary artery occlusion [38, 39]. Following laparotomy, a catheter was positioned in the abdominal cavity to allow intra peritoneal infusions of pentobarbital for maintenance of anaesthesia. Subsequently the anterior mesenteric artery was dissected free and

a suture was placed around the artery for sham operation or to facilitate later a mesenteric artery occlusion with an atraumatic clamp. After the ischemic stimulus was applied, the abdomen was closed. The sham operated rats (Sham), as well the MAO rats as the CAO rats which underwent the classical ischemic preconditioning protocol were subjected to the same procedures. Body core temperature was continuously measured rectally using an electric thermometer (Electromedics Inc.) Temperatures were maintained at 36.5-37.5°C using a coverage of the rat with aluminum foil and heating pads. When rats encountered ventricular fibrillation during ischemia or reperfusion, they were allowed to complete the experimental protocol when conversion of ventricular fibrillation occurred spontaneously within 1 minute or when resuscitation by gentle thumping on the thorax, was successful within 2 minutes after the onset of fibrillation. Occlusion as well as reperfusion were visually verified by appearance and disappearance of myocardial or small intestinal cyanosis.

Experimental groups

Left ventricular free wall of anesthetized rats was isolated after a 25-minute sham period in the Sham group (n=10) or after 15 minutes CAO and 10 minutes reperfusion in the CAO group (n=10) or after 15 minutes MAO and 10 minutes reperfusion in the MAO group (n=10) (Figure 1).

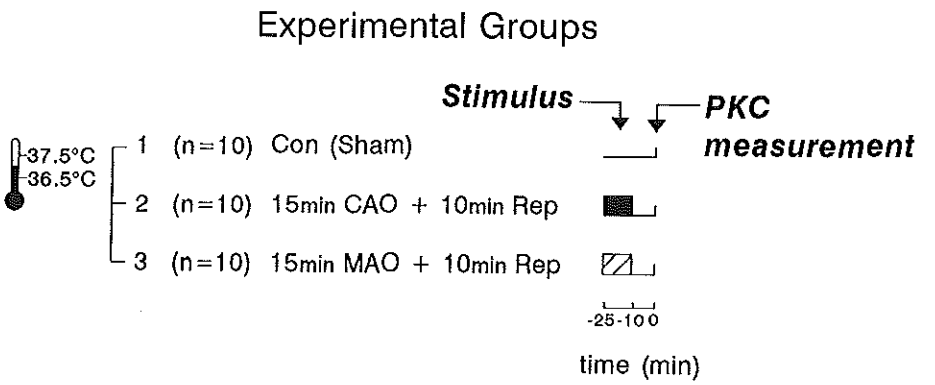

Figure 1. Schematic presentation of the 3 experimental groups in which the left ventricular free wall was isolated for protein kinase C analysis (immunoreactivity and enzyme activity). PKC=protein kinase C; Sham=control; CAO=left anterior descending coronary artery occlusion (closed bars); MAO=anterior mesenteric artery occlusion (hatched bars); Rep=reperfusion.

Protein kinase C-α, -δ, -ϵ and -ζ immuno analysis

Frozen left ventricular tissue samples (5-10 mg wet weight) of 8 animals of each experimental group were pulverized (Braun Mikro Dismembrator) under liquid N_2 temperature

in 200 µl homogenization buffer containing 25 mM Tris/HCl (pH 7.4), 5 mM EGTA, 2 mM EDTA and 5 mM dithiothreitol (DTT), thawed and subsequently centrifuged for 20 minutes at 19,000 rpm in a JA 20 rotor (Beckman Model J2-21 Centrifuge) at 4°C and the obtained supernatants (cytosolic fractions) were stored frozen (-80°C) for the ultimate protein kinase C (immunore)activity measurements. The sediments were resuspended in 200 µl homogenizing buffer containing 1% (v/v) Triton-X-100. Next, the suspensions were incubated for 60 minutes on ice and centrifuged again as above. The supernatants containing the solubilized membranes (membrane fractions) were used for protein kinase C analysis and the sediment discarded. Protein levels in 15 µl of the cytosolic and solubilized membrane fractions were determined using the standard Bradford procedure. Protein kinase C-α, -δ, -ϵ and -ζ of the cytosolic and solubilized membrane fractions were separated on sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAAGE) and detected by immunoblotting following the procedure for protean II mini gel set up of Biorad with a 10% gel (Western-blot) and detection of the immunoreactivity by the ECL™ kit (Supersignal™ CL-HRP Substrate System GS-363 from Biorad (USA)) on the CL-screen in the Molecular Imager (GS-363) [28]. In each gel that was run, samples of the cytosolic as well as membrane fraction of one experiment of the Sham (control), CAO and MAO group were applied so that relative chemiluminescence data were normalized to the total protein content of the cytosolic and membrane fractions and these could all be expressed as percentage of the counts of the cytosolic and membrane samples of the Sham group.

Protein kinase C activity measurements by histon III-S phosphorylation

In 7 animals of each experimental group protein kinase C was assayed in both the cytosolic and membrane fractions. The reaction medium for the basal activity of protein kinase C contained 10 µl of the reaction medium without Ca^{2+} (100 mM Tris/HCl (pH 7.5) and 25 mM $MgCl_2$), 5 µl of 100 mM β-mercaptoethanol, 1 µl of 0.05% (w/v) histon III-S, 2.5 µl of 4 µM phosphoprotein phosphatase inhibitor okadaic acid, 21.5 µl water, 5 µl of 100 µM γ^{32}P-ATP (50-100 cpm/pmol) and 5 µl tissue sample. The reaction medium for the Ca^{2+}, phosphatidylserine (PtdSer) and (1,2)DAG dependent activity of protein kinase C contained 10 µl of the reaction medium with Ca^{2+} (100 mM Tris/HCl (pH 7.5), 25 mM $MgCl_2$, and 2.5 mM $CaCl_2$), 5 µl of 100 mM β-mercaptoethanol, 5 µl of an ultrasonified mixture of 0.016% (w/v) phosphatidylserine (PtdSer) and 0.004% (w/v) (1,2)DAG, 1 µl of 0.05% (w/v) histon III-S, 2.5 µl of 4 µM okadaic acid, 16.5 µl water, 5 µl of 100 µM γ^{32}P-ATP (50-100 cpm/pmol) and 5 µl tissue sample. The assay mixtures were preincubated at 30°C for 2 minutes and the reactions were started by adding 5 µl of the tissue samples and stopped after 5 minutes by spotting 25 µl reaction mixture directly on Whatman P81 paper. Protein kinase C activity was measured as ^{32}P incorporation using the Molecular Phosphor Imager (GS-363) [28]. The activity was then expressed as pmol ^{32}P per min per µg protein.

It should be noted that protein kinase C isozyme (immunore)activity distribution was only determined in cytosolic and solubilized membrane fractions. After solubilization of the membranes a particulate fraction which likely contains cell organelles such as mitochondria, myofibrils and nuclei. We were not able to measure (immunore)activity of protein kinase C isozymes accurately in this particulated fractions. The reason was the low activities and the inhomogeneity of the particulated suspensions.

Data analysis

Comparisons between groups were analyzed using ANOVA and a post hoc Student - t - test. Results were described as mean±SEM and a P-value <0.05 was considered significant.

Results

Hemodynamic measurements

Heart rate and mean arterial blood pressure

Under normothermic (36.5-37.5°C) baseline conditions heart rate and mean arterial blood pressure were not significantly different between the Sham (control), CAO and MAO groups (Table 1). Heart rate and mean arterial bloodpressure did not change during the 15-minute CAO and MAO and the subsequent 10 minutes of reperfusion.

Table 1. Systemic hemodynamics in rats during the intervention of Sham, CAO and MAO

	BL	Organ Ischemia (15 min)	Organ Rep (10 min)
Sham (n=7)			
HR	373±7	370±9	364±6
MAP	98±6	95±7	88±6
15 min CAO + 10 min Rep (n=7)			
HR	369±11	377±8	373±12
MAP	93±7	88±8	83±8
15 min MAO + 10 min Rep (n=8)			
HR	369±8	363±8	356±8
MAP	98±7	109±5	95±6

BL=baseline; Rep=reperfusion; CAO=coronary artery occlusion; Sham=control group without ischemia stimulus; HR=heart rate (beats/min); MAP=mean arterial blood pressure (mmHg); MAO=mesenteric artery occlusion. There were no significant differences between the preconditioning groups (CAO and MAO) and the corresponding sham group for both heart rate and mean aortic pressure. All data are mean ± SEM.

Protein kinase C isozyme measurements by immunoreactivity

Protein kinase C isozymes -α, -δ, -ϵ and -ζ were measured by immunoreactivity. In the CAO group immunoreactivity of the cytosolic and the membrane fractions of protein kinase C isozymes -α, -δ, and -ϵ tended to decrease, and -ζ tended to increase. However, only the decrease of protein kinase C isozyme-δ immunoreactivity in the cytosolic fraction is significantly different (P=0.01 and P=0.001 respectively) (table 2). In addition, cytosolic protein kinase C-δ decreased in the MAO group but this was not significant (P=0.11 and P=0.067, respectively). Protein kinase C-α, -δ, -ϵ and -ζ immunoreactivity in the membrane fractions remained unchanged. The ratio membrane over cytosolic protein kinase C isozymes-α, -δ, -ϵ and -ζ also did not show significant differences between the 3 groups. On the basis of these measurements it may be concluded that protein kinase C-δ translocates/disappears from the cytosol but the disappearance of immunoreactivity is not accompanied by an increase in the membrane fraction after a CAO period.

Protein kinase C activity measurements by histon III-S phosphorylation

No evidence of translocation of protein kinase C activity by histon III-S phosphorylation from the cytosol to the membrane was found (table 3). Moreover, Ca^{2+}- and (1,2)DAG dependent activity was virtually absent in the membrane fractions, which was rather unexpected based upon the immunoreactivity measurements. Furthermore, the observed changes in immunoreactivity of the cytosolic protein kinase C-δ in CAO did not lead to changes in protein kinase C activity by histon III-S phosphorylation in the cytosolic fractions.

Discussion

Protein kinase C, first identified in the brain [40-43], is present in all organs and some of its functions in the myocardium have now been recognized [19, 44]. In myocardial cells, protein kinase C regulates processes such as contractility, ion channel function, energy metabolism, specific gene expression and hypertrophic cell growth [45]. The specificity of action of the protein kinase isozymes most likely depends on their intracellular location. The translocation is guided by the architecture and intracellular localization of anchor proteins, the so-called receptors for activated C kinase (RACKs) [46, 47]. After activation, protein kinase C isozymes translocate to other cellular compartments such as the sarcolemma where they may have their actions. Mitochondria, myofibrils, the sarcoplasmatic reticulum or the perinuclear zone are other possible action sites. The translocation process may occur via the cytoskeleton or by active transport directed by microtubuli [48].

The protein kinase C isozymes can be divided into 3 subgroups. The classical protein kinase C-α is activated by phosphatidylserine (PtdSer), Ca^{2+} and (1,2)DAG. The novel protein kinase isozymes C-δ and -ϵ require PtdSer and (1,2)DAG for their activation but are Ca^{2+} independent

Table 2. Protein kinase C isozymes (α, δ, ϵ and ζ) in cytosolic and membrane fractions and the ratio measured by immunoreactivity.

	PKC-α			PKC-δ			PKC-ϵ			PKC-ζ		
Groups	Cytosol	Membrane	Ratio	Cytosol	Membrane	Ratio	Cytosol	Membrane	Ratio	Cytosol	Membrane	Ratio
Sham (n=8)	88±6	12±3	12.0±3.4	51±6	49±6	1.2±0.3	76±8	24±8	6.0±2.9	86±6	14±6	3.5±0.7
CAO (n=8)	77±24	10±2	10.9±2.5	21±4*	33±13	1.0±0.3	49±29	26±14	2.8±1.4	59±29	77±42	4.8±4.5
MAO (n=8)	95±23	12±3	10.8±2.9	34±6	42±9	1.0±0.2	72±14	41±12	2.1±0.4	68±31	11±4	3.2±1.6

PKC=protein kinase C. Values are mean chemiluminescence counts (immunoreactivity) as percent of Sham (Control) ± SEM, * P<0.05 vs Sham.

Table 3. Protein kinase C activity (basal and Ca^{2+}+(1,2) diacylglycerol (DAG) activated) in cytosolic and membrane fractions measured as histon III-s phosphorylation.

	Cytosol		Membrane	
Groups	Basal	Ca^{2+}+(1,2) DAG	Basal	Ca^{2+}+(1,2) DAG
Sham (n=7)	35±5	30±8	42±7	-9±5
CAO (n=7)	35±7	49±12	35±4	-2±2
MAO (n=8)	37±7	35±7	37±5	-1±8

Values are mean pmol ^{32}P/min/μg protein ± SEM (histon III-S phosphorylation).

and the atypical protein kinase C-ζ only need PtdSer, but no Ca^{2+} and (1,2)DAG. Weinbrenner *et al* [49] showed that protein kinase C-α, -δ, -ϵ and -ζ are the most prominent isoforms in the rat heart which were also found in cultured neonatal [53] and adult rat cardiomyocytes [54]. Weinbrenner *et al* [49] also showed a rapid translocation of the Ca^{2+} independent protein kinase C isozymes -δ, -ϵ and -ζ and of the Ca^{2+} dependent protein kinase C-α to the membrane occurring after a brief ischemic period. Prolongation of the ischemic period led to increased expression of Ca^{2+} independent forms of protein kinase C-δ and -ϵ in the cytosol. Thus, in these studies it appears that distribution of protein kinase C isozymes-α, -δ, -ϵ and -ζ was altered by brief ischemia. On the basis of the previously reported presence of these isozymes in the rat we have chosen to investigate myocardial protein kinase C isozymes-α, -δ, -ϵ and -ζ in classic ischemic preconditioning and cardioprotection by remote organ ischemia. For translocation measurements of protein kinase C isozymes from cytosol to membrane we have used two methods, both immunoblot analysis using protein kinase C isozyme-specific antibodies [28, 49, 50] and the assay of protein kinase C activity by Ca^{2+} and/or (1,2)DAG-dependent ^{32}P incorporation from $\gamma^{32}P$ labelled ATP into histon III-S [22, 28, 51, 52].

On the basis of our immunoreactivity measurements we conclude that the cytosolic immunoreactivity of protein kinase C isozyme-δ in the CAO group was decreased but that the decrease was not accompanied by a rise in immunoreactivity in the membrane fraction. In addition it was also shown that the total immunoreactivity of the cytosolic and the membrane fraction is lower than in the Sham group. This could mean that a small part of protein kinase C-δ is translocated e.g. to the myofibrils, mitochondria and the perinucleus in which protein kinase C (immunore)activity could not accurately be measured (See Materials and Methods). A part of the activated (translocated) enzyme molecules in the membrane could also disappeared due to degradation. Although cytosolic protein kinase C-δ decreased and so could first have been translocated (activated) by the ischemic preconditioning stimulus, the (in)direct involvement of this isozyme in the eventually protective effect is not proven by this finding. For the latter purpose it should become possible to specifically inhibit protein kinase C-δ. At present no method is available. In the MAO group protein kinase C-δ only tended to disappear from the cytosolic fraction. We therefore tempt to conclude that protein kinase C isozymes-α, -δ, -ϵ and -ζ are not involved in the mechanism of the cardioprotective effect of MAO. In the latter the cardioprotection could involve another pathway, which could directly interact with the common protective pathway of ischemic preconditioning beyond the protein kinase C cascade or could be a totally different pathway besides the one of ischemic preconditioning leading to protection.

The basal protein kinase C activity and the maximum excitable activity in the presence of Ca^{2+}, PtdSer and (1,2)DAG with histon III-S as substrate were measured in the cellular subfractions. The protein kinase C activity measurements did not show any differences between the Sham, CAO and the MAO group. Thus, at least the observed changes in protein kinase C-δ

by immunoreactivity in CAO have not led to changes in total protein kinase C activity by histon III-S phosphorylation. The δ isozyme is not dependent on Ca^{2+} but even the Ca^{2+} and (1,2)DAG independent activities were showing no differences. It should be noted that the immunoreactivity measurements are reflecting the individual protein kinase C isozymes while the activity measurements are a summation of the activities of all protein kinase C isozymes together, a part being increased while another part being constant or decreased. It was also remarkable that there was virtually no Ca^{2+} dependent activity in the membrane fractions, whereas the presence of the Ca^{2+} dependent isozyme α in this fraction was shown by immunoreactivity.

Methodological considerations.

The methods used for protein kinase C analysis may be limited in establishing which of the protein kinase C isozymes are translocated and definitively activated. Redistribution or inactivation (proteolysis) could have occurred during the preparation of subfractions and analysis of protein kinase C (immunore-)activity [28]. It is also possible that the samples taken from the myocardium for measuring the activity were heterogenous in being preconditioned or not. The problem of the protein kinase C isozymes (immunore)activity measurement in the particulated fraction containing myofibrils, mitochodria, nuclei etc. could not be solved at this time. The material can be disolved at high ionic strength but the effect of this treatment on protein kinase C activity is not known. The interpretation of the immunoreactivity data of the protein kinase C isozymes applied on tissue fractions may be very preliminar, because generally spoken the antibodies do surely not distinguish between inactive and active form enzymes. (For further details see chapter 8)

Conclusion

After brief CAO cytosolic protein kinase C-δ decreased. Thus, the cytosolic protein kinase C-δ may be translocated to an unknown subcompartment, and was at least not kept in the membrane fraction. This could mean that it is translocated to the myofibrils, mitochondria and/or the perinucleus. It may also be decreased due to partial degradation of the activated enzyme. Although protein kinase C-δ was decreased in the cytosol by the ischemic preconditioning stimulus, its involvement in the eventually protective effect is far from proven. Specific inhibition of this isozyme is needed. Unfortunately, at present this is technically not possible. In the MAO group no alterations of protein kinase C-δ were found to occur in this form of cardioprotection. Therefore, we conclude that protein kinase C-δ may be involved in either ischemic preconditioning and that the cardioprotective effect of MAO likely involves another pathway, which could directly interact with the common protective pathway of ischemic preconditioning beyond the protein kinase C cascade or could be a totally different pathway besides the one of ischemic preconditioning leading to protection.

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References

1. Murry CE, Jennings RB, Reimer KA. Preconditioning with ischemia: a delay of lethal cell injury in ischemic myocardium. *Circulation* 1986;75:1124-1136.
2. Lawson CS, Downey JM. Preconditioning: state of the art myocardial protection. *Cardiovasc Res* 1993;27:542-550.
3. Zager RA, Baltes LA, Sharma HM, Jurkowitz MS. Responses of the ischemic acute renal failure kidney to additional ischemic events. *Kidney Int* 1984;26:689-700.
4. Kitagawa K, Matsumoto M, Tagaya M, Hata R, Ueda H, Niinobe M, Handa N, Fukunaga R, Kimura K, Mikoshiba K, Kamada T. 'Ischemic tolerance' phenomenon found in the brain. *Brain Res* 1990;528:21-24.
5. Lloris-Carsi JM, Cejalvo D, Toledo-Pereyra LH, Calvo MA, Suzuki S. Preconditioning: effect upon lesion modulation in warm liver ischemia. *Transplant Proc* 1993;25:3303-3304.
6. Mounsey RA, Pang CY, Forrest C. Preconditioning: a new technique for improved muscle flap survival. *Otolaryngology-Head Neck Surg* 1992;107:549-552.
7. Neely CF, Keith IM. A_1 adenosine receptor antagonists block ischemia-reperfusion injury of the lung. *Am J Physiol* 1995;268:L1036-L1046.
8. Hotter G, Closa D, Prados M, Fernández-Cruz L, Prats N, Gelpí E, Roselló-Catafau J. Intestinal preconditioning is mediated by a transient increase in nitric oxide. *Biochem Biophys Res Commun* 1996;222:27-32.
9. Liu Y, Downey JM. Ischemic preconditioning protects against infarction in the rat heart. *Am J Physiol* 1992;263:H1107-H1112.
10. Cohen MV, Lui GS, Downey JM, Preconditioning causes improved wall motions as well as smaller infarcts after transient coronary occlusion in rabbits. *Circulation* 1991;84:341-349.
11. Schott RJ, Rohmann S, Braun ER, Schaper W. Ischemic preconditioning reduces infarct size in swine myocardium. *Circ Res* 1990;66:1133-1142.
12. Verdouw PD, Gho BCG, Duncker DJ. Ischemic preconditioning: is it clinically relevant? *Eur Heart J* 1995;16:1169-1176.
13. Kloner RA, Shook T, Antman EM, Cannon CP, Przyklenk K, McCabe CH, Braunwald E, TIMI 9 Investigators. A prospective analysis on the potential preconditioning effect of preinfarct angina in TIMI 9. *Circulation* 1996;94:I-611 (abstract).
14. Gho BCG, Van den Doel MA, Schoemaker RG, Duncker DJ, Verdouw PD. Myocardial protection by transient ischemia in non-cardiac tissue. *Circulation* 1996;47:1193-2200.
15. Thornton J, Liu GS, Olsson RA, Downey JM. Intravenous pretreatment with A1-selective adenosine analogues protects the heart against infarction. *Circulation* 1992;85:659-665.
16. Toombs CF, McGee DS, Johnston WE, Vinten-Johanson J. Myocardial protective effects of adenosine: infarct size reduction with pretreatment and continued receptor stimulation during ischemia. *Circulation* 1992;86:986-994.
17. Rohmann S, Weygandt H, Schelling P, Soei LK, Verdouw PD, Lues I. Involvement of ATP-sensitive potassium channels in preconditioning protection. *Basic Res Cardiol* 1994;89:563-576.
18. Gross GJ, Auchampach JA. Blockade of ATP-sensitive potassium channels prevents myocardial preconditioning in dogs. *Circ Res* 1992;70:223-233.
19. De Jonge HW, Van Heugten HAA, Lamers JMJ. Review. Signaltransduction by the phosphatidylinositol cycle in myocardium. *J Mol Cell Cardiol* 1995;27:93-106.
20. Eskildsen-Helmond YEG, van Heugten HAA, Lamers JMJ. Regulation and functional significance of phospholipase D in myocardium. *Mol Cell Biochem* 1996;157:39-48.
21. Van Heugten HAA, Eskildsen-Helmond YEG, De Jonge HW, Bezstarosti K, Lamers JMJ. Phosphoinositide-generated messengers in cardiac signal transduction. *Mol Cell Biochem* 1996;157:5-14.
22. Kloner RA, Przyklenk K, Whittaker P, Hale S. Preconditioning stimuli and inadvertent preconditioning. *J Mol Cell Cardiol* 1995;27:743-747.
23. Tsuchida A, Liu Y, Liu GS, Cohen MV, Downey JM. α_1-Adrenergic agonists precondition rabbit ischemic myocardium independent of adenosine by direct activation of protein kinase C. *Circ Res* 1994;75:576-585.
24. Liu Y, Tsuchida A, Cohen MV, Downey JM. Pretreatment with angiotensin II activates protein kinase C and limits myocardial infarction in isolated rabbit hearts. *J Mol Cell Cardiol* 1995;27:883-892.
25. Mitchell MB, Meng X, Ao L, Brown JM, Harken AH, Banerjee A. Preconditioning of isolated rat heart is mediated by protein kinase C. *Circ Res* 1995;76:73-81.

26. Ytrehus K, Liu Y, Downey JM. Preconditioning protect the ischemic rabbit heart by protein kinase C activation. *Am J Physiol* 1994;266:H1145-H1152.
27. Speechly-Dick ME, Mocanu MM, Yellon DM. Protein kinase C. Its role in ischemic preconditioning in the rat. *Circ Res* 1994;75:586-590.
28. Eskildsen-Helmond YEG, Gho BCG, Bezstarosti K, Dekkers DHW, Soei LK, Van Heugten HAA, Verdouw PD, Lamers JMJ. Exploration of the possible roles of phospholipase D and protein kinase C in the mechanism of preconditioning in the myocardium. In:Myocardial Preservation, Preconditioning, and Adaptation. Eds: Das DK, Engelman RM, Cherian KM. *Ann N Y Acad Sci* 1996;793:210-225.
29. Li Y, Whittaker P, Kloner RA. The transient nature of the effect of ischemic preconditioning on myocardial infarct size and ventricular arrhythmia. *Am Heart J* 1992;123:346-353.
30. Kuzuya T, Hoshida S, Yamashita N, Fuji H, Oe H, Hori M, Kamada T, Tada. Delayed effects of sublethal ischemia on the acquisition of tolerance to ischemia. *Circ Res* 1993;72:1293-1299.
31. Marber MS, Latchman DS, Walker M, Yellon DM. Cardiac stress protein elevation 24 hours after brief ischemia or heat stress is associated with resistance to myocardial infarction. *Circulation* 1993;88:1264-1272.
32. Baxter GF, Goma FM, Yellon DM. Duration of the "second window of protection" following ischaemic preconditioning in the rabbit. *J Mol Cell Cardiol* 1995;27:A162 (abstract).
33. Downey JM, Cohen MV, Ytrehus K, Liu Y. Cellular mechanisms in ischemic preconditioning: The role of adenosine and protein kinase C. In: Cellular, Biochemical, and Molecular Aspects of Reperfusion Injury. Ed: Das DK; *Ann NY Acad Sci* 1994;723:882-898.
34. Gho BCG, Eskildsen-Helmond YEG, Lamers JMJ, Verdouw PD. Does potein kinase C play a pivotal role in the mechanisms of ischemic preconditioning? *Cardiovasc Drugs Ther* 1996 (in press)
35. Kitakaze M, Node K, Minamino T, Komamura K, Funaya H, Shinozaki Y, Chujo M, Mori H, Inoue M, Hori M, Kamada T. Role of activation of protein kinase C in the infarct size-limiting effect of ischemic preconditioning through activation of ecto-5'-nucleotidase. *Circulation* 1996;93:781-791.
36. Johnson JA, Adak S, Mochly-Rosen D. Prolonged phorbolester treatment down-regulates protein kinase C isozymes and increases contraction rate in neonatal cardiac myocytes. *Life Sciences* 1995;57:1027-1038.
37. Brooks G, Hearse DJ. Role of protein kinase C in ischemic preconditioning: player or spectator? *Circ Res* 1996;79:627-630.
38. Fishbein MC, MacLean D, Marokko PR. Experimental myocardial infarction in the rat. *Am J Pathol* 1978;90:57-70.
39. Schoemaker RG, Urquhart J, Debets JJM, Struyker-Boudier HAJ, Smits JFM. Acute haemodynamic effects of coronary artery ligation in conscious rats. *Basic Res Cardiol* 1990;85:9-20.
40. Nishizuka Y. Intracellular signalling by hydrolysis of phospholipids and activation of protein kinase C. *Science* 1992;258:607-614.
41. Hug H, Sarre TF. Protein kinase C isozymes: divergence in signal transduction. *Biochem J* 1993;291:329-343.
42. Dekker LV, Parker PJ. Protein kinase C - a question of specificity. *Trends Biochem Sci* 1994;19:73-77.
43. Azzi A, Boscoboinik D, Hensey C. The protein kinase C family. *Eur J Biochem* 1992;208:547-557.
44. Pucéat M, Brown JH. Protein kinase C in the heart. In: Kuo JF, ed. Protein kinase C. London: Oxford University Press 1994;249-268.
45. Sugden PH, Bogoyevitch MA. Intracellular signalling through protein kinases in the heart. *Cardiovasc Res* 1995;30:478-492.
46. Mochly-Rosen D, Khaner H, Lopez J. Identification of intracellular receptor proteins for activated protein kinase C. *Proc Natl Acad Sci USA* 1991;88:3997-4000.
47. Ron D, Mochly-Rosen D. An autoregulatory region in protein kinase C: the pseudoanchoring site. *Proc Natl Acad Sci USA* 1995;92:492-496.
48. Pears C, Stabel S, Cauzabon S, Parker P. Studies on the phosphorylation of protein kinase C-α. *Biochem J* 1992;283:515-518.
49. Weinbrenner CE, Simonis G, Marquetant R, Strasser RH. Selective regulation of calcium-dependent and calcium-independent subtypes of protein kinase C in acute and prolonged myocardial ischemia. *Circulation* 1993;88(Suppl 1):I-101 (Abstract).
50. Vogt A, Barancik M, Weihrauch D, Arras M, Podzuweit T, Schaper W. Protein kinase C inhibitors reduce infarct size in pig hearts in vivo. *Circulation* 1994;90(suppl. I):I-647 (Abstract).
51. Przyklenk K, Sussman MA, Simkhovich BZ, Kloner RA. Does ischemic preconditioning trigger translocation of protein kinase C in the canine model? *Circulation* 1995;92:1546-1557.

52. Schaap D, Parker PJ. Expression, purification, and characterization of protein kinase C-ϵ. *J Biol Chem* 1990;265:7301-7307.
53. Lamers JMJ, Eskildsen-Helmond YEG, Resink AM, De Jonge HW, Bezstarosti K, Sharma HS, Van Heugten HAA. Endothelin-1-induced phospholipase C-ß and D and protein kinase C isoenzyme signalling leading to hypertrophy in rat cardiomyocytes. *J Cardiovasc Pharmacol* 1995;26(suppl. 3):S100-S103.
54. Bogoyevitch MA, Parker PJ, Sugden PH. Characterization of protein kinase C isotype expression in adult rat heart. *Circ Res* 1993;72:757-767.

Chapter 9

Summary, general discussion and future perspectives

The original observation by Murry *et al* [1] that in dogs myocardial infarct size produced by a 45-minute coronary artery occlusion is reduced from 29% to 7% of the area at risk when the 45-minute occlusion is preceded by four sequences of 5-minute occlusions and 5 minutes of reperfusion initiated numerous efforts for the search of the mechanism of cardioprotection by ischemic preconditioning and the description of the experimental conditions under which cardioprotection can be demonstrated. Major conclusions of these studies are that (i) ischemic preconditioning has been demonstrated in every species, including rat, rabbit and swine, in which the phenomenon has been investigated [2], (ii) the phenomenon is not restricted to the heart but also occurs in other organs such as kidney [3], skeletal muscle [4], brain [5], liver [6], lung [7] and the intestine [8], although in these organs other endpoints than infarct size limitation have been used and (iii) the molecular mechanism leading to cardioprotection is still not fully understood although several hypotheses have been forwarded based on endogenous production of adenosine, activation of K^+_{ATP} channels and/or protein kinase C activation [9, 10].

During the last number of years we have investigated cardioprotection in a number of animal models in order to mimic the clinical situation more closely and to investigate whether stimuli which do not lead to myocardial ischemia can also protect the myocardium [11-17]. More specifically, we investigated whether partial coronary artery occlusions without intervening reperfusion, rapid ventricular pacing and brief ischemia in other organs alter myocardial infarct size produced by a prolonged coronary artery occlusion. Clinical parallels to the latter is stenosis of the renal artery or chronic reduced arterial circulation to the lower extremities (claudication). Furthermore the possible involvement of protein kinase C in ischemic preconditioning and remote cardioprotection was investigated. Experiments were performed in anesthetized pigs and rats, in which area at risk and infarct size were determined using established techniques. Pigs were used in those studies in which we also studied regional myocardial function and metabolism in detail.

Ischemic preconditioning by partial coronary artery occlusion without intervening reperfusion

In chapter 3 we addressed the possibility to precondition myocardium with partial occlusions without an intervening reperfusion period. This model is similar to the two-stage occlusion model used by Harris [18]. By occluding a coronary artery partially for 30 minutes prior to a complete occlusion, Harris showed that the incidence of ventricular fibrillation was markedly reduced. We hypothesized that the reduction in ventricular fibrillation after the coronary artery was completely occluded might be the consequence of a preconditioning effect during the partial occlusion [12, 13]. In the clinical setting a brief abrupt total coronary artery occlusion is usually not followed by complete reperfusion before that artery is occluded for a sustained period and an infarction develops. If stringent conditions, such as abrupt brief total coronary artery occlusion

and intervening complete reperfusion, would be required to precondition the myocardium, it is very unlikely that ischemic preconditioning has a clinical analog (chapter 2). Several groups of investigators have investigated the requirements of brief total ischemia and complete reperfusion and shown that myocardium can also be protected by moderate partial coronary artery occlusions in the presence of adrenergic stimulation (demand ischemia) or endothelial injury [19, 20]. Furthermore, Ovize *et al* [21] showed that a sufficiently severe coronary artery occlusion can precondition the myocardium, provided that a period of complete reperfusion separated the partial and total occlusions. From their study this group of investigators concluded that intervening complete reperfusion is a prerequisite of ischemic preconditioning by a partial occlusion. We have challenged the generalization of this conclusion because it is based on only one degree of flow reduction. We hypothesized that partial occlusions might be able to precondition myocardium and limit infarct size during a subsequent complete occlusion without an intervening reperfusion period, but that the severity and the duration of the partial coronary occlusions could play a role. We have investigated this hypothesis in open-chest anesthetized pigs which were instrumented for measurement of global and regional myocardial performance (chapter 3, figure 1).

In this study we revealed that flow reductions of 70% but not of 30% were capable to reduce infarct size (chapter 3, figures 3 and 4). Because a flow reduction will affect perfusion of the inner half of the myocardium more severely than the outer half we hypothesized that the degree of protection could be different for the inner (endocardial) and outer (epicardial) halves of the myocardium. Analysis of our results showed that protection with the 70% flow reduction was more pronounced in the epicardial than in the endocardial half. Based on earlier observation with radioactive microspheres, we may assume that with the 30% flow reduction, the endocardium had at least become as ischemic as the epicardial half with the 70% flow reductions. Nevertheless we did not observe any protection in the endocardial half with the 30% flow reductions. The reason is yet unclear, but may be related to the lesser degree of ischemia during the 30% flow reduction thereby providing a subthreshold stimulus. Another explanation for this observation could be that the protection involves K^+_{ATP} channels and that these channels are not homogeneously distributed throughout the myocardial wall but that they are predominantly located in the epicardial layer of the myocardial wall. The latter is recently shown in a study by Miyoshi *et al* [22] in which they reported a different response of the epicardial and endocardial layers to K^+_{ATP} channel modulators which suggested a lower threshold for the activation and/or a denser distribution of K^+_{ATP} channels or other K^+ channels at the epicardial layer. The finding that partial occlusions protect the myocardium against the development of irreversible damage during a subsequent total occlusion may be useful to the clinical condition that a coronary stenosis becomes totally occluded by a thrombus. The results of these studies if applicable to man, imply that studies evaluating the effectiveness of pharmacological agents in thrombolysis

trials should take into account the existence and the severity of preexisting stenosis as ischemic preconditioning could be a confounding factor. In the present study we have only investigated partial occlusions which lasted 30 minutes or longer. It is of interest to study whether partial occlusions of much shorter duration can precondition the myocardium without intervening reperfusion period.

Infarct size limiting effect of rapid ventricular pacing

From several studies it has become clear that a variety of stimuli, which do not produce myocardial ischemia, are also capable to limit infarct size during a subsequent sustained coronary artery occlusion. We further addressed this issue by applying rapid ventricular pacing (RVP) prior to a 60-minute coronary artery occlusion in anesthetized pigs. Ventricular pacing was chosen as a stimulus because ventricular pacing prior to a coronary artery occlusion (CAO) reduces the incidence of ventricular arrhythmias and fibrillation [23]. In that study it was assumed that ventricular pacing (at 300 bpm in dogs) produced myocardial ischemia in view of the occurrence of ST segment changes [23]. However, no attempts were made to confirm the presence of myocardial ischemia by abnormalities in myocardial contraction and metabolism. In chapter 4 we therefore first detailed the functional and metabolic changes that occurred in anesthetized pigs when the left ventricle was paced at 200 bpm [14] and concluded from these studies that ventricular pacing did not produce myocardial ischemia because (i) transmural myocardial blood flow 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, (iii) postsystolic shortening did not develop, and (iv) there were no changes in myocardial ATP and phosphocreatine levels, energy charge, and arterial or coronary venous pH concentrations. Other signs that ischemia did not develop during ventricular pacing were (v) the absence of reactive hyperemia after pacing was stopped and (vi) the immediate recovery of systolic segment shortening indicating that postischemic myocardial stunning did not occur.

It proved that when 10-minute RVP and 60-minute CAO were separated by 15 minutes of normal sinus rhythm (NSR), infarct area over area at risk (IA/AR) was not different from the control group but when the duration of RVP was extended to 30 minutes a small protection was observed (chapter 4, figure 4). Without a period of normal sinus rhythm between the 30-minute RVP and the 60-minute CAO IA/AR was markedly limited but the protection was less than with ischemic preconditioning (chapter 4, figure 5). The finding that the 15 minutes of NSR already attenuated protection by 30-minute RVP also implies that the time course of protection by ventricular pacing is different from that by ischemic preconditioning. K^+_{ATP} channel activation proved to play a role in the protection by rapid ventricular pacing as pretreatment with glibenclamide abolished the protection. The observation that administration of glibenclamide after the period of ventricular pacing was terminated did not attenuate the protection strongly

suggests that K^+_{ATP} channels did not have to remain activated after the protective stimulus had been applied. This is at variance with observations in ischemic preconditioning experiments as in these experiments glibenclamide abolished the protection independent whether administration occurred prior or after the brief coronary artery occlusion (preconditioning stimulus). The results of this study confirm that myocardium does not first have to be ischemic for a brief period in order to increase its tolerance to the development of irreversible damage during a sustained coronary artery occlusion and suggests that the myocardium is able to protect itself against the development of irreversible damage by a large number of stimuli prior to sustained coronary artery occlusion and which may or may not produce ischemia in the jeopardized myocardium. It remains of interest that the protection by RVP appears not only to be less potent than by ischemic preconditioning, but also that the protection has a much narrower time window. In some preliminary studies we have shown that RVP leads to activation of protein kinase C. In future studies it would therefore be interesting to relate the time course of protein kinase C activation to activation of K^+_{ATP} channels.

Cardioprotection by brief ischemia in remote organs and the infarct size limiting effect of low body temperature

In chapter 5 we hypothesised that brief ischemia in remote organs might also limit infarct size in the myocardium, because a brief coronary artery occlusion does not only limit infarct size inside but also outside its distribution territory [24].

In the first series of experiments we investigated in anesthetized male Wistar rats whether brief ischemia in remote organs limited myocardial infarct size (chapter 5 figure 1). To this end rats were subjected to either a 15-minute (left) renal artery occlusion (RAO) or a 15-minute mesenteric artery occlusion (MAO) 10 minutes prior to a 60-minute left anterior descending coronary artery occlusion and 180 minutes of reperfusion. Experiments were performed at 36.5°C-37.5°C (normothermia) and 30°C-31°C (hypothermia). Our study reveals that (i) in control animals the relation between IA and AR is highly linear without a positive intercept on the AR-axis unlike in pigs in which there is a positive intercept on the AR-axis in the IA-AR-relation [25], (ii) in control animals development of the infarct areas was the same in the two temperature ranges (chapter 5 figure 4 and table 1). These data appear to contradict those reported for rabbits [26] and swine [27]. Thus, Chien *et al* [26] reported a steep relation between body core temperature in the "normothermic" range (35°-42°C) and myocardial infarct size in rabbits subjected to a 30-minute CAO and 3 hours of reperfusion, so that an increase of 1°C resulted in 12% infarction of the area at risk with no infarction occurring at a body core temperature of 34.5°C. Duncker *et al* [27] showed an even steeper relation between body core temperature and infarct size produced by a 45 minute coronary artery occlusion and four hours of reperfusion in swine as 20% of the area at risk became infarcted with a 1°C increase in temperature in the range

of 35°C to 39°C. However, in those last studies different temperature ranges were used, while the coronary artery occlusions were also shorter (30 min - 45 min). The importance of the duration of the coronary artery occlusion in studying the temperature-dependency of infarct development is supported by the study from McClanahan *et al*, who showed that the infarct area produced by a 60-minute CAO was not affected when body temperature was decreased from 37°C to 35°C [28]. We addressed this hypothesis in chapter 6 and demonstrated that the infarct size limiting effect of hypothermia depends on the duration of the coronary artery occlusion. (For details see further).

Our studies in chapter 6 also revealed that (iii) protection by 15-minute CAO was much more pronounced during hypothermia than normothermia. Because temperature had no effect on infarct area in the control animals, we conclude that the 15-minute CAO stimulus was more effective during hypothermia than during normothermia, (iv) the 15-minute MAO limited myocardial infarct size both in the normothermic and hypothermic temperature ranges. The protection during hypothermia was, at variance with the classical ischemic preconditioning stimulus, only slightly larger than during normothermia, (v) the 15-minute RAO was not protective during normothermia but limited myocardial infarct size when experiments were performed during hypothermia (chapter 5 table 1). We can only speculate on the reason why the 15-minute MAO was effective and the 15-minute RAO ineffective during normothermia. An argument could be that the 15-minute RAO produced less severe ischemia than the 15-minute MAO as only approximately 10% of renal artery flow (compared to 90% of mesenteric flow) is needed for nutritional flow. Thus, the less severe ischemia produced by the 15-minute RAO may therefore not have been severe enough to trigger myocardial protection.

In the following series of experiments we investigated whether the mechanism of cardioprotection by remote brief organ ischemia differed from that by brief myocardial ischemia. Because protection by 15-minute MAO occurred during normothermia as well as hypothermia we selected this stimulus for investigation of its mechanism. We first evaluated whether a neurogenic mechanism could be involved in the protection by 15-minute MAO. To this end infarct size was determined after neurogenic blockade with hexamethonium. We showed that hexamethonium neither affected IA/AR in the control animals nor in the animals preconditioned with 15-minute CAO, regardless of whether the experiments were performed during normothermia or hypothermia. In contrast protection by 15-minute MAO was completely abolished after pretreatment with hexamethonium (chapter 5 figure 3 and 5 and table 1).

These data demonstrate that activation of the neurogenic pathway was involved in the protection by 15-minute MAO but not by 15-minute CAO. In the next series of experiments we investigated whether activation of the neurogenic pathway had occurred during early ischemia or during the 10-minute intervening reperfusion period, which separated the period from brief intestinal ischemia from the sustained coronary artery occlusion. We therefore studied the effect

of a permanent mesenteric artery occlusion starting 25 min prior to the onset of the 60-minute CAO on myocardial infarct size. In these experiments, MAO failed to limit infarct size produced by 60-minute CAO and the neurogenic pathway appears therefore to be triggered during early reperfusion of the mesenteric artery (chapter 5 figure 3 and 6 and table 1). In addition to these experiments we performed experiments to answer the question whether it is possible to protect the myocardium with MAO when the neurogenic pathway (and a possible downstream cascade) triggered during reperfusion of the intestinal bed arrived the myocardium after the onset of the long-term coronary artery occlusion. Thus in these experiments the mesenteric artery occlusion was terminated 1 minute after the onset of the long-term coronary artery occlusion. It was revealed that, MAO failed to limit infarct size and it seemed that the stimulus via the neurogenic pathway (cascade) must arrive at the myocardium before the onset of the myocardial ischemic period.

From our experiments we can not conclude that protection by 15-minute RAO also involves a neurogenic pathway. Furthermore, although protection by 15-minute MAO appears to differ from that by 15-minute CAO we cannot exclude the possibility that these two approaches to cardiac protection have a common endpoint such as the intracellular activation of protein kinase C (chapter 7 and 8).

The myocardial infarct size limiting effect of low body temperature in rats depends on the duration of the coronary artery occlusion.

As already stated recently, Chien *et al* [26] and Duncker *et al* [27] showed a steep relation between body core temperature and myocardial infarct size. In contrast to these studies our study in rats described in chapter 6 did not show a protective effect of hypothermia on infarct size determined 3 hours after a 60-minute CAO [17]. In chapter 6 we demonstrated that the infarct size limiting effect of hypothermia depends on the duration of the coronary artery occlusion. Thus, when in rats the coronary artery was occluded for 30 minutes, hypothermia was protective (5.2% of the area at risk per 1°C) , but when the duration of the CAO was extended to 60 minutes the protective effect of hypothermia could not be detected (chapter 6 figure 4).

There are several hypotheses by which hypothermia could exert protection. For instance a role myocardial oxygen demand at the onset of the coronary artery occlusion as a determinant of infarct size is postulated but is controversial. A positive correlation [29, 30] as well as no relation [30-33] between the rate-pressure product and infarct size have been reported. Nienaber *et al* [34] produced bradycardia in dogs with a synthetic opiate to lower the metabolic demand at the onset of a 24 hour coronary artery occlusion thereby producing a smaller infarction compared to a group of animals with a high metabolic demand at the onset of coronary artery occlusion. It cannot be excluded that the obtained protection by bradycardia was actually a direct result of μ-opioid receptor stimulation [35]. In collateral deficient species such as rabbit and swine infarct size

does not appear to be correlated with the rate-pressure product [12, 33]. In the study by Duncker *et al* [27] univariate or stepwise multivariate regression analysis did not reveal a significant correlation between temperature and systemic hemodynamic variables at baseline or myocardial blood flow under baseline conditions, suggesting that temperature did not exert its effect by altering myocardial oxygen demand at the onset of occlusion. Similarly, in rabbit hearts [26] and rat hearts (chapter 6) the infarct size limiting effect of hypothermia was unmitigated when hypothermia-induced bradycardia was prevented. It is likely that during coronary artery occlusion when contraction ceases, energy utilization is no longer reflected by the rate-pressure product. The present findings therefore lend further support to those studies that could not find a relation between the development of infarct size and energy demand at the time of occlusion as originally proposed.

The role of protein kinase C in ischemic preconditioning and cardioprotection by ischemia in other organs

Activation of receptors by exogenously administered stimuli such as adenosine [36, 37], bradykinin [38, 39], noradrenaline [40, 41], acetylcholine [42, 43], endothelin-1 [44] or opiates [45] mimic myocardial protection by ischemic preconditioning. This led to the hypothesis that intracellular signalling by these stimuli, via GTP-binding-protein-linked receptors and phospholipase C-β and possibly phospholipase D [46, 47], leads to activation of one or more isozymes of the protein kinase C family which ultimately phosphorylate putative target proteins directly involved in cardioprotection [10, 48]. Consistent with the hypothesis is that direct activators of protein kinase C, phorbol esters, have been reported to mimic ischemic preconditioning [49] and inactivators of protein kinase C, such as staurosporin or chelerythrine can block ischemic preconditioning [49, 50] (chapter 7). Possible target proteins of protein kinase C could be those that regulate opening of K^+_{ATP} channels [51-53], activate ecto-5'-nucleotidase [54] (during the first window of protection (FWOP) [55]) or modulate transcriptional regulation of the expression of heat shock proteins [56, 57] (during the second window of protection (SWOP) [58-60]). For instance, K^+_{ATP} channels are opened when an ischemic preconditioning stimulus is applied, while blockade of K^+_{ATP} channels prevents ischemic preconditioning [51, 52]. However, blockade of the action potential shortening by dofetilide does not abolish protection by ischemic preconditioning [61] therefore it is likely that modulation of K^+_{ATP} channels in the mitochondria, sarcoplasmic reticulum or the nucleus are involved in the mechanism of the protection. The presence of these channels in the mitochondria [62, 63] or other types of K+ channels in the sarcoplasmic reticulum [64, 65] or the nucleus are shown [66] but their role in ischemic preconditioning is not yet investigated.

Since protein kinase C can be activated via various receptors linked to phospholipase C- and possibly phospholipase D-mediated signalling pathways, these receptors may act synergistically [10]. Opening of K^+_{ATP} channels by pharmacological substances lowers the threshold for ischemic

preconditioning [67], which is consistent with the hypothesis that K^+_{ATP} channels are target proteins for protein kinase C. Kitakaze *et al* [54] reported that ischemic preconditioning increased ecto-5'-nucleotidase activity and that activation of protein kinase C increases ecto-5'-nucleotidase activity in isolated rat cardiomyocytes, supporting the candidacy of ecto-5'-nucleotidase as another target protein of protein kinase C.

At present myristoylated-alanine-rich-C-kinase-substrate (MARCKS) is the only endogenous target protein for protein kinase C that has been shown to be phosphorylated in preconditioned rabbit myocardium. However, the former is believed to be an intracellular location site rather than a protein factor intimately involved in the protective response [68]. Irrespective of the target protein(s) we are dealing with or its (their) covalently bound phosphates must be relatively stable during the 2 to 3 hours in which the cardioprotection is present (FWOP). Furthermore, the precise time point at which protein kinase C is maximally translocated (activated) during ischemia or reperfusion (preconditioning stimulus) is unknown and consequently also the time point at which the enzyme reaches the target proteins for catalysing their phosphorylation. It is quite feasible that protein kinase C is removed from its translocation site or proteolytically degraded after performing its action and thereafter the enzyme is not longer detectable by immunoreactivity or activity measurements. It is therefore mandatory to determine the time course of translocation/activation and subsequent relocalization/inactivation or proteolytic degradation of the protein kinase C isozyme and the time course(s) of phosphorylation and dephosphorylation or proteolytic degradation of the target protein(s). Because the time course of weaning of the protective effect of the FWOP is roughly known, the time course of dephosphorylation/inactivation of the target protein could be correlated to the time course of weaning of protection. Candidate target proteins of protein kinase C involved in the FWOP are e.g. the K^+_{ATP} channel [51, 52] and/or the ecto-5'-nucleotidase [54], but experimental evidence for phosphate incorporation into channel proteins and/or enzymes or regulating proteins is lacking. If the K^+_{ATP} channel or the ecto-5'-nucleotidase are its targets the most likely translocation site for the protein kinase C isozyme(s) involved in the FWOP is the sarcolemma.

Protein kinase C is involved in the agonist-receptor interaction induced changes in gene expression of many cells [47, 69-74]. If we take into account the time required for inducing heat shock/stress proteins [56, 57] the induced changes by protein kinase C may only play a role in the SWOP. Therefore, a transcription factor involved in the regulation of expression of heat shock/stress proteins could be another potential target protein of protein kinase C. If true, the nucleus is the most likely translocation site for the protein kinase C isozyme(s) in causing the protection.

The protein kinase C isozymes can be divided into 3 subgroups. The classical protein kinase C-α is activated by phosphatidylserine (PtdSer), Ca^{2+} and (1,2)DAG. The novel protein kinases C-δ and -ϵ require PtdSer and (1,2)DAG for their activation but are Ca^{2+} independent and the

atypical protein kinase C-ζ only needs PtdSer, but no Ca^{2+} and (1,2)DAG. Weinbrenner *et al* [75] showed that protein kinases C-α, -δ, -ε and -ζ are the most prominent isozymes in the rat heart which were also found in cultured neonatal [76] and adult rat cardiomyocytes [77]. Weinbrenner *et al* [75] also showed a rapid translocation of the Ca^{2+} independent isozymes, namely protein kinases C-δ, -ε and -ζ and the Ca^{2+} dependent protein kinase C-α to the membrane after a brief ischemic period. An extension of the ischemia period led to increased expression of Ca^{2+} independent forms of protein kinases C-δ and -ε in the cytosol. Thus, in these studies it appears that the distribution of protein kinase C isozymes-α, -δ, -ε and -ζ were altered. Therefore we investigated in chapter 8 myocardial protein kinase C isozymes-α, -δ, -ε and -ζ in classic ischemic preconditioning and cardioprotection by remote organ ischemia (chapter 8, figure 1). For the measurements of the translocation of protein kinase C isozymes from cytosol to membrane we have applied two methods, namely immunoblot analysis using protein kinase C isozyme-specific antibodies [69, 75, 78] (chapter 8, table 1) and assay of protein kinase C activity by Ca^{2+} and/or (1,2)DAG-dependent phosphate incorporation into histon III-S [69, 76, 79, 80] (chapter 8, table 2).

In the CAO cytosolic protein kinase C-δ disappeared, but was not found back in the membrane fraction. It could mean that protein kinase C-δ translocates to the myofibrils, mitochondria or the perinucleus and/or the activated enzyme is not detectable anymore due to degradation of the enzyme in the membrane fraction. Although protein kinase C-δ was found to be decreased in the cytosol, the involvement of this event in the ultimate protective effect is not proven. Specific inhibition of protein kinase C-δ is needed for the latter which is at present not possible. In the MAO group protein kinase C-δ tended to disappear from the cytosolic fraction.

Thus when it is concluded that protein kinase C-δ is involved in ischemic preconditioning, the cardioprotective effect of MAO must involves another pathway, which could directly interact with the common protective pathway of ischemic preconditioning beyond the protein kinase C cascade or could be a totally different pathway besides the one of ischemic preconditioning leading to protection.

Future perspectives

The neurogenic and other mediators of remote cardioprotection

In chapter 5 the phenomenological characteristics of the myocardial infarct size limiting effect of ischemia in the small intestines were determined. Briefly, (i) infarct size limitation afforded by brief ischemia in the small intestines is temperature dependent, (ii) it involves a neurogenic pathway as it could be blocked by ganglion blockade (hexamethonium), (iii) this neurogenic pathway is activated during reperfusion of the ischemic small intestines, (iv) the signal must arrive in the myocardium before it becomes ischemic as the infarct size limiting effect could not be shown when reperfusion of the small intestines was induced 1 minute after the onset of the coronary artery occlusion (preliminary experiments not described in this thesis). The infarct size limiting effect of low body temperature depends on the duration of the coronary artery occlusion (chapter 6) and the infarct size limiting effect of prior brief ischemia in the kidney is temperature dependent; this implicates that the latter also depends on the duration of the coronary artery occlusion. The involvement of a neurogenic pathway which is activated during reperfusion of the ischemic small intestines and the observation that the signal must arrive before occlusion of the coronary artery suggests a two step mechanism in which a circulating mediator could be involved. It may be possible that autocoids trigger a neurogenic pathway during the intervening reperfusion of the small intestines and this neurogenic signal could trigger the release of a circulating mediator which reaches the myocardium during the intervening reperfusion period. Another possibility is that processing of the received signal by the myocardium that leads to the protective effect demands energy, which is only available during the intervening reperfusion period. In future studies (i) the infarct size limiting effect of brief ischemia in other organs has to be further characterised by determining the window of protection (ii) the existence of a "second window of protection" has to be investigated 24 hours after the stimulus has been given; (iii) the organ specificity of this phenomenon has to be studied and (iv) it has to be shown whether the infarct size limiting effect in the myocardium is also seen in other organs such as brains. In addition, (v) studies must be performed to determine more precisely the mechanism by investigating the role of substances such as bradykinin, oxygen radicals, and adenosine in the ischemic organ or the heart in a donor-recipient model in which the circulation of the organ that has to become ischemic is shunted to another animal so that the circulation of the heart and the target organ is separated from each other to permit specific application of pharmacological inhibitors/activators to the target organ or to the heart.

The cellular mechanism of preconditioning

In chapters 7 and 8 we already addressed the potential role of protein kinase C in the mechanisms of ischemic preconditioning. We also delineated the limitations and the difficulties

of the procedures used. Thus interpretation of the data obtained must be done with caution. The role of protein kinase C in the mechanism of ischemic preconditioning remains therefore a controversial issue (see chapter 7). One of the reasons is that protein kinase C represents multiple isozymes (more than 12). It is not possible to measure the translocation of each isozyme by activity individually. Thus, activity measurements show only the activity of protein kinase C isozymes all together. Moreover in all studies only one time point was chosen to determine translocation of (usually only a few) isozymes and activity of total protein kinase C. The time course of translocation is therefore still not known. Furthermore, measurements (phosphorylation/dephosphorylation/inhibiting) on potential target proteins have not yet been done. So in future studies the time course and the subcellular compartment to which several protein kinase C isozymes translocates must be determined by isozyme and activity measurements in sequential/serial biopsies taken at different time points during the experiment. Furthermore, it will be necessary to develop specific blockers for isozymes of protein kinase C to see whether these can inhibit cardioprotection induced by CAO and/or MAO. In addition the place of the opening of the K_{ATP} channel in the cascade of intracellular signal transduction pathway should be investigated by simultaneously inhibiting this channel by glibenclamide and protein kinase C isozyme protein and activity measurements.

The mechanism of the infarct size limiting effect of low body temperature

The infarct size limiting effect of low body temperature depends on the duration of the coronary artery occlusion. Thus in rats for body temperatures around 30°C the protective effect could only be shown between 20 and 40 minutes of coronary artery occlusion. This implicates that the effect is only a delay in the infarct size development and not an absolute abolishment of infarction. Evidence suggests that myocardial cell death due to ischemia is mostly determined to be apoptotic cell death [81] (see below). This is rather unexpected because apoptosis is an energy dependent process. The enzymatic reactions involved are, as any other, temperature dependent. Thus, in our experiments the lower body core temperature of the rats has reduced the rate of the genome-programmed process of apoptosis. Another factor is the known effect of temperature changes on the membrane fluidity. Some enzymes involved in apoptosis, are membrane bound and so their catalytic function dependent on the fluidity of the membranes.

The role of apoptosis in myocardial ischemia-reperfusion injury and preconditioning

The ultimate protection of the myocardium against ischemia is still the termination of ischemia by restoring the blood flow to the jeopardized myocardium. However, reperfusion after an infarction inducing ischemic period may act as a double edged sword as it will not only lead to salvage of jeopardized tissue but may also promote an extension of the infarcted area [82-84] ("reperfusion injury" [85,86]). In the setting with myocardial infarction as endpoint and reperfusion

injury thus refers to the death of myocytes that are still viable at the onset of reperfusion. However "reperfusion injury" has been questioned by several groups of investigators [87, 88]. Jennings and Reimer have ascribed this extension to a "delayed ischemic injury" i.e. injury that is induced during the ischemic period but comes at first to expression during reperfusion [89]. Pure ischemic injury and combined ischemic-reperfusion injury have different histological characteristics i.e. the amount of coagulation necrosis and contraction band necrosis is different between the pure ischemic injury and the combined ischemic-reperfusion injury [90]. In infarctions produced by an 8 hour coronary artery occlusion without reperfusion, 98% of the infarction consists of coagulation necrosis. In contrast, the infarctions produced by a 1 hour occlusion period with 1 to 7 hours of reperfusion show 30% contraction band necrosis and 70% coagulation necrosis. It is not known how the ratio between coagulation necrosis and contraction band necrosis varies with the duration of the ischemic period. These different morphological characteristics could reflect a different modality of cell death and the mechanism in causing these different forms of cell death could be different. It is quite feasible that apoptosis plays a role in ischemic-reperfusion injury. Apoptosis as a mode of cell death has been recognized in the mid 70's and is currently acknowledged to exist in normal tissue to maintain balance between cell homeostasis and cell proliferation and differentiation. The characteristic events of apoptotic cell death which occur in a fixed sequence are cell shrinkage, loss of environmental contact of the cell, dense chromatin condensation of the nucleus, cellular budding and fragmentation, and rapid phagocytosis by macrophages or adjacent cells [91, 92]. Gottlieb *et al* [93] have recently shown that reperfusion injury induces apoptosis in rabbit cardiomyocytes. Apoptotic cells are programmed to die in a certain manner and the authors ascribed to the reperfusion after the ischemic period as the trigger because they could not detect any signs of apoptosis when the myocytes were subjected only to 30 min or 4.5 hours of ischemia. In this study there was no group which received a shorter period of reperfusion after the ischemic period. More recently Kajstura *et al* [81] shows that apoptotic and necrotic myocyte cell deaths are independent contributing variables of infarct size in rats. In these experiments rats were subjected to 20 minutes to 7 days of coronary artery occlusion. The contribution of myocyte necrotic and apoptotic cell death to the myocardial infarction was assessed. Apoptotic cell death represented the major independent form of myocyte cell death and peaked at 4.5 hours which is rather unexpected and difficult to understand because this genome directed programmed process needs energy-rich phosphates at many reaction steps (e.g. transcription and translation). Therefore, programmed cell death occurring during reperfusion is more plausible, but even during this period myocardial energy charge will remain reduced for a long period. From these studies we conclude that if apoptosis plays a major role in ischemic myocardial death it must be in the ischemia-reperfusion injury. The level of energy-rich phosphates is progressively reduced during ischemia. Furthermore, if apoptosis is responsible for the major part of the infarction, the infarct size limiting effect of

ischemic preconditioning and K^+_{ATP} channel activation may interfere with this form of myocardial cell death. It is of interest to know whether the infarct size limiting effect of these stimuli/strategies has its effect on ischemic injury or ischemia-reperfusion injury and whether apoptosis or necrosis is limited by these interventions. If apoptosis is involved we possibly could interfere in this active process of cell death once we know the mechanism of apoptosis. Because both apoptosis and preconditioning are biological processes, possibly existing in all tissues these two phenomena could be (inter)related. The aim of future studies could be to attempt to distinguish reperfusion injury from delayed ischemic injury in an in-situ rat or porcine model for acute myocardial infarction and try to detect, quantify and localize apoptotic cardiomyocytes at the end of the induction of ischemic injury induced by a 1 hour period of ischemia and after a 1 hour period of ischemia and 10 min or 3 hours of reperfusion. From the results of these experiments we hopefully can conclude whether apoptosis is involved in ischemic-reperfusion injury or whether this is a modality of its own. If apoptosis is involved, and if the extension of infarction during the reperfusion period could be ascribed to apoptosis or cell death induced during the ischemic period then the extension is delayed ischemic injury. If the extension could be ascribed to apoptosis induced during reperfusion the extension is indeed reperfusion injury. If the apoptotic cells lie beyond the "necrotic area" it is a modality of its own.

In view of the ischemic preconditioning phenomenon one should investigate whether infarct size limitation is due to limitation of ischemic injury, limitation of reperfusion injury or a combination of both. In addition, one should also investigate whether the infarct size limiting effect of preconditioning could be ascribed to a limitation of cell death due to apoptosis.

Concluding remarks

In the effort to search for the ideal therapy to protect ischemic myocardium one should keep in mind that finally perfusion must be reinstalled. The current view is that the mechanism of myocardial cell death due to ischemia and reperfusion is multifactorial. Thus, during reperfusion the myocardium is also injured, which therefore is another point to intervene and to protect the myocardium at risk. If protection mechanisms in ischemic and ischemic-reperfused myocardium act in a different way on the damaged myocardium, one could theoretically combine these protective interventions and create the best approach to save myocardium at risk. However, even when we are able to make the ideal anti-ischemic therapy, we should keep in mind that this will only be a delay for myocardial death as long as we are not able to reperfuse the myocardium at risk in time.

References

1. Murry CE, Jennings RB, Reimer KA. Preconditioning with ischemia: a delay of lethal cell injury in ischemic myocardium. *Circulation* 1986;75:1124-1136.
2. Lawson CS, Downey JM. Preconditioning: state of the art myocardial protection. *Cardiovasc Res* 1993;27:542-550.
3. Zager RA, Baltes LA, Sharma HM, Jurkowitz MS. Responses of the ischemic acute renal failure kidney to additional ischemic events. *Kidney Int* 1984;26:689-700.
4. Mounsey RA, Pang CY, Forrest C. Preconditioning: a new technique for improved muscle flap survival. *Otolaryngology-Head Neck Surg* 1992;107:549-552.
5. Kitagawa K, Matsumoto M, Tagaya M, Hata R, Ueda H, Niinobe M, Handa N, Fukunaga R, Kimura K, Mikoshiba K, Kamada T. 'Ischemic tolerance' phenomenon found in the brain. *Brain Res* 1990;528:21-24.
6. Lloris-Carsi JM, Cejalvo D, Toledo-Pereyra LH, Calvo MA, Suzuki S. Preconditioning: effect upon lesion modulation in warm liver ischemia. *Transplant Proc* 1993;25:3303-3304.
7. Neely CF, Keith IM. A1 adenosine receptor antagonists block ischemia-reperfusion injury of the lung. *Am J Physiol (Lung Cell Mol Physiol 12)* 1995;268:L1036-L1046.
8. Hotter G, Closa D, Prados M, Fernández-Cruz L, Prats N, Gelpí E, Roselló-Catafau J. Intestinal preconditioning is mediated by a transient increase in nitric oxide. *Biochem Biophys Res Commun* 1996;222:27-32.
9. Yellon DM, Alkhulaifi AM, Browne EE, Pugsley WB. Ischaemic preconditioning limits infarct size in the rat heart. *Cardiovasc Res* 1992;26:983-987.
10. Downey JM, Cohen MV, Ytrehus K, Liu Y. Cellular mechanisms in ischemic preconditioning: The role of adenosine and protein kinase C. In: Cellular, Biochemical, and Molecular Aspects of Reperfusion Injury. Ed: Das DK; *Ann NY Acad Sci* 1994;723:882-898.
11. Koning MMG, De Zeeuw S, Nieukoop S, De Jong JW, Verdouw PD. Is myocardial infarct size limitation by ischemic preconditioning an "all or nothing" phenomenon ? In: Cellular, Biochemical and Molecular Aspects of Reperfusion Injury. Ed: Das DK. *Ann N Y Acad Sci* 1994;723:333-336.
12. Koning MMG, Simonis LAJ, de Zeeuw S, Nieukoop S, Post S, Verdouw PD. Ischaemic preconditioning by partial occlusion without intermittent reperfusion. *Cardiovasc Res* 1994;28:1146-1151.
13. Koning MMG, Gho BCG, Van Klaarwater E, Duncker DJ, Verdouw PD. Endocardial and epicardial infarct size after preconditioning by a partial coronary artery occlusion without intervening reperfusion. Importance of the degree and duration of flow reduction. *Cardiovasc Res* 1995;30:1017-1027.
14. Koning MMG, Gho BCG, Van Klaarwater E, Opstal RLJ, Duncker DJ, Verdouw PD. Rapid ventricular pacing produces myocardial protection by non-ischemic activation of K^+_{ATP} channels. *Circulation* 1996;93:178-86.
15. Verdouw PD, Gho BCG, Duncker DJ. Cardioprotection by organs in stress or distress. *Basic Res Cardiol* 1996;91:44-46.
16. Verdouw PD, Gho BCG, Schoemaker R, Duncker DJ. Cardioprotection by ischemic and non-ischemic myocardial stress and ischemia in remote organs: Implications for the concept of ischemic preconditioning. In: Myocardial Preservation, Preconditioning, and Adaptation. Eds: Das DK, Engelman RM, Cherian KM. *Ann NY Acad Sci* 1996;793:27-42.
17. Gho BCG, Schoemaker RG, van den Doel MA, Duncker DJ, Verdouw PD. Myocardial protection by brief ischemia in non cardiac tissue. *Circulation* 1996;94:2193-2200.
18. Harris AS. Delayed development of ventricular ectopic rhythms following experimental coronary occlusion. *Circulation* 1950;1:1318-1328.
19. Iwamoto T, Bai XJ, Downey HF. Preconditioning with supply-demand imbalance limits infarct size in dog heart. *Cardiovasc Res* 1993;27:2071-2076.
20. Ovize M, Kloner RA, Hale SL, Przyklenk K. Coronary cyclic flow variations "precondition" ischemic myocardium. *Circulation* 1992;85:779-789.
21. Ovize M, Przyklenk K, Kloner RA. Partial coronary stenosis is sufficient and complete reperfusion is mandatory for preconditioning the canine heart. *Circ Res* 1992;71:1165-1173.
22. Miyoshi S, Myazaki T, Moritani K, Ogawa S. Different responses of epicardium and endocardium to K_{ATP} channel modulators during regional ischemia. *Am J Physiol (Heart Circ Physiol 40)* 1996;271:H140-H147.
23. Vegh A, Szekeres L, Parratt JR. Transient ischaemia induced by rapid cardiac pacing results in myocardial preconditioning. *Cardiovasc Res* 1991;25:1051-1053.
24. Przyklenk K, Bauer B, Ovize M, Kloner RA, Whittaker P. Regional ischemic 'preconditioning' protects remote

virgin myocardium from subsequent sustained coronary occlusion. *Circulation* 1993;87:893-899.
25. Gho BCG, Koning MMG, Opstal RLJ, Van Klaarwater E, Duncker DJ, Verdouw PD. Novel approaches to myocardial preconditioning in pigs. In: Myocardial Preconditioning. Editors: Parratt JR and Wainwright CL; Landes, R.G, Co., Austin, Texas. 1996:97-113.
26. Chien GL, Wolff RA, Davis RF, Van Winkle DM. "Normothermic range" temperature affects myocardial infarct size. *Cardiovasc Res* 1994;28:1014-1017.
27. Duncker DJ, Klassen CL, Herrlinger SH, Pavek TJ, Ishibashi Y, Bache RJ. Effect of temperature on myocardial infarction in swine. *Am J Physiol (Heart Circ. Physiol. 39)* 1996;270:H1189-H1199.
28. McClanahan TB, Mertz TE, Martin BJ, Gallagher KP. Pentostatin reduces infarct size in pigs only when combined with mild hypothermia. *Circulation* 1994;90:I478(Abstract)
29. Garcia-Dorado D, Theroux P, Elizaga J, Aviles FF, Alonso J, Solares J. Influence of tachycardia and arterial hypertension on infarct size in the pig. *Cardiovasc Res* 1988;22:620-626.
30. Reimer KA, Jennings RB, Cobb FR, Murdock RH, Greenfield Jr. JC, Becker LC, Bulkley BH, Hutchins GM, Schwartz RP, Bailey KR, Passamani ER. Animal models for protecting ischemic myocardium: results of the NHLBI Cooperative Study. Comparison of unconscious and conscious dog models. *Circ Res* 1985;56:651-665.
31. Lowe JE, Reimer KA, Jennings RB. Experimental infarct size as a function of the amount of myocardium at risk. *Am J Pathology* 1978;90:363-380.
32. Miura, T., D.M. Yellon, D.J. Hearse, M. Downey. Determinants of infarct size during permanent occlusion of a coronary artery in the closed chest dog. *J Am Coll Cardiol* 1987;9:647-654.
33. Miura T, Ogawa T, Iwamoto T, Shimamoto K, Iimura O. Dipyridamole potentiates the myocardial infarct size-limiting effect of ischemic preconditioning. *Circulation* 1992;86:979-985.
34. Nienaber Ch, Gottwik M, Winkler B, Schaper W. The relationship between the perfusion deficit, infarct size and time after experimental coronary artery occlusion. *Basic Res Cardiol* 1983;78:210-225.
35. Schultz JEJ, Hsu A, Gross GJ. Morphine-induced cardioprotection is mediated via the ATP-sensitive potassium (K_{ATP}) channel in the rat heart. *FASEB J* 1996;10:A12(abstract)
36. Thornton J, Liu GS, Olsson RA, Downey JM. Intravenous pretreatment with A1-selective adenosine analogues protects the heart against infarction. *Circulation* 1992;85:659-665.
37. Toombs CF, McGee DS, Johnston WE, Vinten-Johanson J. Myocardial protective effects of adenosine: infarct size reduction with pretreatment and continued receptor stimulation during ischemia. *Circulation* 1992;86:986-994.
38. Wall TM, Sheehy R, Hartman JC. Role of bradykinin in myocardial preconditioning. *J Pharmacol Exp Ther* 1994;270:681-689.
39. Goto M, Liu Y, Yang X-M, Ardell JL, Cohen MV, Downey JM. Role of bradykinin in protection of ischemic preconditioning in rabbit heart. *Circ Res* 1995;77:611-621.
40. Hu K, Nattel S. Mechanisms of ischemic preconditioning in rat hearts. Involvement of alpha 1B-adrenoceptors, pertussis toxin-sensitive G proteins, and protein kinase C. *Circulation* 1995;92(8):2259-2265.
41. Strasser RH, Marquetant R. Sensitization of the beta-adrenergic system in acute myocardial ischaemia by a protein kinase C-dependent mechanism. *Eur Heart J* 1991;12 Suppl F:48-53.
42. Yao Z, Gross GJ. Acetylcholine mimics ischemic preconditioning via a glibenclamide-sensitive mechanism in dogs. *Am J Physiol (Heart Circ Physiol* 33) 1993;264:H2221-H2225.
43. Yao Z, Gross GJ. Role of nitric oxide, muscarinic receptors, and the ATP-sensitive K^+ channel in mediating the effects of acetylcholine to mimic preconditioning in dogs. *Circ Res* 1993;73:1193-1201.
44. Wang P, Gallagher KP, Downey JP, Cohen MV. Pretreatment with endothelin-1 mimics ischemic preconditioning against infarction in isolated rabbit heart. *J Mol Cell Cardiol* 1996;28:579-588.
45. Schultz JEJ, Hsu A, Gross GJ. Morphine-induced cardioprotection is mediated via the ATP-sensitive potassium channel in the rat heart. *FASEB J* 1996;10:A12, 68 (abstract).
46. Sugden PH, Bogoyevitch MA. Intracellular signalling through protein kinases in the heart. *Cardiovasc Res* 1995;30:478-492.
47. De Jonge HW, Van Heugten HAA, Lamers JMJ. Review. Signaltransduction by the phosphatidylinositol cycle in myocardium. *J Mol Cell Cardiol* 1995;27:93-106.
48. Cohen MV, Downey JM. Preconditioning during ischemia: basic mechanism and potential clinical applications. *Cardiol Rev* 1995;3:137-149.
49. Liu Y, Ytrehus K, Downey LM. Evidence that translocation of protein kinase C is a key event during ischemic preconditioning of rabbit myocardium. *J Mol Cell Cardiol* 1994;26:661-668.

50. Liu Y, Cohen MV, Downey JM. Chelerythrine, a highly selective protein kinase C inhibitor, blocks the anti-infarct effect of ischemic preconditioning in rabbit hearts. *Cardiovasc Drugs Ther* 1994;8:881-882.
51. Gross GJ, Auchampach JA. Blockade of ATP-sensitive potassium channels prevents myocardial preconditioning in dogs. *Circ Res* 1992;70:223-233.
52. Rohmann S, Weygandt H, Schelling P, Soei LK, Verdouw PD, Lues I. Involvement of ATP-sensitive potassium channels in preconditioning protection. *Basic Res Cardiol* 1994;89:563-576.
53. Hu K, Duan D, Nattel S. Protein kinase C-mediated activation of ATP-sensitive potassium current - The missing link in ischemic preconditioning. *Circulation* 1995;92:2259-2265.
54. Kitakaze M, Node K, Minamino T, Komamura K, Funaya H, Shinozaki Y, Chujo M, Mori H, Inoue M, Hori M, Kamada T. Role of activation of protein kinase C in the infarct size-limiting effect of ischemic preconditioning through activation of ecto-5'-nucleotidase. *Circulation* 1996;93:781-791.
55. Li YW, Whittaker P, Kloner RA. The transient nature of the effect of ischemic preconditioning on myocardial infarct size and ventricular arrhythmia. *Am Heart J* 1992;123:346-353.
56. Donnely TJ, Sievers RE, Visseren FLJ, Welch WJ, Wolfe CL. Heat shock protein induction in rats. A role for improved myocardial salvage after ischemia reperfusion. *Circulation* 1992;85:769-778.
57. Yellon DM, Pasini E, Cargnoni A, Marber MS, Latchman DS, Ferrari R. The protective role of heat stress in the ischaemic and reperfused rabbit myocardium. *J Mol Cell Cardiol* 1992;24:895-907.
58. Kuzuya T, Hoshida S, Yamashita N, Fuji H, Oe H, Hori M, Kamada T, Tada. Delayed effects of sublethal ischemia on the acquisition of tolerance to ischemia. *Circ Res* 1993;72:1293-1299.
59. Marber MS, Latchman DS, Walker M, Yellon DM. Cardiac stress protein elevation 24 hours after brief ischemia or heat stress is associated with resistance to myocardial infarction. *Circulation* 1993;88:1264-1272.
60. Baxter GF, Goma FM, Yellon DM. Duration of the "second window of protection" following ischaemic preconditioning in the rabbit. *J Mol Cell Cardiol* 1995;27:A162 (Abstract).
61. Sleph P, D'Alonzo A, Dzwonczyk S, Parham C, Darbenzio R, Grover GJ. Preconditioning is not abolished by the delayed rectifier K'(IKR) blocker dofetilide. *FASEB J* 1996;10:A319-1841.
62. Garlid KD, Paucek P, Yarov-Yarovoy V, Sun X, Schindler PA. The mitochondrial K_{ATP} channel as a receptor for potassium channel openers. *J Biol Chem* 1996;271:8796-8799.
63. Paucek P, Mironova G, Mahdi F, Beavis AD, Woldegioris G, Garlid KD. Reconstitution and partial purification of the glibenclamide-sensitive, ATP-dependent K^+ channel from rat liver and beef heart mitochondria. *J Biol Chem* 1992;267:26062-26069.
64. Rousseau E, Chabot H, Beaudry C, Muller B. Reconstitution and regulation of cation-selective channels from cardiac sarcoplasmic reticulum. *Mol Cell Biochem* 1992;114:109-117.
65. Shen WK, Hill JA Jr, Rasmusson R, Strauss HC. Reconstitution of the K channel of cardiac sarcoplasmic reticulum. *Prog Clin Biol Res* 1990;334:205-230.
66. Bustamente JO. Nuclear ion channels in cardiac myocytes. *Pflugers Arch* 1992;421:473-485.
67. Yao Z, Gross GJ. Activation of ATP-sensitive potassium channels lowers threshold for ischemic preconditioning in dogs. *Am J Physiol* 1994;267:H1888-H1894.
68. Brooks G, Walsh R, Downey J. Phosphorylation of 80K/MARCKS, a specific substrate of protein kinase C occurs after 5' ischemia but only in preconditioned hearts. *Circulation* 1993;88(SuppII):101(abstract).
69. Nishizuka Y. Intracellular signalling by hydrolysis of phospholipids and activation of protein kinase C. *Science* 1992;258:607-614.
70. Pucéat M, Brown JH. Protein kinase C in the heart. In: Protein kinase C. Ed: Kuo JF; London: Oxford University Press 1994;249-268.
71. Steinberg SF, Goldberg M, Rybin VO. Protein kinase C isoform diversity in the heart. *J Mol Cell Cardiol* 1995;27:141-153.
72. Harrington EO, Ware JA. Diversity of the protein kinase C gene family. Implications for cardiovascular disease. *Trends Cardiovasc Med* 1995;5:193-198.
73. Hug H, Sarre TF. Protein kinase C isozymes: divergence in signal transduction. *Biochem J* 1993;291:329-343.
74. Dekker LV, Parker PJ. Protein kinase C - a question of specificity. *Trends Biochem Sci* 1994;19:73-77.
75. Weinbrenner CE, Simonis G, Marquetant R, Strasser RH. Selective regulation of calcium-dependent and calcium-independent subtypes of protein kinase C in acute and prolonged myocardial ischemia. *Circulation* 1993;88(Suppl 1):I-101(abstract).
76. Lamers JMJ, Eskildsen-Helmond YEG, Resink AM, De Jonge HW, Bezstarosti K, Sharma HS, Van Heugten HAA. Endothelin-1-induced phospholipase C-ß and D and protein kinase C isoenzyme signalling leading to hypertrophy in rat cardiomyocytes. *J Cardiovasc Pharmacol* 1995;26(suppl. 3):S100-S103.

77. Bogoyevitch MA, Parker PJ, Sugden PH. Characterization of protein kinase C isotype expression in adult rat heart. *Circ Res* 1993;72:757-767.
78. Przyklenk K, Sussman MA, Simkhovich BZ, Kloner RA. Does ischemic preconditioning trigger translocation of protein kinase C in the canine model? *Circulation* 1995;92:1546-1557.
79. Kloner RA, Przyklenk K, Whittaker P, Hale S. Editorial. Preconditioning stimuli and inadvertent preconditioning. *J Mol Cell Cardiol* 1995;27:743-747.
80. Schaap D, Parker PJ. Expression, purification, and characterization of protein kinase C-ϵ. *J Biol Chem* 1990;265:7301-7307.
81. Kajstura J, Cheng W, Reiss K, Clark WA, Sonnenblick EH, Krajewski S, Reed JC, Olivetti G, Anversa P. Apoptotic and necrotic myocyte cell deaths are independent contributing variables of infarct size in rats. *Lab Invest* 1996;74:86-107.
82. Braunwald E, Kloner RA. Myocardial reperfusion: a double-edged sword? *J Clin Invest* 1985;76:1713-1719.
83. Farb A, Kolodgie FD, Jones RM, Jenkins M, Virmani R.Early detection and measurement of experimental myocardial infarcts with horseradish peroxidase. *J Mol Cell Cardiol* 1993;25:343-353.
84. Farb A, Kolodgie FD, Jenkins M, Virmani R. Myocardial infarct extension during reperfusion after coronary artery occlusion: pathological evidence. *J Am Coll Cardiol* 1993;21:1245-1253.
85. Rosenkranz ER, Buckberg GD. Myocardial protection during surgical coronary reperfusion. *J Am Coll Cardiol* 1983;1:1235-1246.
86. Jolly SR, Kane WJ, Bailie MB, Abrams GD, Lucchesi BR. Canine myocardial reperfusion injury: its reduction by the combined administration of superoxide dismutase and catalase. *Circ Res* 1984;54:277-285.
87. Ganz W, Watanabe I, Kanamasa K, Yano J, Han DS, Fishbein MC. Does reperfusion extend necrosis? a study in a single territory of myocardial ischemia-halfreperfused and half not reperfused. *Circulation* 1990;82:1020-1033.
88. Miura T. Does reperfusion induce myocardial necrosis? *Circulation* 1990;82:1070-1072.
89. Jennings RB, Reimer KA. Lethal myocardial ischemic injury. *Am J Pathol* 1981;102:241-255.
90. Fujiwara H, Onodera T, Tanaka M, Miyazaki S, Wu DJ, Matsuda M, Kawamura A, Ishida M, Takemura G, Fujiwara Y, Fujiwara T, Kawai C. Acceleration of cell necrosis following reperfusion after ischemia of the pig heart without collateral circulation. *Am J Cardiol* 1989;63:14E-18E.
91. Kerr JFR, Wyllie AH, Currie AR. Apoptosis: a basic biological phenomenon with wide ranging implications in tissue kinetics. *Br J Cancer* 1972;26:239-257.
92. Majno G, Joris I. Apoptosis, oncosis and necrosis. *Am J Pathol* 1995;146:3-15.
93. Gottlieb RA, Burleson KO, Kloner RA, Babior BM, Engler RL. Reperfusion injury induces apoptosis in rabbit cardiomyocytes. *J Clin Invest* 1994;94:1621-1628.

Chapter 10

Samenvatting

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In ischemische preconditionerings studies word(en) één of meer korte, abrupte, totale coronair arterie occlusie - reperfusie periodes gebruikt om het myocard te beschermen tegen irreversibele schade veroorzaakt door een langdurige coronair arterie occlusie. Bewijs stapelt zich nu op dat partiële coronair arterie occlusies, met of zonder een toename in de zuurstofvraag, ook het myocard kan preconditioneren. Bovendien, in tegenstelling tot wat er eerder werd gerapporteerd, blijkt een partiële coronaire arterie occlusie ook het myocard te kunnen preconditioneren, wanneer er geen tussenliggende periode van reperfusie plaatsvindt (hoofdstuk 4). Echter de flow reductie moet ernstig genoeg zijn. De ernst van de flow reductie tijdens de eerste fase (preconditionerings stimulus) is kritieker voor de cardioprotectie tegen de tweede fase (langdurige occlusie) dan de duur van de flow reductie. In hoofdstuk 4 hebben we aangetoond dat zowel een 30 minuten als een 90 minuten durende flow reductie van 30% faalde het myocard te preconditioneren, maar dat zowel een 30 minuten als een 90 minuten durende flow reductie van 70% de infarctgrootte, veroorzaakt door een 60 minuten totale coronair arterie afsluiting, wel kon beperken. Daarentegen toonden Schulz *et al* aan dat infarcering, veroorzaakt door een langdurige periode van lage-flow ischemie, beperkt wordt wanneer het onmiddelijk wordt voorafgegaan door een korte totale occlusie zonder een tussenliggende periode van reperfusie. Deze partiële occlusie studies bootst meer de klinische omstandigheden na dan zo'n abrupte occlusie-reperfusie prikkel in voorgaande studies. Een aantal observaties tonen aan die suggereren dat myocardiale bescherming getriggerd kan worden door (patho)fysiologische stimuli welke zelf geen myocardiale ischemie veroorzaken.

Przyklenk *et al* toonden in honden aan dat na een kortdurende coronair arterie occlusie de infarctgrootte niet alleen was afgenomen in het gepreconditioneerde myocard, maar ook in het aangrenzende "virgin" myocard ("remote intracardiac preconditioning"). Hun studie was de eerste die suggereerde dat het myocard niet direct aan ischemie bloodgesteld hoefde te worden om beschermd te raken tegen irreversibele schade veroorzaakt door een langdurige coronair arterie occlusie.

De observaties van Pryklenk *et al* zijn de aanzet geweest voor verschillende studies die de vraag of het myocard niet alleen beschermd kan worden door *remote intracardiac* ischemische preconditionering maar ook door *interorgan* ischemische preconditionering proberen te beantwoorden. In een voorlopige studie toonden we aan in geanesthetiseerde ratten, dat na een 15 minuten durende nier arterie occlusie en een 10 minuten durende tussenliggende reperfusie, het infarct veroorzaakt door een 60 minuten coronair arterie occlusie kleiner is dan het controle infarct. Kort hierna rees het vermoeden, dat de resultaten van onze laatste experimenten

waarschijnlijk beïnvloed zijn door de lichaamstemperatuur. Daarom hebben we in hoofdstuk 6 onze experimenten herhaald onder strengere temperatuur controle. Nu hebben we gevonden dat een 15 minuten nier arterie occlusie voorafgaand aan de 60 minuten coronair arterie afsluiting, de infarctgrootte tot 30% wordt beperkt onder hypothermische omstandigheden (30-31°C), maar dat het geen effect had op de infarctgrootte onder normothermische omstandigheden (36.5-37.5°C). Een 15 minuten durende darm arterie afsluiting, had hetzelfde beperkende effect op de infarctgrootte als een 15 minuten coronair arterie afsluiting tijdens normothermie.

In verschillende studies is aangetoond dat andere vormen van stress het myocard kan beschermen tegen infarcering *zonder* dat ischemie eraan te pas moet komen. Ovize *et al* demonstreerde dat stretch en verhoging van de wand stress van de linker ventrikel door acute volume belasting ook infarctgrootte kon beperken. Deze observatie suggereert, dat stretch van de "remote" myocard veroorzaakt door dyskinesis in het ischemische myocard heeft meegewerkt tot het ontstaan van remote intracardiac preconditionering. Het mechanisme van interorgaan preconditionering is nog steeds speculatief, want myocard stretch lijkt een onwaarschijnlijke factor om bijdrage te geven in interorgan preconditionering.

Ter ondersteuning van de observaties door Ovize *et al* dat niet-ischemische stimuli ook het myocard kan beschermen, hebben we in hoofdstuk 5 in varkens geobserveerd dat 30 minuten van ventrikel pacen op 200 slagen per minuut gevolgd door 15 minuten van normale sinus ritme, dit ook de infarctgrootte, veroorzaakt door 60 minuten coronair arterie occlusie, kan beperken van 84±7% (mean±SD) van de Area at Risk in de controle dieren naar 71±6%. Er werden zelfs kleinere infarctgroottes (63±13%) geobserveerd, wanneer 30 minuten van ventrikel pacen onmiddellijk wordt voorafgegaan aan de 60 minuten coronair occlusie zonder een tussenliggende periode van normale sinus ritme. Het beschermende effect van ventrikel pacen werd opgeheven door voorbehandeling met de K^+_{ATP} kanaal blokker glibenclamide. K^+_{ATP} kanaal aktivatie trad niet op als gevolg van myocard ischemie welke aangetoond kon worden door metabole en contractiliteits (functionele) metingen zoals de afwezigheid van post-systolische segment verkorting, een normale distributie van de transmurale bloeddoorstoming en de onveranderde myocardiale ATP-niveaus gedurende het ventrikel pacen en de afwezigheid van reactieve hyperemie en de onmiddellijke herstel van de systolische segment verkorting na het stoppen van het ventrikel pacen. Toediening van glibenclamide na de 30 minuten van ventrikel pacen op het moment van 15 minuten van normaal sinus ritme, kon niet de bescherming blokkeren, waaruit we kunnen concluderen dat K^+_{ATP} kanalen niet zelf een bijdrage leveren aan de bescherming, dat nog steeds aanwezig was 15 minuten na het stoppen van de ventrikel pacen. Deze bevindingen wijzen erop dat K^+_{ATP} kanaal aktivatie nodig was om de bescherming te triggeren, maar dat continuering van de activatie niet nodig was op het moment dat het myocard al in beschermende

staat verkeert. Dit laatste is in tegenstrijd met wat in ischemische preconditionering wordt gezien.

In tegen stelling tot Chien *et al* en Duncker *et al* vonden we in hoofdstuk 6 geen verschil in infarctgrootte tussen de lage temperatuurs groep (30°C-31°C) en de normale temperatuurs groep (36.5°C-37.5°C). We verklaarden dit door het verschil in de duur van de periode van coronaire arterie occlusie. In hoofdstuk 7 toonden we aan dat het infarctgrootte beperkende effect van lage lichaamstemperaturen inderdaad afhangt van de duur van de coronaire arterie occlusie. We konden dit beschermende effect alleen maar aantonen wanneer de duur van de coronaire arterie occlusie tussen de 20 en de 40 minuten was.

In hoofdstuk 8 beschrijven we de problematiek rondom de interpretatie van de studies die proberen de rol van de signaal transductie eiwit, protein kinase C, in de mechanismen van ischemische preconditionering aan te tonen of uit te sluiten. In een poging om een bijdrage te leveren aan het antwoord voor deze vraagstelling hebben we in hoofstuk 9 protein kinase C isozymen-α, -δ, -ϵ en -ζ en de totale protein kinase C activiteit bepaald in ratte myocard welke onderworpen werden aan een controle stimulus, een periode van myocardiale ischemie en reperfusie en een periode van dunne darm ischemie en reperfusie. Resultaten van deze studie tonen aan dat er inderdaad een translokatie/activatie van het δ isozyme van het cytosol plaatsvindt, echter deze is niet in de membraan fractie aan te tonen en de activiteits metingen kunnen deze bevinding ook niet ondersteunen.

In conlusie kunnen we zeggen dat de huidige studies suggereren dat het myocard beschermd kan worden door vormen van stress anders dan myocardiale ischemie. Deze houden onder andere stimuli in welke stress van het hart veroorzaken, maar dat niet leidt tot ischemie (stretch, ventrikel pacen) en stimuli welke distress (ischemie) veroorzaken in andere organen anders dat het hart. Ischemie in "remote organs" zoals nier, hersens, skeletspier en lever, kunnen ook deze organen preconditioneren. Het is duidelijk dat verschillende vormen van stress of distress in staat zijn processen te triggeren, welke de organen kan beschermen die zowel hebben blootgestaan aan deze stimulus, maar ook andere organen op afstand. Het zou er dus op kunnen lijken dat ischemische myocardiale preconditionering slechts één van de aspecten van een groter fenomeen is dat resulteert in orgaan bescherming. Toekomstige studies moeten daarom het ontrafelen van het mechanisme dat leidt tot orgaan bescherming welke dan farmacologisch nagebootst kan worden als doel hebben. Het is ook belangrijk om in gedachte te houden, dat niet alle vormen van (dis)stress (myocardiale hibernation, roken) resulteren in myocard bescherming. Ten slotte, zouden deze nieuwe gevonden beschermende stimuli, samen met de problemen van infarctgrootte determinatie in patiënten zoals ergens anders vermeld, verwarrende factoren

kunnen zijn in de het vinden van definitieve bewijs voor het voorkomen van ischemische myocardiale preconditionering in mensen. Als laatste wil ik nog vermelden dat de beste bescherming van het ischemische myocard nog altijd de beëindiging is van de ischemisch toestand door reperfusie van het bedreigde myocard.

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1 Duncker DJGM, Sassen LMA, Gho BCG and Verdouw PD. The central and regional cardiovascular responses to intravenous administration of the potassium channel opener EMD 52692. *Eur J Pharmacol* 1990;183(4): 1263-1264.

2 Sassen LMA, Duncker DJGM, Gho BCG and Verdouw PD. Haemodynamic profile of the novel potassium channel activator EMD 52692. *Br J Pharmacol* 1990;101:605-614.

3 McFalls EO, Duncker DJ, Sassen LMA, Gho BCG and Verdouw PD. The effect of antianginal therapy on coronary flow reserve and the maximal flow-pressure relationship in anesthetized swine, *J Cardiovasc Pharmacol* 1991;18:827-836.

4 Masuda M, Chang-Chun C, Gho BCG, Flameng W. Coronary reserve and contractile reserve in crystalloid- and blood-perfused rabbit hearts. *Heart and Vessels* 1994;9:175-182.

5 Sharma HS, Tang ZH, Gho BCG and Verdouw PD. Nucleotide sequence and expression of the porcine vascular endothelial growth factor. *Biochim Biophys Acta* 1995;1260:235-238.

6 Gho BCG, Koning MMG, Duncker DJ, Verdouw PD. Ischemic preconditioning: modalities and clinical relevance. *J Applied Cardiopulm Pathophysiol* 1995;5:117-193. (review).

7 Gho BCG, Koning MMG, Opstal RLJ, Van Klaarwater E, Duncker DJ, Verdouw PD. Novel Approaches to myocardial preconditioning in pigs. In: Myocardial Preconditioning. Editors: Wainwright CL, Parratt JR; Landes, R.G, Co., Austin, Texas. 1996;97-113.

8 Verdouw PD, Gho BCG, Duncker DJ. Ischemic preconditioning: is it clinically relevant? *Eur Heart J* 1995;16:1169-1176. (review)

9 Koning MMG, Gho BCG, Duncker DJ, Verdouw PD. Endocardial and epicardial infarct size after preconditioning by partial coronary artery occlusion without intervening reperfusion. *Cardiovasc Res* 1995;30:1017-1027.

10 Koning MMG, Gho BCG, Van Klaarwater E, Opstal RLJ, Duncker DJ, Verdouw PD. Rapid ventricular pacing produces myocardial protection by non-ischemic activation of K^+_{ATP}channels. *Circulation* 1996;93:178-186.

11 Gho BCG, Koning MMG, Duncker DJ, Verdouw PD. Myocardial protection by rapid ventricular pacing: a role for non-ischemic activation of K^+_{ATP}channels. *Thoraxcenter J* 1995;7:20-22.

12 Verdouw PD, Gho BCG, Duncker DJ. Cardioprotection by organs in stress in distress. *Basic Research in Cardiology* 1996;91:44-46.

13 Verdouw PD, Gho BCG, Duncker DJ. Cardioprotection by organs in stress in distress. In: New Paradigms of Coronary Artery Disease. Hibernation, Stunning, Ischemic Preconditioning. 1996;162-164. Editors: Heusch G and Schulz R; Steinkopff/Springer, Darmstadt, Germany.

14 Sharma HS, Maulik N, Gho BCG, Das DK, Verdouw PD. Coordinated expression of heme oxygenase-1 and ubiquitin in the porcine heart subjected to ischemia and reperfusion. In: Biochemistry of signal transduction in myocardium. Editors: Lamers JMJ and Verdouw PD; Kluwer Academic Publishers, Dordrecht/Boston/London. *Mol Cell Biochem* 1996;157:111-116.

15 Gho BCG, Schoemaker RG, van den Doel MA, Duncker DJ, Verdouw PD. Myocardial protection by transient ischemia in non-cardiac tissue.*Circulation* 1996;94:2193-2200.

16 Verdouw PD, Gho BCG, Schoemaker RG, Koning MMG, Duncker DJ. Cardioprotection by ischemic and nonischemic myocardial stress and ischemia in other organs. Implication for the concept of ischemic preconditioning. In: Myocardial Preservation, Preconditioning, and Adaptation. Eds: Das DK, Engelman RM, Cherian KM. *Ann NY Acad Sci* 1996;793:27-42.

17 Eskildsen-Helmond YEG, Gho BCG, Beztarosti K, Dekkers DHW, Soei LK, van Heugten HAA, Verdouw PD, Lamers JMJ. Exploration of the possible roles of phospholipase D and protein kinase C in the mechanism of ischemic preconditioning in the myocardium. In: Myocardial Preservation, Preconditioning, and Adaptation. Eds: Das DK, Engelman RM, Cherian KM. *Ann NY Acad Sci* 1996;793:210-225.

18 Gho BCG, Koning MMG, van den Doel MA, Duval SY, de Zeeuw S, Schoemaker RG, Duncker DJ, Verdouw PD. Cardiac adaptation in response to ischemic and non-ischemic stress. *Clin Exp Cardiol* 1996 (in press)

19 Gho BCG, Eskildsen-Helmond YEG, Lamers JMJ, Verdouw PD. Does potein kinase C play a pivotal role in the mechanisms of ischemic preconditioning. (In press)

20 Gho BCG, van den Doel MA, Duncker DJ, Schoemaker RG, Verdouw PD. Myocardial infarct size limiting effect of low body temperature in rats depends on the duration of the coronary artery occlusion.*Circulation* 1997. (Submitted as letter to the editor).