Brief Ischemia: a double-edged sword

Kortdurende Ischemie: het tweesnijdende zwaard

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Voor mijn ouders
Voor John

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1
Introduction

Myocardial ischemia

The heart is an organ that strongly depends on the supply of oxygen to preserve its viability and contractile function. Thus, even under optimal conditions anaerobic metabolism can only provide 7% of the ATP production that is required to maintain a normal contractile function. Since the oxygen storage capacity of the heart is minimal, a constant supply of oxygen by the arterial blood is mandatory. Under basal conditions, the extraction of oxygen from the blood is already 70-80% (compared to 20-40% in most organs), so that an increase in oxygen demand (up to five-fold during heavy exercise) simply cannot be met by an increase in oxygen extraction. Consequently, the increased oxygen demand is principally accommodated by an increase in flow of oxygenated blood to the myocardium. In the normal heart the vasodilator capacity of the coronary bed allows sufficient increases in flow, so that increases in oxygen demand do not result in anaerobic metabolism, even during strenuous exercise.[1] However, when an obstruction of the coronary artery impedes coronary blood flow and hence myocardial substrate and oxygen supply, the energy demand of the heart cannot be met and ischemia ensues. Acute myocardial ischemia results in anaerobic metabolism and loss of contractile function of the ischemic area and may lead to cardiac arrhythmias, including ventricular fibrillation, and loss of viability (myocardial necrosis) when left untreated.[2]

In virtually all people who develop myocardial ischemia, the underlying cause is coronary artery disease, due to atherosclerosis, for which smoking, high cholesterol and hypertension are some of the important risk factors. Acute myocardial ischemia is often caused by thrombus formation at the site of a partially stenosed artery. Thus, platelet aggregation at the site of the stenosis (often induced by plaque rupture) can occlude the coronary artery. When a thrombus (unstable angina pectoris) or vasospasm (Prinzmetal's angina pectoris) or a severe stenosis impairs blood flow so that the energy demand cannot be met at rest, ischemia results principally as a consequence of an impaired supply and is therefore termed *supply ischemia*. Conversely, a mild stenosis, which does not result in ischemia at rest, can become flow limiting during increased myocardial oxygen demand caused by exercise or positive chronotropic and/or inotropic stimulation of the heart. Since the resultant myocardial ischemia principally occurs during an increased oxygen demand, it is often referred to as *demand ischemia*.

Novel insights into the consequences of myocardial ischemia

Already many centuries ago clinical symptoms of myocardial ischemia, like chest pain and pain in shoulders and stomach, were noted in patients. At that time, however, these symptoms were not associated with disease of the heart, but rather related to disease of the stomach.[3] It was not until the early years of the twentieth century that Herrick[4] found a relation between persistent coronary occlusion and the pathological findings of myocardial necrosis. In

1935, Tennant and Wiggers[5] observed that following a short period (<3 min) of occlusion, contractile function recovered almost immediately upon reperfusion. The next 40 years researchers maintained the idea that myocardial ischemia, dependent on its duration, could only lead to two distinct consequences. If the ischemic period lasted only a few minutes, the damage was reversible and recovery of function was immediate. If, on the other hand, the ischemic period was prolonged, myocardium became irreversible damaged, resulting in myocardial necrosis and sustained impaired function. In 1975, these straightforward ideas were overhauled by the findings of Heyndrickx et al.[6], who found that after a 15 min period of myocardial ischemia in awake dogs contractile function remained depressed for several hours despite an immediate restoration of coronary blood flow and a normal electrocardiogram within minutes after reperfusion. Similar observations of delayed recovery of function in anesthetized dogs were initially received with scepsis and even considered an artefact.[7] It was not until seven years later, when Braunwald and Kloner[8] coined the term "*Myocardial Stunning*" to describe this phenomenon of "delayed recovery of regional myocardial contractile function after reperfusion despite the absence of irreversible damage and despite restoration of normal flow", that the idea that myocardial damage could be reversible and that impaired contractile function does not necessarily reflect necrosis became fully appreciated.[9]

Three different patterns in the responses to total coronary artery occlusions are currently delineated, which depend on the duration of occlusion. First, a coronary artery occlusion that lasts less than 2 min produces no damage at all, so that cardiac metabolism and contractile function return to normal almost immediately upon reperfusion.[5] Second, an occlusion that lasts between 2 and approximately 20 min results in reversible damage characterized by a delayed recovery of function upon reperfusion despite normal supply of oxygen and nutrients. The degree of stunning depends on the ischemic burden, i.e. the duration and severity of ischemia. After 5 min of myocardial ischemia produced by a total coronary artery occlusion in dogs, contractile function remained depressed for 3 hours, whereas after 15 min of myocardial ischemia stunning lasted up to 6 hours.[6] The severity of ischemia comprises both energy supply during ischemia (residual flow) and energy demand of the myocardium at the onset of ischemia. Several studies showed an inverse relationship between collateral blood flow during ischemia and myocardial stunning after a 10-min coronary artery occlusion occlusion in conscious dogs.[10,11] Moreover, in patients the presence of residual flow within jeopardized myocardium during ischemia correlates with the rapid functional recovery from myocardial stunning.[12] In pigs, which lack a native collateral circulation, 15 min of total coronary artery occlusion resulted in a complete loss of wall thickening,[13] whereas a 70% flow reduction lasting 1 hour resulted in a decrease of only 50% loss of systolic wall thickening.[14] Thus, residual flow during ischemia either via collaterals or a subtotal coronary artery occlusion attenuates stunning. The importance of energy demand at the onset of ischemia (often represented by the rate-pressure product) was suggested by observations that postischemic

contractile dysfunction is more pronounced in pentobarbital-anesthetized open-chest dogs (with heart rates of 150-175 beats/min) than in awake dogs (with heart rates of 100-110 beats/min).[15] However, the effect of heart rate may be due to modification of collateral blood flow, as Bolli et al.[11] found that although the rate-pressure product was related to the degree of stunning, when collateral blood flow was taken into account the influence became insignificant.

While in intact hearts ischemia lasting no longer than about 20 min results only in reversible damage, an ischemic period lasting more than 20 min produces cell death and myocardial infarction develops. One of the most prominent features of cell death is membrane disruption, indicating that ischemic injury has entered the lethal phase, and may be caused by phase separation of the phospholipidbilayer and activity of either Ca^{2+}-activated phospholipases and proteases and/or osmotic imbalance resulting in cell swelling.[2] It has also been suggested that norepinephrine, which is extensively released in the myocardial interstitium during ischemia, may be cardiotoxic.[16] Administration of high systemic doses of isoproterenol produced focal necrotic lesions in normal rat hearts.[17] In contrast, depletion of cardiac norepinephrine stores by reserpination, did not limit myocardial infarct size in rabbits[18] and dogs.[19] This hypothesis, however, is still controversial.

The fraction of the jeopardized myocardium that becomes infarcted depends on the duration of ischemia and the presence of collateral blood flow (fig. 1). In guinea pigs the collateral system is so well developed that proximal occlusion of a major coronary branch does not result in any myocardial necrosis. On the other hand, in rats, rabbits and pigs collaterals are virtually absent, resulting in complete infarction within 1-1½ hours.[20] In humans a moderate and variable number of collaterals (which have probably developed during adulthood) is found and infarct development is therefore more gradual. After 2 hours of ischemia 50% of the endangered area is infarcted and infarction is complete after 6 hours of ischemia.[21] Myocardial oxygen demand at the onset of ischemia was originally thought to be an important determinant of infarct size, as some studies suggested that myocardial oxygen demand before ischemia influences infarct size.[22-24] Nevertheless, most of these studies are done in dogs and the results can be explained by the presence of collateral flow, which is affected by both heart rate and bloodpressure.[25] Moreover, in studies by the group of Schaper the bradycardia-induced decrease in myocardial oxygen consumption was achieved by either cholinergic stimulation[23] or infusion of a synthetic opiate,[24] which were later shown to be cardioprotective (see preconditioning).

Body core temperature also exerts an important effect on myocardial infarct size, as in pigs an increase of the temperature of 1°C increased infarct size by 20%.[25] Although hypothermia produces significant decreases in heart rate in rodents, the decrease in myocardial oxygen demand is not related to the positive relation between infarct size and temperature.[26,27] The mechanism by which temperature influences infarct size is uncertain, but occurs most

likely via reduction of the basal metabolism with consequent slowing of high energy phosphate depletion, as all reaction rates are highly sensitive to temperature. Other mechanisms that may be responsible for the hypothermia-mediated protection, include reduced acidosis, and/or maintenance of ion homeostasis during ischemia and reperfusion.[25]

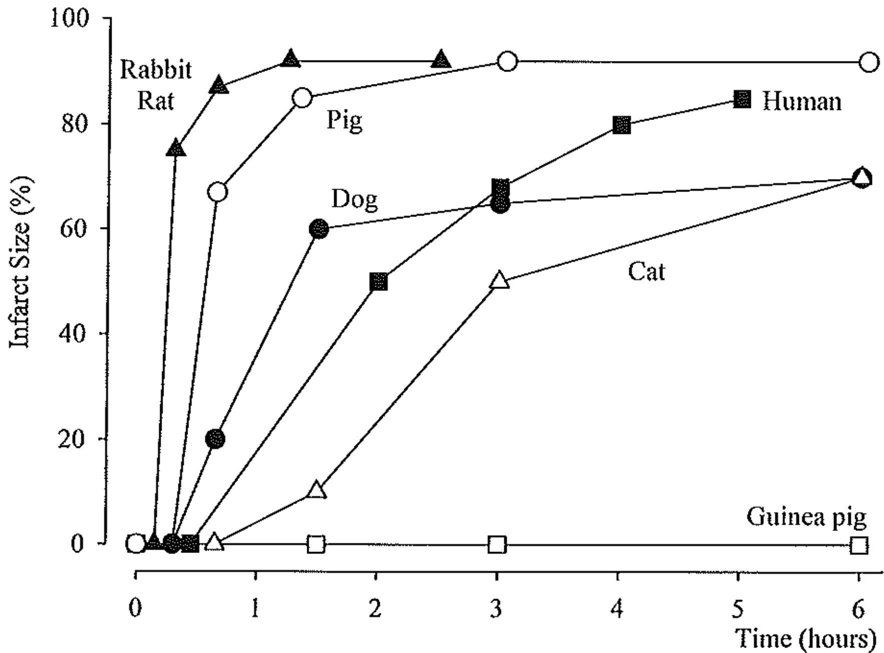

Figure 1 Development of irreversible myocardial damage during total coronary artery occlusion in different species (modified from Schaper et al.[20] and Arnold et al.[21]).

It was long believed that sustained periods of myocardial ischemia always resulted in irreversible myocardial damage. However, with the advent of revascularization therapy, evidence accumulated that chronically impaired contractile dysfunction was not always the consequence of infarction. Thus, restoration of blood flow by bypass grafting could restore myocardial function in patients with chronically depressed function due to coronary artery disease.[28] This phenomenon of chronic left ventricular dysfunction in the absence of ongoing metabolic evidence of ischemia may represent a self-protective downregulation in myocardial function and metabolism in order to abort evolution toward cell death despite chronic hypoperfusion. Diamond et al.[29] introduced this concept and Rahimtoola et al.[30] later popularized this as *myocardial hibernation*. During the first few minutes of ischemia the energy supply-demand imbalance results in a decrease of CrP contents, lactate consumption

and myocardial contractile function. Myocardial contraction decreases slightly further which then in turn downregulates the energy demand, leading to a gradual recovery of CrP content and lactate consumption to preischemic levels while contractile function remains depressed and necrosis does not occur.[31,32] Hibernating myocardium retains its responsiveness to inotropic stimulation, however, at the expense of metabolic recovery, resulting in a new supply-demand imbalance.[33] In the experimental setting hibernation can be achieved by severe flow reduction for several hours, for example 50% flow reduction for 5 hours results in a 40% decrease in contractile function and delayed but complete recovery of function within 1 week of reperfusion.[31] This short-term hibernation can be distinguished from stunning by measuring blood flow, which is reduced in hibernation and normal in stunning, or measuring metabolic changes associated with inotropic stimulation, which is at the expense of metabolic recovery in hibernation, while in stunning no metabolic deterioration occurs.[34] Long-term hibernation can only be inferred from clinical studies and is associated with ultrastructural alteration in cardiomyocytes, including loss of contractile material within the remaining cardiomyocytes, glycogen deposits, depletion of sarcoplasmic reticulum and increased interstitial fibrosis and possibly loss of cardiomyocytes.[35] Until now, an experimental model for chronic hibernation has not been found because of the difficulty of excluding repetitive episodes of exercise-induced ischemia providing an alternative explanation for the observed chronic contractile dysfunction during persistent coronary stenosis.[34] On the other hand, it should be questioned whether a model of pure chronic hibernation is necessary as in patients coexistence of hibernation and stunning often occurs. It is more likely that coronary blood flow will fluctuate, because severe coronary artery stenosis causes loss of coronary autoregulation, which will result in brief periods of ischemia.[36] At the present time, the mechanisms responsible for the development of short-term hibernation are not clear. Alterations in ß-adrenoceptor density or affinity, activation of ATP-sensitive K^+ channels or endogenous adenosine have all been excluded.[37,38] It has been suggested that Ca^{2+} transients are reduced[39] or that the Ca^{2+} responsiveness of the myofilaments is decreased, due to a decrease in maximal developed force.[40]

Patients with angina pectoris who develop myocardial infarction often experience ischemia in the preceding hours to days. It was initially believed that these preceding periods of ischemia added to the ischemic burden possibly resulting in a larger infarct size. Paradoxically, however, Murry et al.[41] observed that when dogs were subjected to a 40 min coronary artery occlusion 29% of the ischemic area was infarcted, but only 7% when the sustained period of ischemia was preceded by four cycles of 5 minutes of coronary artery occlusion and 5 min of reperfusion. This cardioprotective effect of short periods of ischemia was termed *ischemic preconditioning*, which also explains earlier findings of attenuated metabolic ischemia during a second flow reduction in the swine heart. Not also has the discovery of this phenomenon revived interest in the development of novel cardioprotective

strategies, but also shows that brief periods of myocardial ischemia can be either detrimental (stunning) or protective (ischemic preconditioning) to the heart. In this thesis both stunning and ischemic preconditioning are studied.

Stunning

Stunning, although originally described in a conscious animal model, has been the focus of numerous studies in particular in intact anesthetized animal models and isolated (rat) heart studies. Evidence for the occurrence of stunning in man has been found in a variety of conditions. For example, using positive inotropic agents, myocardial dysfunction was succesfully restored in patients after open heart surgery.[42,43] With the current knowledge on the pathogenesis of dysfunction it is logical to assume that stunning may be the underlying cause, because stimulation with positive inotropic agents in the presence of ischemia would more often lead to irreversible damage and to chronic impairment of function. In addition, after unstable angina Wijns et al.[44] found that flow values are normal and identical between normally contracting and dysfunctional segments. Stunning also coexists with myocardial infarction as thrombolysis or coronary angioplasty in patients with myocardial infarction salvaged a significant amount of myocardium.[45] Therefore, despite a lower sensitivity of blood flow and contractile function measurements in man compared to animal models, there is sufficient evidence that stunning does occur in humans. Several clinical investigators consider stunning not as a serious clinical problem, as the postischemic myocardial dysfunction eventually restores without pharmacological intervention. However, it has also been suggested that repeated episodes of ischemia with subsequent recurrent stunning can induce myocardial hibernation[46] and hence stunning does appear to be of clinical importance.

Pathogenesis of myocardial stunning

The observations by Heyndrickx et al.[6] triggered tremendous research efforts to unravel the mechanisms of stunning which have greatly contributed to our current knowledge about the role of many of the cellular processes during and after ischemia. Over the years a number of mechanisms have been forwarded to explain stunning, including loss of and reduced ability to synthesize high-energy phosphates, impairment of microvascular perfusion, impairment of sympathetic neural responsiveness, generation of oxygen derived free radicals, activation of leukocytes, reduction in the activity of creatine kinase, and disturbances in Ca^{2+} homeostasis.[47] At the present time, the release of oxygen free radicals and Ca^{2+} overload are considered to be key events in the pathogenesis of stunning. However, there are several controversies regarding these triggers and modulators that remain to be solved.

During ischemia metabolites such as lactate accumulate, resulting in a decrease in pH. Under normal conditions the michondria neutralize the increase in intracellular H^+. However,

during ischemia the Na^+/H^+ exchanger is the primary mechanism to restore intracellular pH, resulting in high intracellular Na^+. Because less intracellular ATP is available during ischemia, the Na^+/K^+ pump is unable to adequately eliminate the increased Na^+. Consequently, the Na^+/Ca^+ exchanger reverses its function and exchanges Na^+ for Ca^{2+}, resulting in increased intracellular Ca^{2+}. This mechanism, however, is of short duration as massive amounts of extracellular H^+ inhibit the Na^+/H^+ exchanger. At the onset of reperfusion, the extracellular fluid is replaced by a more alkalotic media, resulting in a decreased extracellular H^+ concentration and inhibition of the Na^+/H^+ exchanger is no longer occurring.[48] The role of the Na^+/Ca^+ exchanger is supported by the finding that Na^+/H^+ antiporters given only during reperfusion can attenuate contractile dysfunction.[49,50] Thus, upon reperfusion the Ca^{2+} levels do not immediately recover, but even increase further via the above mentioned mechanism of restoration of intracellular H^+ concentration.

The role of voltage dependent Ca^{2+} channels for the Ca^{2+} overload at the onset of reperfusion is studied using Ca^{2+} antagonists mainly before ischemia and at the onset of reperfusion. Ca^{2+} antagonists administered either before ischemia or prior to reperfusion attenuate myocardial stunning in isolated perfused hearts.[51] *In vivo* studies also support a role for Ca^{2+} in myocardial stunning since numerous studies have shown a beneficial effect of Ca^{2+} antagonists.[51,52] However, *in vivo* studies could not show a beneficial effect when Ca^{2+} antagonists were administered just prior to or at the onset of reperfusion, questioning the importance of an increase in cytosolic Ca^{2+} during early reperfusion.[52] Explanations for these findings could be that the residual flow via collaterals is not sufficient enough to get adequate levels of Ca^{2+} antagonists in the myocardium, or that the voltage dependent Ca^{2+} channels are less important for an increase in Ca^{2+} during early reperfusion than Na^+/Ca^{2+} exchanger.

During the initial minutes of reperfusion, not only the intracellular free Ca^{2+} concentration increase further but also oxygen free radicals are produced. Administration of anti-oxidants during occlusion or just prior to reperfusion diminishes myocardial stunning, whereas anti-oxidants administered 1 min after the onset of reperfusion have no effect on myocardial dysfunction.[53] Direct measurement by spin trap and electron paramagnetic resonance spectroscopy has also shown production of oxygen free radicals in the initial minutes of reperfusion.[54] With these techniques a linear positive relation was noted between the magnitude of free radical production and the severity of stunning, dependent on the duration of ischemia and the amount of collateral blood flow. In dogs xanthine oxidase may be a source of oxygen free radicals, but its role in rabbits, swine and humans has been questioned as these species reportedly have negligible levels of this enzyme.[55] The role of oxygen free radicals is also confirmed by the observation that ACE inhibitors may attenuate stunning by direct scavenging of oxygen derived free radicals and bradykinin-mediated release of prostacyclin.[56] The exact mechanism whereby oxygen free radicals cause stunning is still in debate, but is thought to be mainly targeted on proteins and lipids, leading eventually to a

decrease in the Ca^{2+} responsiveness of myofilaments by two distinct mechanisms. First, directly by oxidative modification of contractile proteins. Secondly, indirectly by damage to the sarcolemma and sarcoplasmic reticulum which results in transient Ca^{2+} overload.[57] Sarcolemmal damage results in loss of selective permeability, impairment of Ca^{2+}-stimulated ATPase activity and Ca^{2+} transport out of the cell, and impairment of Na^+/K^+ ATPase activity with consequently Na^+ overload and Ca^{2+} overload. In the sarcoplasmic reticulum, impairment of the Ca^{2+}-stimulated ATPase activity and Ca^{2+} uptake results in decreased Ca^{2+} sequestration and Ca^{2+} overload (for detailed references see Bolli and Marban[58]).

The consequences of oxygen free radicals and Ca^{2+} overload are still in debate and involve disturbance in Ca^{2+} homeostasis and decreased responsiveness of the myofilaments to Ca^{2+}. Evidence for a decreased availability of Ca^{2+} is controversial as *in vitro* studies showed either decreased[59], unchanged[60,61] or increased[62,63] Ca^{2+} transients. Studies on reduced Ca^{2+} responsiveness are more conclusive. The increase in intracellular Ca^{2+}, either caused by the Na^+ overload or the oxygen free radicals, activates Ca^{2+}-activated proteases (calpains), which damages the contractile proteins, resulting in a decrease in myofilament Ca^{2+} responsiveness. This was supported by the study of Gao et al.[64], who subjected skinned trabeculae 20 minutes to Calpain I. Force-[Ca^{2+}] relations showed a reduced F_{max} and an increased Ca_{50} after exposure, which could be prevented by calpastatin, a specific calpain inhibitor. Other *in vitro* studies also showed a decrease in maximal force of stunned myocardium[60,61] and additionally a decrease in the Ca^{2+} sensitivity.[60] Thus, *in vitro* studies show a decreased Ca^{2+} responsiveness of the myofilaments, however *in vivo* evidence is still lacking.[40]

Treatment of stunning

Although contractile function of stunned myocardium eventually recovers completely, it may temporarily produce critical impairment of global left ventricular function when the stunned area encompasses a large region of the heart or when the heart is already failing. Considering the role of oxygen free radicals, it is not surprising that agents exhibiting free radical scavenging properties attenuate stunning.[53] Also agents that limit Ca^{2+} overload during ischemia and early reperfusion, such as Ca^{2+} antagonists and Na^+/H^+ exchange inhibitors, can diminish postischemic dysfunction.[49-52] These agents, however, should ideally be adminstered before ischemia, but always before reperfusion. Once stunning has been established only positive inotropic agents (β-adrenoceptor agonists, phosphodiesterase (PDE)-III inhibitors, Ca^{2+} and Ca^{2+} agonists) result in complete recruitment of contractile function. However, after infusion of these positive inotropic agents stops, contractile function returns to pre-infusion levels, indicating that contractility is increased without modifying the mechanism that underlies stunning, but also indicating that β-agonists are capable of recruiting contractile reserve of stunned myocardium without deleterious effects.[65]

Conventional inotropic agents, however, also possess some disadvantages. The increase in Ca^{2+} transients after treatment with PDE-III inhibitors, Ca^{2+} or Ca^{2+} agonists may lead to arrhythmias. Moreover, the positive inotropic capacity of PDE-III inhibitors is diminished in heart failure, probably due to reduced cAMP production by the adenylyl cyclase.[66,67] Because of this negative outcome, Ca^{2+}sensitizers which should be devoid of the risk of Ca^{2+} overload-induced arrhythmias have received increasing interest. As it is thought that stunning is caused by a decrease in the Ca^{2+} sensitivity of the myofilaments, restoration of the contractile function of stunned myocardium by Ca^{2+} sensitizers seemed a logical approach. The advantage of Ca^{2+} sensitizers over conventional agents is that Ca^{2+} sensitizers increase force without an increase in the Ca^{2+} transients, thereby avoiding Ca^{2+} overload-induced arrhythmias. In addition, Ca^{2+} sensitizers do not increase energy consumption, because the rate of Ca^{2+} uptake into the sarcoplasmic reticulum and myofibrillar ATPase, two processes that use ATP, are unchanged. Conversely, a concern is that an increase in the Ca^{2+} sensitivity could adversely affect diastolic function, including slowed relaxation and increased end-diastolic stiffness, which results in impairment of left ventricular filling and hence cardiac pump function. Addition of mild PDE inhibition could therefore be favorable as an increase in cAMP improves relaxation by facilitating the release of Ca^{2+} from troponin C and stimulation of Ca^{2+} uptake by the sarcoplasmic reticulum.

Until now, numerous Ca^{2+} sensitizers have been developed, which all have different sites of action. Ca^{2+} sensitizers can modify the binding of Ca^{2+} to troponin C by increasing the affinity or prolong the duration of the Ca^{2+}-induced conformation (pimobendan, levosimendan, MCI-154 and EMD 60263), modify the interaction between the various components of the myofilaments (pimobendan, MCI-154) or alter actin-myosin cross-bridge kinetics (EMD 57033). Next to the Ca^{2+} sensitizing properties, most of these agents also have other properties, like PDE-III inhibition (pimobendan, levosimendan and MCI-154) or inhibition of the delayed inward rectifier K^+ current (EMD 60263).

Preconditioning

Ischemic preconditioning

Following the experimental discovery of ischemic preconditioning the existence of this phenomenon has been verified in several animal species including the pig[68], rat[69,70] and rabbit[71]. Direct evidence for the occurrence of ischemic preconditioning in patients is not available, because of the inability to accurately determine the different occlusion and reperfusion phases and the extent of collateralization. Also, infarct size cannot be measured directly, but is assessed by indirect parameters such as enzyme leakage, survival or left ventricular function.[72] Although the existence of ischemic preconditioning in humans is difficult to demonstrate several *in vitro*[73,74] and *in vivo*[75,76] studies have confirmed this

ischemic syndrome in humans. Based on total enzyme release, improved left ventricular function and a lower in-hospital mortality rate, it has been shown that patients who experience angina before acute myocardial infarction have smaller infarct sizes.[77-79] Ischemic preconditioning can be a confounding factor in clinical studies assessing the effectiveness of reperfusion therapy on infarct size, as the infarct size can be influenced by previous periods of ischemia. Moreover, insights into the mechanisms underlying ischemic preconditioning could potentially help to develop pharmacological agents which mimic preconditioning.[72]

Initially, it was thought that stunning was involved in the mechanism underlying preconditioning. Because of the depressed contractile function, the energy requirements were decreased, which would result in a reduced rate of energy untilization. However, the study by Murry et al.[80] showed that in dogs after 15 min coronary artery occlusion followed by 120 min reperfusion, myocardial stunning still persisted despite attenuation of preconditioning.

Downey et al.[81] were the first to propose that adenosine plays a role in cardioprotection, after which a lot of studies were done focussing on cellular mechanisms. For example adenosine, bradykinin and norepinephrine, which are released during myocardial ischemia, all have their actions via activation of G-protein coupled receptors, leading to activation of protein kinase C. The latter is thought to play a central role in ischemic preconditioning. However, the role of protein kinase C is still in debate as controversial evidence is found in several species. Activation of protein kinase C is believed to lead to opening of the ATP-sensitive K^+ channels in the sarcolemma and mitochondria that protects the heart against a subsequent ischemic period. Opening of the sarcolemmal ATP-sensitive K^+ channels result in shortening of the action potential duration and consequently less Ca^{2+} overload.[82] Opening of the ATP-sensitive K^+ channels in the inner mitochondrial membrane leads to membrane depolarization, matrix swelling, slowing of the ATP synthesis and accelerated respiration.[83] The role of ATP-sensitive K^+ channels in ischemic preconditioning is supported by several studies in which ischemic preconditioning was abolished after administration of glibenclamide or 5-hydroxydecanoate, which block the ATP-sensitive K^+ channels in several species.[84-89]

In experimental models, cardioprotection was afforded by total coronary occlusion followed by an abrupt reperfusion, a rare feature in patients. Ovize et al.[90] determined whether moderate ischemia by partial occlusion could also protect the heart and found that a flow reduction of 50% for 15 min could reduce infarct size, provided that complete reperfusion was given between the partial occlusion and the subsequent sustained occlusion. Based on the two-stage Harris model, Koning et al.[91] showed that 30 or 90 min of severe (70%), but not mild (30%) flow reduction protected against myocardial infarction without intermittent reperfusion. Thus, the degree of ischemia has to be severe enough to induce cardioprotection. The hypothesis of a threshold to protect the heart was proposed by the group of Downey[92]. They suggested that during one cycle of ischemic preconditioning several metabolites are released such as adenosine and bradykinin, which play a major role in triggering protection by having

additive effects on protein kinase C stimulation. Blockade of bradykinin results in a subthreshold for cardioprotection, which can be overruled by more preconditioning stimuli resulting in additional release of adenosine and other metabolites.

Non-ischemic preconditioning

Besides brief myocardial ischemia, several stimuli that are not associated with ischemia have been shown to afford cardioprotection. For example, stretching the heart by volume overload protects the heart against sustained myocardial ischemia via activation of stretched activated channels, likely through a mechanism that involves downstream activation of protein kinase C, adenosine receptors, and/or ATP sensitive K^+ channels.[93,94] Also, in pigs increasing the heart rate to 200 bpm for a period of 30 minutes without myocardial ischemia protected the heart, and could be blocked by glibenclamide, suggesting a mechanism via opening of the ATP sensitive K^+ channels[95]. Domenech et al.[96] could mimic preconditioning by 5 brief periods of tachycardia in dogs, which was not due to changes in collateral flow nor activity or translocation of protein kinase C, but mediated by adenosine. Additionally, the role of ATP-sensitive K^+ channels has also been confirmed in cardioprotection by heat stress.[97-99] Thus, the mechanism by which nonischemic stimuli give cardioprotection is comparable to the mechanism of ischemic preconditioning.

Not surprisingly pharmacological agents that stimulate one or more of the cellular components involved in the mechanism of ischemic preconditioning can also produce cardioprotection. Infusion of the ATP sensitive K^+ channel opener before the sustained ischemic period decreased infarct size in anesthetized pigs, whereas infusion after the onset of ischemia and during reperfusion had no effect.[87,100] Other pharmacological agents that can protect the heart are protein kinase C activators[101,102], norepinephrine[103] and adenosine.[104,105] The role of the latter has been confirmed in several species like pigs and rabbits, but its role in rats is still controversial.

Remote preconditioning

In the above mentioned studies the preconditioning stimulus and the sustained period of ischemia involved the same region of the heart. However, cardioprotection can also be provoked by ischemia in other regions of the heart or even other organs, which is known as *remote preconditioning*. Przyklenk et al.[106] reported that ischemia in the area perfused by the left circumflex coronary artery could also protect the adjacent area perfused by the left anterior descending coronary artery. This can be explained by activation of stretch-activated channels, because severe myocardial dysfunction leads to stretching of the adjacent non-ischemia myocardium.[93] Also, ischemia in other organs such as kidneys, intestines (abdominal angina) or skeletal muscles prior to a sustained occlusion of the coronary artery also limited infarct development, most likely via a neurogenic pathway activated during reperfusion, but also

involving adenosine and ATP-sensitive K^+ channels.[107-109] Moreover, the combination of flow restriction in the femoral artery and stimulation of the gastrocnemius muscle, a clinical analogue in patients with claudicatio intermittens, resulted in demand ischemia and consequently cardioprotection in the heart.[110]

Studies on the interaction between the brain and the heart are also available and has shown that brain ischemia, induced by subarachnoidal hemorrhage or transient ischemic attacks (TIA), causes a transient decrease in myocardial contractile function,[111,112] also known as neurogenic stunning. However, the effect of brain ischemia on the development of myocardial infarct size during coronary artery occlusion has not yet been determined.

Aim and outline of the thesis

The general aim of this thesis is to study the various aspects of brief periods of myocardial ischemia on the heart muscle. Several animal models for the study of myocardial ischemia are available. Therefore, in *chapter 2* the models that can be used to study myocardial ischemia are extensively discussed: ischemia versus hypoxia, global versus regional, *in vitro* versus *in vivo*. Also, in this review the three different ischemic syndromes stunning, preconditioning and hibernation are outlined in more detail.

Rational therapy of established myocardial stunning consists of administration of inotropic agents, in particular Ca^{2+} sensitizers. However, several *in vitro* studies reported a Ca^{2+} sensitization induced impairment of relaxation in normal[113-115] or stunned[116] myocardium. Importantly, in *in vivo* studies showing no adverse effect of Ca^{2+} sensitizing agents on diastolic function, the agents used not only possess Ca^{2+} sensitizing but also phosphodiesterase III (PDE-III) inhibitory properties. Currently, there are no *in vivo* studies in which the effect of Ca^{2+} sensitizing agents without PDE-III inhibitory properties on diastolic function of regionally stunned myocardium has been evaluated. Consequently, in *chapter 3* we describe the effects of the Ca^{2+} sensitizer EMD 60263, which is devoid of PDE-III inhibitory properties, on diastolic function of stunned myocardium. The study focuses mainly on early diastolic changes in regional myocardial segment dynamics. However, since EMD 60263 decreases heart rate via blockade of the delayed inward rectifier K^+ current, experiments were performed at spontaneous sinus rhythm, but also after restoration of heart rate via atrial pacing. Moreover, since disturbances in diastolic function are more likely to be aggravated at decreased diastolic intervals we also studied the effects of EMD 60263 after a further increase in heart rate.

It has been suggested that the inotropic response to Ca^{2+} sensitizers may differ between stunned and normal myocardium. For instance, it has been shown that after infusion of EMD 60263 the increase in regional systolic shortening in stunned myocardium of anesthetized pigs was much more pronounced than that in normal myocardium.[117] However, systolic shortening

is strongly load-dependent, even more so in stunned myocardium, and does not necessarily reflect myocardial contractility[118] Therefore, in *chapter 4,* we employed the regional LV pressure-segment length relationship in analogy to the time varying elastance concept to examine both systolic and diastolic function in a load-independent manner. In *chapter 3* we observed that the inhibition of the delayed inward rectifier current by EMD 60263 via prolongation of the action potential duration modified systolic and diastolic responses. Therefore, we used the Ca^{2+} sensitizer EMD 57033, which is devoid of the delayed inward rectifier K^+ current blocking properties. However, EMD 57033 does exert some PDE-III inhibitory actions in addition to its Ca^{2+} sensitizing effects.

In several *in vitro* and *in vivo* studies (including the studies of *chapter 3 and 4*) the beneficial effects of Ca^{2+} sensitizing agents on stunned myocardium have been confirmed, but in none of these studies it was actually shown that during stunning the myocardial responsiveness to added Ca^{2+} was altered and that subsequent administration of Ca^{2+} sensitizers restored the responsiveness to added Ca^{2+}. Therefore, in *chapter 5* we studied the effect of EMD 57033 on the responsiveness of normal and stunned myocardium to intracoronary Ca^{2+} infusions *in vivo.*

Brief periods of ischemia not only result in stunning, but also protects the heart against a subsequent long period of ischemia (ischemic preconditioning). In *chapter 6* an overview is given of ischemic preconditioning. Several aspects are discussed like non-ischemic stimuli, remote preconditioning and the effect of temperature on cardioprotection. A possible trigger for preconditioning is adenosine, which is a breakdown product of ATP and released during ischemia. The role of adenosine in ischemic preconditioning has been confirmed in several species like pigs, dogs and rabbits.[119] Three adenosine receptor subtypes (A_1, A_3 and A_2) have been characterized to date, of which A_1 and A_3 receptors are thought to be involved in ischemic preconditioning.[120-123] In rats, evidence on the role of adenosine and receptor subtypes is still controversial.[124,125] Therefore in *chapter 7* the role of adenosine A_1 and A_3 receptors in ischemic preconditioning in anesthetized rats is studied.

Norepinephrine, released during myocardial ischemia, is also one of the mediators involved in the signaling pathway leading to ischemic preconditioning. The release mechanism of catecholamines during regional ischemia was studied using a relatively new technique, called microdialysis. With this technique, based on diffusion of molecules through a semi-permeable membrane, the local interstitial levels of norepinephrine can be measured. The advantage of this method above the conventional method of coronary venous sampling and biopsies is that coronary venous sampling is an indirect measurement and cannot be performed during total coronary occlusion and biopsies comprise not only interstitial fluid, but also cardiomyocytes and sympathetic nerve endings. Microdialysis measured within the myocardium is not dependent on blood flow and thus can be used during total coronary occlusion. In addition, this technique makes it possible to measure continuously and locally.

Using an *in vivo* porcine model, the time course and magnitude of changes in interstitial concentrations of norepinephrine during severe myocardial ischemia and reperfusion was investigated (*chapter 8*).

Remote preconditioning of the heart has been observed to be induced by ischemia in the kidneys, small intestines and skeletal muscle. Brief periods of ischemia also occur in the brain, better known as transient ischemic attacks. As cerebral ischemia has been reported to cause an excessive release of catecholamines from sympathetic nerve endings in normal myocardium, the question can be raised whether transient cerebral ischemia prior to a prolonged period of myocardial ischemia may also be cardioprotective. In *chapter 9* the effect of cerebral ischemia on infarct development is tested with special focus on norepinephrine as possible mediator of this remote preconditioning. As the role of norepinephrine has not yet been investigated in pigs, it was also tested whether exogenous administration of norepinephrine can protect the heart.

References

1. Laughlin MH, Korthuis RJ, Duncker DJ, Bache RJ. Control of blood flow to cardiac and skeletal muscle during exercise. In: Rowell L, Shepherd J, eds. *Handbook of Physiology*. New York: Oxford University Press; 1996:705-769.
2. Reimer KA, Jennings RB. Myocardial Ischemia, Hypoxia and Infarction. In: Fozzard AF, Haber E, Jennings RB, Katz AM, Morgan HE, eds. *The Heart and Cardiovascular System*. New York: Raven Press; 1992:1875-1973.
3. Heusch B, Guth B, Heusch G. A brief history of angina pectoris: change of concepts and ideas. In: Heusch G, ed. *Pathophysiology and rationale pharmacotherapy of myocardial ischemia*. Darmstadt: Steinkopff Verlag; 1990:1-9.
4. Herrick JB. Clinical features of sudden obstruction of the coronary arteries. *Jama*. 1983;250:1757-1765.
5. Tennant R, Wiggers C. The effect of coronary occlusion on myocardial contraction. *Am J Physiol*. 1935;112:351-361.
6. Heyndrickx GR, Millard RW, McRitchie RJ, Maroko PR, Vatner SF. Regional myocardial functional and electrophysiological alterations after brief coronary artery occlusion in conscious dogs. *J Clin Invest*. 1975;56:978-985.
7. Lerch R, Villiger B, Riedhammer H, Meier W, Rutishauser W. Left ventricular function and creatine phosphate content during reperfusion (abstract). *Circulation*. 1975;51 (supplement II):II-208.
8. Braunwald E, Kloner RA. The stunned myocardium: prolonged, postischemic ventricular dysfunction. *Circulation*. 1982;66:1146-1149.
9. Duncker DJ, Schulz R, Ferrari R, Garcia-Dorado D, Guarnieri C, Heusch G, Verdouw PD. "Myocardial stunning" remaining questions. *Cardiovasc Res*. 1998;38:549-558.
10. Wouters PF, Van de Velde M, Van Aken H, Flameng W. Ischemic event characteristics determine the extent of myocardial stunning in conscious dogs. *Basic Res Cardiol*. 1996;91:140-146.
11. Bolli R, Zhu WX, Thornby JI, O'Neill PG, Roberts R. Time course and determinants of recovery of function after reversible ischemia in conscious dogs. *Am J Physiol*. 1988;254:H102-H114.
12. Lim YJ, Masuyama T, Mishima M, Fukui O, Nakatani D, Kawano S, Sakata Y, Kodama K, Hori M. Effect of pre-reperfusion residual flow on recovery from myocardial stunning: a myocardial contrast echocardiography study. *J Am Soc Echocardiogr*. 2000;13:18-25.
13. de Zeeuw S, Trines SAIP, Krams R, Verdouw PD, Duncker DJ. Cardiovascular profile of the calcium sensitizer EMD 57033 in open-chest anaesthetized pigs with regionally stunned myocardium. *Br J Pharmacol*. 2000;129:1413-1422.
14. Fallavollita J, Trojan C, Canty JJ. Transmural distribution of FDG uptake in stunned myocardium. *Am J Physiol*. 2000;279:H102-H109.

15. Triana JF, Li XY, Jamaluddin U, Thornby JI, Bolli R. Postischemic myocardial "stunning". Identification of major differences between the open-chest and the conscious dog and evaluation of the oxygen radical hypothesis in the conscious dog. *Circ Res.* 1991;69:731-747.
16. Rona G. Catecholamine cardiotoxicity. *J Mol Cell Cardiol.* 1985;17:291-306.
17. Rona G, Boutet M, Huttner I, Peters H. Pathogenesis of isoproterenol-induced myocardial alterstions. Functional and morphological correlates. In: Dhalla N, ed. *Recent advances in studies on cardiac structure and metabolism.* Baltimore: University Park Press; 1975:507-525.
18. Toombs CF, Wiltse AL, Shebuski RJ. Ischemic preconditioning fails to limit infarct size in reserpinized rabbit myocardium. Implication of norepinephrine release in the preconditioning effect. *Circulation.* 1993;88:2351-2358.
19. Vander Heide RS, Schwartz LM, Jennings RB, Reimer KA. Effect of catecholamine depletion on myocardial infarct size in dogs: role of catecholamines in ischemic preconditioning. *Cardiovasc Res.* 1995;30:656-662.
20. Schaper W, Gorge G, Winkler B, Schaper J. The collateral circulation of the heart. *Prog Cardiovasc Dis.* 1988;31:57-77.
21. Arnold AE, Simoons ML. 'Expected infarct size without thrombolysis', a concept that predicts immediate and long-term benefit from thrombolysis for evolving myocardial infarction. *Eur Heart J.* 1997;18:1736-1748.
22. Reimer KA, Jennings RB, Cobb FR, Murdock RH, Greenfield Jr. JC, Becker LC, Bulkley BH, Hutchins GM, Schwartz Jr. RP, Bailey KR, et al. 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.
23. 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.
24. Nienaber C, 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;78:210-226.
25. Duncker DJ, Klassen CL, Ishibashi Y, Herrlinger SH, Pavek TJ, Bache RJ. Effect of temperature on myocardial infarction in swine. *Am J Physiol.* 1996;270:H1189-H1199.
26. Chien GL, Wolff RA, Davis RF, van Winkle DM. "Normothermic range" temperature affects myocardial infarct size. *Cardiovasc Res.* 1994;28:1014-1017.
27. van den Doel MA, Gho BC, Duval SY, Schoemaker RG, Duncker DJ, Verdouw PD. Hypothermia extends the cardioprotection by ischaemic preconditioning to coronary artery occlusions of longer duration. *Cardiovasc Res.* 1998;37:76-81.
28. Rahimtoola SH. A perspective on the three large multicenter randomized clinical trials of coronary bypass surgery for chronic stable angina. *Circulation.* 1985;72:V123-V135.
29. Diamond GA, Forrester JS, deLuz PL, Wyatt HL, Swan HJ. Post-extrasystolic potentiation of ischemic myocardium by atrial stimulation. *Am Heart J.* 1978;95:204-209.
30. Rahimtoola SH. The hibernating myocardium. *Am Heart J.* 1989;117:211-221.
31. Matsuzaki M, Gallagher KP, Kemper WS, White F, Ross Jr. J. Sustained regional dysfunction produced by prolonged coronary stenosis: gradual recovery after reperfusion. *Circulation.* 1983;68:170-182.
32. 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.
33. 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.
34. Heusch G, Schulz R. Features of short-term myocardial hibernation. *Mol Cell Biochem.* 1998;186:185-193.
35. Flameng W, Suy R, Schwarz F, Borgers M, Piessens J, Thone F, Van Ermen H, De Geest H. Ultrastructural correlates of left ventricular contraction abnormalities in patients with chronic ischemic heart disease: determinants of reversible segmental asynergy postrevascularization surgery. *Am Heart J.* 1981;102:846-857.
36. Bolli R. Myocardial 'stunning' in man. *Circulation.* 1992;86:1671-1691.
37. Schulz R, Rose J, Martin C, Brodde OE, Heusch G. Development of short-term myocardial hibernation. Its limitation by the severity of ischemia and inotropic stimulation. *Circulation.* 1993;88:684-695.
38. Schulz R, Rose J, Post H, Heusch G. Regional short-term myocardial hibernation in swine does not involve endogenous adenosine or K^{+}_{ATP} channels. *Am J Physiol.* 1995;268:H2294-H2301.
39. Marban E. Myocardial stunning and hibernation. The physiology behind the colloquialisms. *Circulation.* 1991;83:681-688.
40. Heusch G, Rose J, Skyschally A, Post H, Schulz R. Calcium responsiveness in regional myocardial short-term hibernation and stunning in the *in situ* porcine heart. Inotropic responses to postextrasystolic potentiation and intracoronary calcium. *Circulation.* 1996;93:1556-1566.

41. Murry CE, Jennings RB, Reimer KA. Preconditioning with ischemia: a delay of lethal cell injury in ischemic myocardium. *Circulation.* 1986;74:1124-1136.
42. Keogh B, Priddy R, Morgan C, Gillbe C. Comparative study of the effects of enoximone and dobutamine in patients with impaired left ventricular function undergoing cardiac surgery. *Cardiology.* 1990;77:58-61.
43. Peterzen B, Lonn U, Babic A, Granfeldt H, Casimir-Ahn H, Rutberg H. Postoperative management of patients with Hemopump support after coronary artery bypass grafting. *Ann Thorac Surg.* 1996;62:495-500.
44. Wijns W, Vanoverschelde J-LJ, Gerber BL, Bartunek J, de Bruyne B, Heyndrickx GR, Melin JA. Evidence that myocardial stunning occurs in humans following unstable angina (abstract). *J Am Coll Cardiol.* 1995;25:811-812.
45. Ito H, Tomooka T, Sakai N, Higashino Y, Fujii K, Katoh O, Masuyama T, Kitabatake A, Minamino T. Time course of functional improvement in stunned myocardium in risk area in patients with reperfused anterior infarction. *Circulation.* 1993;87:355-362.
46. Vanoverschelde JL, Wijns W, Depre C, Essamri B, Heyndrickx GR, Borgers M, Bol A, Melin JA. Mechanisms of chronic regional postischemic dysfunction in humans. New insights from the study of noninfarcted collateral-dependent myocardium. *Circulation.* 1993;87:1513-1523.
47. Bolli R. Mechanism of myocardial "stunning". *Circulation.* 1990;82:723-738.
48. Levitsky J, Gurell D, Frishman WH. Sodium ion/hydrogen ion exchange inhibition: a new pharmacologic approach to myocardial ischemia and reperfusion injury. *J Clin Pharmacol.* 1998;38:887-897.
49. Meng HP, Pierce GN. Protective effects of 5-(N,N-dimethyl)amiloride on ischemia-reperfusion injury in hearts. *Am J Physiol.* 1990;258:H1615-H1619.
50. Myers ML, Karmazyn M. Improved cardiac function after prolonged hypothermic ischemia with the Na^+/H^+ exchange inhibitor HOE 694. *Ann Thorac Surg.* 1996;61:1400-1406.
51. Opie L. Myocardial stunning: a role for calcium antagonists during reperfusion? *Cardiovasc Res.* 1992;26:20-24.
52. Ehring T, Bohm M, Heusch G. The calcium antagonist nisoldipine improves the functional recovery of reperfused myocardium only when given before ischemia. *J Cardiovasc Pharmacol.* 1992;20:63-74.
53. Bolli R, Jeroudi MO, Patel BS, Aruoma OI, Halliwell B, Lai EK, McCay PB. Marked reduction of free radical generation and contractile dysfunction by antioxidant therapy begun at the time of reperfusion. Evidence that myocardial "stunning" is a manifestation of reperfusion injury. *Circ Res.* 1989;65:607-622.
54. Bolli R, Jeroudi MO, Patel BS, DuBose CM, Lai EK, Roberts R, McCay PB. Direct evidence that oxygen-derived free radicals contribute to postischemic myocardial dysfunction in the intact dog. *Proc Natl Acad Sci U S A.* 1989;86:4695-4699.
55. de Jong JW, van der Meer P, Nieukoop AS, Huizer T, Stroeve RJ, Bos E. Xanthine oxidoreductase activity in perfused hearts of various species, including humans. *Circ Res.* 1990:770-773.
56. Duncker DJ, Soei LK, Verdouw PD. Pharmacological modulation of myocardial stunning. In: Heyndrickx G, Vatner S, Wijns W, eds. *Stunning, Hibernation, and Preconditioning.* Philadelphia: Lippincott-Raven Publishers; 1997:229-251.
57. Steenbergen C, Murphy E, Levy L, London RE. Elevation in cytosolic free calcium concentration early in myocardial ischemia in perfused rat heart. *Circ Res.* 1987;60:700-707.
58. Bolli R, Marban E. Molecular and cellular mechanisms of myocardial stunning. *Physiol Rev.* 1999;79:609-634.
59. Krause SM, Jacobus WE, Becker LC. Alterations in cardiac sarcoplasmic reticulum calcium transport in the postischemic "stunned" myocardium. *Circ Res.* 1989;65:526-530.
60. Gao WD, Atar D, Backx PH, Marban E. Relationship between intracellular calcium and contractile force in stunned myocardium. Direct evidence for decreased myofilament Ca^{2+} responsiveness and altered diastolic function in intact ventricular muscle. *Circ Res.* 1995;76:1036-1048.
61. Carrozza Jr. JP, Bentivegna LA, Williams CP, Kuntz RE, Grossman W, Morgan JP. Decreased myofilament responsiveness in myocardial stunning follows transient calcium overload during ischemia and reperfusion. *Circ Res.* 1992;71:1334-1340.
62. Kusuoka H, Koretsune Y, Chacko VP, Weisfeldt ML, Marban E. Excitation-contraction coupling in postischemic myocardium. Does failure of activator Ca^{2+} transients underlie stunning? *Circ Res.* 1990;66:1268-1276.
63. Lamers JM, Duncker DJ, Bezstarosti K, McFalls EO, Sassen LM, Verdouw PD. Increased activity of the sarcoplasmic reticular calcium pump in porcine stunned myocardium. *Cardiovasc Res.* 1993;27:520-524.
64. Gao WD, Liu Y, Mellgren R, Marban E. Intrinsic myofilament alterations underlying the decreased contractility of stunned myocardium. A consequence of Ca^{2+}-dependent proteolysis? *Circ Res.* 1996;78:455-465.

65. Becker LC, Levine JH, DiPaula AF, Guarnieri T, Aversano T. Reversal of dysfunction in postischemic stunned myocardium by epinephrine and postextrasystolic potentiation. *J Am Coll Cardiol.* 1986;7:580-589.
66. Schmitz W, von der Leyen H, Meyer W, Neumann J, Scholz H. Phosphodiesterase inhibition and positive inotropic effects. *J Cardiovasc Pharmacol.* 1989;14:S11-S14.
67. Schmitz W, Scholz H, Erdmann E. Effects of alpha-and beta-adrenergic agonists, phosphodiesterase inhibitors and adenosine on isolated human heart muscle preparations. *Trends Pharmacol Sci.* 1987;8:447-450.
68. Schott RJ, Rohmann S, Braun ER, Schaper W. Ischemic preconditioning reduces infarct size in swine myocardium. *Circ Res.* 1990;66:1133-1142.
69. 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.
70. Liu Y, Downey JM. Ischemic preconditioning protects against infarction in rat heart. *Am J Physiol.* 1992;263:H1107-H1112.
71. Iwamoto T, Miura T, Adachi T, Noto T, Ogawa T, Tsuchida A, Iimura 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.
72. Verdouw PD, Gho BC, Duncker DJ. Ischaemic preconditioning: is it clinically relevant? *Eur Heart J.* 1995;16:1169-1176.
73. Ikonomidis JS, Tumiati LC, Weisel RD, Mickle DA, Li RK. Preconditioning human ventricular cardiomyocytes with brief periods of simulated ischaemia. *Cardiovasc Res.* 1994;28:1285-1291.
74. Walker DM, Walker JM, Pugsley WB, Pattison CW, Yellon DM. Preconditioning in isolated superfused human muscle. *J Mol Cell Cardiol.* 1995;27:1349-1357.
75. 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.
76. Deutsch E, Berger M, Kussmaul WG, Hirshfeld JW, Jr., Herrmann HC, Laskey WK. Adaptation to ischemia during percutaneous transluminal coronary angioplasty. Clinical, hemodynamic, and metabolic features. *Circulation.* 1990;82:2044-2051.
77. Anzai T, Yoshikawa T, Asakura Y, Abe S, Akaishi M, Mitamura H, Handa S, Ogawa S. Preinfarction angina as a major predictor of left ventricular function and long-term prognosis after a first Q wave myocardial infarction. *J Am Coll Cardiol.* 1995;26:319-327.
78. Kloner RA, Shook T, Przyklenk K, Davis VG, Junio L, Matthews RV, Burstein S, Gibson M, Poole WK, Cannon CP, et al. Previous angina alters in-hospital outcome in TIMI 4. A clinical correlate to preconditioning? *Circulation.* 1995;91:37-45.
79. Ottani F, Galvani M, Ferrini D, Sorbello F, Limonetti P, Pantoli D, Rusticali F. Prodromal angina limits infarct size. A role for ischemic preconditioning. *Circulation.* 1995;91:291-297.
80. Murry CE, Richard VJ, Jennings RB, Reimer KA. Myocardial protection is lost before contractile function recovers from ischemic preconditioning. *Am J Physiol.* 1991;260:H796-H804.
81. Downey JM, Cohen MV, Ytrehus K, Liu Y. Cellular mechanisms in ischemic preconditioning: the role of adenosine and protein kinase C. *Ann N Y Acad Sci.* 1994;723:82-98.
82. Gross GJ, Fryer RM. Sarcolemmal versus mitochondrial ATP-sensitive K^+ channels and myocardial preconditioning. *Circ Res.* 1999;84:973-979.
83. Holmuhamedov EL, Jovanovic S, Dzeja PP, Jovanovic A, Terzic A. Mitochondrial ATP-sensitive K^+ channels modulate cardiac mitochondrial function. Am J Physiol. 1998:H1567-H1576.
84. Auchampach JA, Grover GJ, Gross GJ. Blockade of ischaemic preconditioning in dogs by the novel ATP dependent potassium channel antagonist sodium 5-hydroxydecanoate. *Cardiovasc Res.* 1992;26:1054-1062.
85. Gross GJ, Auchampach JA. Blockade of ATP-sensitive potassium channels prevents myocardial preconditioning in dogs. *Circ Res.* 1992;70:223-233.
86. Hide EJ, Thiemermann C. Limitation of myocardial infarct size in the rabbit by ischaemic preconditioning is abolished by sodium 5-hydroxydecanoate. *Cardiovasc Res.* 1996;31:941-946.
87. 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.
88. Schultz JE, Qian YZ, Gross GJ, Kukreja RC. The ischemia-selective K^+_{ATP} channel antagonist, 5-hydroxydecanoate, blocks ischemic preconditioning in the rat heart. *J Mol Cell Cardiol.* 1997;29:1055-1060.
89. Schulz R, Rose J, Heusch G. Involvement of activation of ATP-dependent potassium channels in ischemic preconditioning in swine. *Am J Physiol.* 1994;267:H1341-H1352.
90. 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.

91. Koning MMG, Gho BC, 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.
92. Goto M, Liu Y, Yang XM, Ardell JL, Cohen MV, Downey JM. Role of bradykinin in protection of ischemic preconditioning in rabbit hearts. *Circ Res.* 1995;77:611-621.
93. Ovize M, Kloner RA, Przyklenk K. Stretch preconditions canine myocardium. *Am J Physiol.* 1994;266:H137-H146.
94. Gysembergh A, Margonari H, Loufoua J, Ovize A, Andre-Fouet X, Minaire Y, Ovize M. Stretch-induced protection shares a common mechanism with ischemic preconditioning in rabbit heart. Am J Physiol. 1998;274:H955-H964.
95. Koning MMG, Gho BC, van Klaarwater E, Opstal RL, Duncker DJ, Verdouw PD. Rapid ventricular pacing produces myocardial protection by nonischemic activation of K^+_{ATP} channels. *Circulation.* 1996;93:178-186.
96. Domenech RJ, Macho P, Velez D, Sanchez G, Liu X, Dhalla N. Tachycardia preconditions infarct size in dogs: role of adenosine and protein kinase C. *Circulation.* 1998;97:786-794.
97. Marber MS, Latchman DS, Walker JM, 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.
98. Pell TJ, Yellon DM, Goodwin RW, Baxter GF. Myocardial ischemic tolerance following heat stress is abolished by ATP-sensitive potassium channel blockade. *Cardiovasc Drugs Ther.* 1997;11:679-686.
99. 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.
100. 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.
101. Ytrehus K, Liu Y, Downey JM. Preconditioning protects ischemic rabbit heart by protein kinase C activation. *Am J Physiol.* 1994;266:H1145-H1152.
102. 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.
103. Thornton JD, Daly JF, Cohen MV, Yang XM, Downey JM. Catecholamines can induce adenosine receptor-mediated protection of the myocardium but do not participate in ischemic preconditioning in the rabbit. *Circ Res.* 1993;73:649-655.
104. Toombs CF, McGee S, Johnston WE, Vinten-Johansen J. Myocardial protective effects of adenosine. Infarct size reduction with pretreatment and continued receptor stimulation during ischemia. *Circulation.* 1992;86:986-994.
105. Thornton JD, Liu GS, Olsson RA, Downey JM. Intravenous pretreatment with A_1-selective adenosine analogues protects the heart against infarction. *Circulation.* 1992;85:659-665.
106. 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.
107. Pell TJ, Baxter GF, Yellon DM, Drew GM. Renal ischemia preconditions myocardium: role of adenosine receptors and ATP-sensitive potassium channels. *Am J Physiol.* 1998;275:H1542-H1547.
108. Takaoka A, Nakae I, Mitsunami K, Yabe T, Morikawa S, Inubushi T, Kinoshita M. Renal ischemia/reperfusion remotely improves myocardial energy metabolism during myocardial ischemia via adenosine receptors in rabbits: effects of "remote preconditioning". *J Am Coll Cardiol.* 1999;33:556-564.
109. Gho BC, Schoemaker RG, van den Doel MA, Duncker DJ, Verdouw PD. Myocardial protection by brief ischemia in noncardiac tissue. *Circulation.* 1996;94:2193-2200.
110. Birnbaum Y, Hale SL, Kloner RA. Ischemic preconditioning at a distance: reduction of myocardial infarct size by partial reduction of blood supply combined with rapid stimulation of the gastrocnemius muscle in the rabbit. *Circulation.* 1997;96:1641-1646.
111. Handlin LR, Kindred LH, Beauchamp GD, Vacek JL, Rowe SK. Reversible left ventricular dysfunction after subarachnoid hemorrhage. *Am Heart J.* 1993;126:235-240.
112. Ohtsuka T, Hamada M, Kodama K, Sasaki O, Suzuki M, Hara Y, Shigematsu Y, Hiwada K. Images in Cardiovascular Medicine. Neurogenic stunned myocardium. *Circulation.* 2000;101:2122-2124.
113. Hajjar RJ, Schmidt U, Helm P, Gwathmey JK. Ca^{++} sensitizers impair cardiac relaxation in failing human myocardium. *J Pharmacol Exp Ther.* 1997;280:247-254.
114. Hgashiyama A, Watkins MW, Chen Z, LeWinter MM. Effects of EMD 57033 on contraction and relaxation in isolated rabbit hearts. *Circulation.* 1995;92:3094-3104.
115. White J, Lee JA, Shah N, Orchard CH. Differential effects of the optical isomers of EMD 53998 on contraction and cytoplasmic Ca^{2+} in isolated ferret cardiac muscle. *Circ Res.* 1993;73:61-70.

116. Korbmacher B, Sunderdiek U, Selcan G, Arnold G, Schipke JD. Different responses of non-ischemic and post-ischemic myocardium towards Ca^{2+} sensitization. *J Mol Cell Cardiol.* 1997;29:2053-2066.
117. Soei LK, Sassen LM, 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.
118. Fan D, Soei LK, Sassen LM, Krams R, Verdouw PD. Mechanical efficiency of stunned myocardium is modulated by increased afterload dependency. *Cardiovasc Res.* 1995;29:428-437.
119. Przyklenk K, Kloner RA. Ischemic preconditioning: exploring the paradox. *Prog Cardiovasc Dis.* 1998;40:517-547.
120. Liu GS, Richards SC, Olsson RA, Mullane K, Walsh RS, Downey JM. Evidence that the adenosine A_3 receptor may mediate the protection afforded by preconditioning in the isolated rabbit heart. *Cardiovasc Res.* 1994:1057-1061.
121. Auchampach JA, Gross GJ. Adenosine A_1 receptors, K^+_{ATP} channels, and ischemic preconditioning in dogs. *Am J Physiol.* 1993;264:H1327-H1336.
122. Schulz R, Rose J, Post H, Heusch G. Involvement of endogenous adenosine in ischaemic preconditioning in swine. *Pflugers Arch.* 1995;430:273-282.
123. 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.* 1994;266:H829-H839.
124. Li Y, Kloner RA. The cardioprotective effects of ischemic 'preconditioning' are not mediated by adenosine receptors in rat hearts. *Circulation.* 1993;87:1642-1648.
125. Headrick JP. Ischemic preconditioning: bioenergetic and metabolic changes and the role of endogenous adenosine. *J Mol Cell Cardiol.* 1996;28:1227-1240.

Animal models in the study of myocardial ischemia and ischemic syndromes

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Introduction

Although myocardial ischemia has been one of the most extensively studied topics in cardiovascular research, its definition is still debated.[1] In the strictest sense 'ischemia' derived from the Greek words, ischō (to restrain) and haima (blood), means insufficient blood, and if we would adhere to this definition, all animal models which lack blood (e.g. isolated heart models) should by definition be termed 'ischemic'. Most investigators prefer to define ischemia as an imbalance between the amount of oxygen and substrates supplied to the heart and the amount needed to perform normal function.[1,2] The rationale behind this definition is that the myocardium strongly depends on oxygen to sustain adequate oxidative phosphorylation, the only metabolic process that is capable of providing sufficient high energy phosphates to maintain normal myocardial contraction. When oxygen supply to the heart becomes impaired there will be inadequate production of high energy phosphates with a resultant decline in myocardial contractility. When this condition develops, the myocardium starts to produce some, but not sufficient, high energy phosphates by anaerobic glycolysis, and lactate which is the end-product of this process starts to accumulate in the myocardium. Myocardial ischemia is thus viewed as a condition in which an imbalance exists between oxygen-supply and oxygen-demand, leading to anaerobic metabolism and reduced contractile function. Central to the definition of ischemia is that coronary flow is not only insufficient to allow adequate energy production, but that the impaired flow also results in impaired removal of metabolic waste products. In the large majority of cases in man myocardial ischemia is confined to specific regions of the myocardium (regional ischemia), because a stenosis in a coronary artery prevents adequate perfusion of the artery's distribution area (supply ischemia). In its early stage of development, the stenosis can be sufficiently mild so that oxygen supply is adequate under resting conditions, but becomes flow-limiting during stress such as exercise. At the moment that ischemia develops during exercise, coronary blood flow may be higher than under resting conditions, but the increase is not sufficient to meet the increased oxygen demand (demand ischemia). The situations that the entire human heart becomes ischemic (global ischemia) are relatively rare, but may be seen during severe hypotension and during open-heart surgery when aortic-cross clamping is required.

Ischemia versus hypoxia

In man at high altitude myocardial oxygen supply may become impaired, not because of a reduced coronary blood flow but because the oxygen content of the blood is decreased. Because coronary blood flow is not restricted, there is no accumulation of myocardial metabolic waste products. This condition of reduced oxygen delivery in the presence of unrestricted coronary inflow, has been termed hypoxia (or anoxia when no oxygen is present). Important in the interpretation of the data from ischemia and hypoxia studies is that in the latter there is adequate removal of metabolic waste products.

Ischemia versus infarction

Myocardial ischemia does not only lead to changes in (global and regional) cardiovascular function and metabolism, but also to changes in homeostasis of electrolytes, neurohormones and ultrastructural features of the myocardium. These changes can be seen within the first few minutes of ischemia and are reversible when perfusion is promptly restored. However, when ischemia is maintained there is a gradual transition from reversible to irreversible injury as infarction develops. The distinction between ischemia and infarction thus concerns the reversibility of the changes.

In this overview we will discuss some of the most frequently used animal models in the study of myocardial ischemia (and hypoxia) and ischemic syndromes, which are the consequence of prior ischemia.

Myocardial ischemia

Regional ischemia

Experimental animal

In man, myocardial ischemia is most commonly regional in nature and this condition has been widely studied in intact animals. In *in vivo* studies the dog has traditionally been the most frequently used animal species, but because of cost and social pressure against the use of dogs, the pigs has become a more favoured animal only in recent years.[3,4] This is surprising because Leonardo da Vinci already used pigs to demonstrate the movement of the heart during the cardiac cycle nearly five centuries ago.[5] However, the pig did not become a widely used animal in biomedical research because of problems with handling of the animal. For instance, Pavlov banned all pigs from the laboratory as he considered all pigs to be hysterical because of their shrieking.[4] In most studies young and thus growing domestic swine are used and the age-dependency of cardiovascular responses should therefore be kept in mind. The age of domestic pigs in acute studies usually ranges from 3 to 5 months, which is sufficiently old for maturation of central nervous system regulation of cardiovascular function.[6,7] An interesting feature is the relation between heart and body weight. In man this ratio is about 5 g/kg, whereas it is twice as high in dogs. For pigs weighing between 25 and 30 kg the ratio is similar to that in man, but for animals exceeding 100 kg it is only half that value. The relative low heart weight in domestic pigs is probably the result of selective breeding during the last two centuries. Nowadays, one year old pigs reach body weights of 150 kg, whereas 200 years ago weights of only 40 kg were attained in twice that life span.[8]

Smaller animals such as the rabbit and the rat have also been used to study regional myocardial ischemia,[9] but instrumentation of *in situ* myocardium for measurement of regional metabolism and function is limited due to the small size of the heart and coronary vessels. An additional problem is that instantaneous reperfusion may not always occur when an occlusion

of a coronary artery is released and because quantitative assessment of phasic coronary blood flow is difficult, one must rely on visual inspection to verify reperfusion (disappearance of cyanosis). Nevertheless, the use of small animals has gained a large popularity in studies of ischemic preconditioning,[10,11] studies employing techniques such as microdialysis[12] and studies of post-infarction remodeling.[13,14]

Collateral circulation

The early response to ischemia depends to a large extent on the existence of a collateral circulation.[15] Especially the group of Schaper has shown that major differences exist in the extent of collateralization of the various species.[16-18] Thus, it is important to realize that dogs may have a well-developed coronary collateral circulation (which may vary considerably among individual animals), while collateral blood flow in pigs and baboons is extremely low (<5% of transmural blood flow).[16,19-21] From the other species used in the study of myocardial ischemia, it has been well established that the collateral circulation of the rat is sparse and that the rabbit may show intraspecies differences.[18,22] The guinea pig has such an extensive collateral network that normal perfusion is maintained after a coronary artery occlusion and infarction does not develop. Cats also have a well-developed collateral network, but this is not sufficient to prevent infarction.[22,23]

In animals that do not have an extensive native collateral circulation, coronary collaterals can be induced by a variety of methods. These include permanent complete coronary artery occlusion, brief (2 min) repeated complete coronary artery occlusions at regular intervals for a number of weeks,[24-26] progressive coronary artery occlusion by means of an ameroid constrictor,[16,27] partial coronary artery occlusion without or in combination with exercise[28-31] (exercise alone does not promote collateral growth,[29,30] or embolization of the microvasculature with non-radioactive microspheres with a diameter of 25 μm.[32] In young growing pigs a mild fixed coronary stenosis becomes flow limiting as the animals grow older and collaterals develop.[28] Bloor et al.[33] have shown that in pigs collateral blood flow can eventually account for 80% of the needed perfusion at baseline and 50% during exercise.

It is quite obvious that when a coronary artery is completely occluded in a species with a sizeable collateral network, determination of residual coronary perfusion is mandatory to appreciate the severity of ischemia. Labeled (radioactive[34,35] or coloured[36,37]) microspheres are the first choice for measuring collateral blood flow, as this technique permits the determination of both total collateral flow and its transmural distribution. The latter is important because from earlier studies we know that, in particular, the subendocardial perfusion is a major determinant of regional function.[38] For species with a negligible collateral circulation regional perfusion data during ischemia are less mandatory. For a description of other methods to determine collateral blood flow and growth the reader is referred to reference 18.

Although pigs and baboons differ from dogs in their collateral blood flow, they both represent distinct groups of patients with coronary artery disease as a large number of these patients may have few collaterals during the early phase of their disease. On the other hand, in patients with a longstanding history of coronary artery lesions, collaterals may develop and dogs may be a more appropriate model for their condition.

Experimental conditions

Because the pathophysiological responses to ischemia and also the response of pharmacological agents may be influenced by the presence of anesthetic agents[39-42] and acute surgical trauma,[43] chronically instrumented awake animal models are the first choice to mimic myocardial ischemia in man. In addition to the possibility of studying the animals without interference of anesthesia and acute surgical trauma, the awake animal model has the major advantage that it can be used in studies requiring long-term interventions or follow-up and during physiological stress as produced by exercise. Furthermore, if only used in short-term studies, a number of different protocols can be performed in the same animal, provided that sufficient time is (can be) taken to prevent interference from previous interventions. The elaborate surgery under sterile conditions, the high cost of the implanted transducers and probes (which have a limited life span due to chronic implantation), the cost of housing, the initial therapy to minimize the unavoidable discomfort related to the recovery from surgery and some of the interventions have limited the widespread use of awake animals.

In the majority of studies, ischemia is therefore produced in anesthetized animals, either open- or closed-chest. The open-chest preparation has the advantage that regional function and metabolism can be studied in detail. In open-chest animals severe ischemia is almost invariably produced by complete occlusion of a coronary artery by inflation of a balloon placed around the vessel. If one wants to avoid thoracotomy, intracoronary devices have to be used to occlude a vessel. The closed-chest model has the advantage that tissue trauma is minimized, but the approach requires fluoroscopy for proper positioning of the obstruction. The most frequently employed method is inflation of a balloon placed at the tip of a catheter.[44]

In order to produce mild or moderate ischemia, the flow in the coronary artery can be reduced to a fixed percentage of resting blood flow. In open-chest animals, this can be achieved by a hydraulic occluder positioned around the coronary artery,[45] tightening a J-shaped screw clamp[46] or other mechanical devices.[47,48] These methods have the disadvantage that control of blood flow is difficult and requires continuous measurement of blood flow with flow probes or the use of microspheres at intervals. In order to circumvent large variations in flow (e.g. by changes in perfusion pressure or movement of the occlusion devices), more advanced methods may be used which control perfusion pressure[49] or perfuse the coronary vascular bed by an extracorporeal system using the animal's own blood.[50,51] Flow reductions can also be achieved by intracoronary placement of a hollow plug attached to a catheter.[52,53]

The lumen of a hole, drilled in the length of the plug, provides the limited perfusion when the catheter is so far advanced into the coronary artery that the plug occludes the vessel. An advantage of this method is that it can also be used in closed chest animals and that the lumen of the catheter allows post-stenotic pressure measurements. A disadvantage is the risk that the lumen of the plug may become obstructed due to formation of blood clots, unless precautions are taken. Furthermore, the site of the obstruction in the artery cannot be determined in advance, which may lead to considerable variations in the size of the ischemic areas between individual animals.

A disadvantage of all above described methods is that they produce concentric stenoses, while in man the majority (~70%) of stenoses are eccentric. At the site of obstruction, vessels with a fixed concentric constriction do not respond to vasodilators, while vessels with eccentric stenoses are capable to increase their narrowed lumen, thereby enlarging coronary blood flow. There have been a few attempts to employ eccentric stenoses by partial inflation of an intraluminal balloon.[54] The drawback of this approach is that it is very difficult to keep the balloon (eccentric stenosis) in place. Movement of the balloon inside the artery will cause variation in the geometry of the residual lumen and thereby in the severity of the obstruction.

The left anterior descending coronary artery is most frequently used to produce regional myocardial ischemia, as it permits selective sampling of the regional coronary vein for metabolic studies. Nevertheless, left circumflex coronary artery occlusions have also been used.[55,56] It is well known that in man and pigs proximal occlusion of the left anterior descending coronary artery leads to a greater impairment of global left ventricular function than occlusion of the left circumflex coronary artery, which is believed to be caused by the greater area at risk. Hoit and Lew[57] have, however, shown that for the same areas at risk the compensatory increase in function in the adjacent non-ischemic area is different for the left anterior descending coronary artery than for the left circumflex coronary artery. This illustrates that not only the size of the area at risk, but also its location within the left ventricle determines the global functional consequences of ischemia.

Blood samples collected from the coronary sinus are quite often used for metabolic studies. These samples are not only contaminated with blood from adjacent non-ischemic myocardium, but in pigs there is also contamination with blood from the left azygous vein, which drains into the coronary sinus. As a result of this, oxygen saturation in the coronary sinus of pigs may be around 65 to 70%, while in the local coronary vein it is usually around 20% or even less.[58] However, the use of coronary venous samples for studying metabolism is decreasing, because of recent developments in microdialysis, NMR spectroscopy and positron emission tomography.[59-61]

In vivo models of coronary arterial thrombosis

In patients, myocardial ischemia develops quite often after formation of a thrombus at the site of a stenosis. Models mimicking this process have been developed in the past to study the process of thrombus formation, its prevention by pharmacological agents or the effectiveness of thrombolytic agents. These models are not very useful for the study of well-defined ischemia and the evaluation of cardioprotective therapies, because of variation in time to occlusive thrombus formation and uncontrollable duration and severity of ischemia. These models are therefore not dealt with in this overview; for an extensive review of these models the reader is referred to reference 62.

Global ischemia (in vitro models)

The consequences of acute myocardial ischemia can often be studied more easily under well-controlled conditions in isolated than in *in situ* heart models. Langendorff has to be credited for being the first to devise a method to investigate the mechanical activity of an isolated heart.[63] The principle has been described extensively[64] and is based on forcing blood or an oxygenated fluid into the coronary arteries through a cannula implanted in the aorta. Initially, Langendorff used the model to study contractile function, but already before the turn of the century he reported on the relationship between phasic coronary blood flow and the rhythmic contraction of the heart.[65] Already very early, it was noted that coronary flow was considerably higher in hearts perfused with saline-like substances, than in hearts perfused with blood at the identical perfusion pressure; the reason being the higher viscosity of blood and consequently the higher coronary vascular resistance. The original Langendorff preparation underwent many modifications, which permitted studies under well-controlled conditions such as constant perfusion, perfusion at constant perfusion pressure or perfusion via a donor animal.[66,67]

A critical point in setting up the model remains the time interval between removing the heart from the animal and fixation in the perfusion system, as ATP and creatine phosphate start to decline within seconds after the heart is removed from the animal. After coronary perfusion with oxygenated blood has been re-established, a stabilization period of at least 15 to 30 minutes must be allowed before baseline values can be taken. The stability of the preparation is usually assessed from heart rate, left ventricular pressure and coronary blood flow (weaning of the reactive hyperemia after the period of ischemia). Since *in vivo* experiments have revealed that brief periods of ischemia may already lead to ischemic syndromes such as stunning and preconditioning and as these phenomena are also studied in isolated heart models, one must be aware that the period between removing the heart from the animal and the re-establishment of coronary perfusion and also the duration of the stabilization period could influence the outcome of such studies.

In comparison with the *in vivo* models, the perfusion pressures of the isolated hearts are lower. In *in vivo* experiments diastolic pressures are usually in the range of 70 to 85 mmHg. When saline-like solutions are used, such high pressures cannot be used in isolated hearts because tissue oedema will develop due to the lower coronary vascular resistance (leading to higher intracapillary filtration pressures), in conjunction with the low colloid osmotic pressure of these solutions. The use of whole blood could circumvent these problems initially, but will lead to complications caused by foaming, clotting, hemolysis and thereby considerably shortens the period during which the preparation remains stable. Most investigators have therefore added substances to their perfusion medium, which increase the osmotic pressure or increase tissue pressure, by immersing the heart in an organ bath filled with perfusion medium. Interstitial oedema contributes to non-uniform perfusion when perfusion is restored after ischemia, which is a limitation when recovery of post-ischemic function or ischemic preconditioning is studied. Microvascular compression does not only result from interstitial oedema but may also result from the development of myocardial contracture.[68,69] The ensuing vascular deformation can partially be prevented by placing a fluid-filled balloon inside the left ventricular cavity.[70]

The isolated heart model is well suited to study the effects of acute myocardial ischemia or hypoxia.[71] Different degrees of ischemia can be produced by completely stopping or partially restricting perfusion, while different degrees of hypoxia can be produced by removing oxygen completely or partially from the perfusion medium. An advantage of the isolated heart model is that one can study in a very simple manner whether the consequences of ischemia are due to either lack of oxygen supply or accumulation of metabolites resulting from anaerobic metabolism.[72] Depending on the hypothesis that is investigated it may either be an advantage or a disadvantage that the influence of the hemodynamic and neurohumoral factors, which are present in the intact animal, are eliminated. However, caution must be exerted when interpreting the effects of pharmacological interventions on acute ischemia. In such studies quite often concentrations of pharmacological agents are used that are not tolerated by the intact animal. For instance, it has been concluded from isolated heart studies that dihydropyridine derivatives protect the myocardium by a negative inotropic action,[73] while *in vivo* experiments clearly demonstrate that the doses used in the isolated heart studies cannot be used in the intact animal because of severe hypotension secondary to their vasodilator actions.[74]

The Langendorff preparation does not permit the left ventricle to eject the perfusate (cardiac output) and is therefore a non-working model. Some 30 years ago Neely et al.[75] developed a work performing isolated heart model, which was capable of ejecting perfusate. In order to do so, the perfusate is now supplied by a cannula inserted into the left atrium and left ventricular outflow can be monitored, while left atrial pressure or aortic pressure can be controlled. The advantage of the working heart model is that it allows the construction of

cardiac function curves using Windkessel like models under a wide variety of conditions such as during the post-ischemic recovery period.

Although functional studies can be performed in isolated heart models, they are particularly useful for the study of metabolic pathways because the content of the substrates in the perfusion solution (usually Krebs-Henseleit bicarbonate buffer) can be altered, while the metabolic products can be determined in the effluent. Finally, at the end of the experiment myocardial tissue is easily accessible for biochemical and histological analysis. Rat and rabbit hearts are most frequently used in isolated heart studies, but the use of hearts from larger animals such as pigs is not uncommon.[76]

In addition to using the whole heart, some models have employed only the intraventricular septum.[77] For a review of this and other simple models the reader is referred to reference 2.

Finally, although isolated heart studies can provide useful information about the events that occurs during ischemia and early reperfusion, it should never be forgotten that extrapolation of the results to what is actually happening in regional ischemia in man should occur with extreme care. For instance, the perfusion medium of the isolated hearts lacks blood components such as leukocytes, which have been implicated in the development of myocardial injury associated with ischemia and reperfusion. Furthermore, with global ischemia one eliminates the interaction between the myocytes of different regions of the heart, which may have an impact on the ultimate function of regionally ischemic myocardium.[78]

Isolated cardiac myocytes

During the last 25 years isolated myocytes have emerged as a new experimental model and their applications are still evolving. Among them are studies under anoxic or ischemic conditions, but routinely isolated myocytes are most frequently used in electrophysiological and biochemical experiments. Because the cells are free from surrounding tissue, all changes can be attributed to processes occurring within the cell. Cardiac myocytes are available as either cultured embryonic or adult cells or freshly isolated adults cells. Details of the different types and isolation procedures have been described extensively (see [79]). An important determinant of the quality of the preparation is the oxygen consumption, which must match that of the intact quiescent heart.[80,81] Contraction of the myocytes can be assessed by optical (e.g. laser diffraction, photodiode array edge detectors, and direct visual imaging) or mechanical detectors.[82-84] Critical for all studies is the viability and stability of the preparation. It is therefore mandatory to determine that the percentages of viable cells at the beginning and at the end of the study do not differ when interventions are performed that do not produce irreversible injury. Membrane damage can be evaluated by release of cytoplasmic enzymes such as lactate dehydrogenase and creatine phosphokinase.[85] Another reason for concern is that functional changes may occur during the isolation procedures. For instance, Linden et al.[86]

have shown that exposing myocytes to catecholamines leads to down-regulation of the number of receptors within minutes.

Ischemic syndromes

Stunning

In 1975 Heyndrickx et al.[87] reported that recovery of regional contractile function after a 10-minute coronary artery occlusion in conscious dogs was not immediate but took hours to days. In contrast, the electrocardiogram normalized almost instantaneously. This delayed recovery of post-ischemic function, which initially received little attention and was even considered to be an experimental artefact,[88] was later termed stunning[89] and the search for its mechanism has greatly contributed to our current knowledge of events occurring during ischemia and reperfusion. By definition, function of stunned myocardium ultimately recovers without intervention. Stunning is therefore only clinically relevant for those patients in which global left ventricular function is already compromised through other cardiovascular conditions such as previous infarction.

Because myocardial stunning is the consequence of a period of reversible ischemia, it follows that every model that is used for the study of brief ischemia can serve as a model for stunning by merely showing that contractile function will recover when the period of reperfusion is extended sufficiently.

In vivo models

Studies in chronically instrumented awake animals (dogs, pigs or baboons) are most suitable for the study of stunning, as the time course of recovery of the post-ischemic function can be followed for periods up to weeks or months in the absence of complicating factors such as anesthesia, artificial ventilation and variations in temperature (see [90]). Because the degree of stunning is determined in part by the ischemic burden i.e. duration and severity of the flow (O_2) deficit,[91,92] determination of residual perfusion during ischemia is mandatory in animal species with an extensive coronary collateral circulation such as the dog. Without collateral flow data, the effect of an intervention on myocardial stunning cannot be properly interpreted. An additional advantage of the conscious animal model is that it can serve as its own control, provided that sufficient long periods are allowed between two successive stunning protocols. However, before pharmacological interventions are evaluated in such models, it is mandatory to establish that the successive occlusions produce the same degree of stunning. With respect to this, one has to keep in mind that Shen and Vatner[93] recently reported significant species differences for the same experimental protocols. Thus, while in pigs the degree of stunning was the same after two consecutive occlusions separated by 24 hours, it was less after the second occlusion in dogs, an observation which might be related to recruitment of collateral

flow in the latter species. Because of cost and complexity of the awake animal models, most investigators use (open or closed chest) anesthetized animals, thereby accepting that full recovery of function cannot be achieved in the limited time span that is available. An important difference between the awake and anesthetized models involves the degree of stunning and the time course of recovery.[42,91] Thus, when the same experimental protocols are used, post-ischemic dysfunction is more severe in pentobarbital anesthetized dogs than in awake dogs,[42] to which the less significant production of oxygen derived free radicals in awake animals may contribute. An explanation might be that pentobarbital anesthesia, in part via altering hemodynamic conditions, results in higher oxygen demand at the onset of ischemia and thus increases the total ischemic burden.[42] The effect of anesthesia on myocardial oxygen demand and its importance for the degree of stunning is supported by canine studies in which halothane[94] and isoflurane[95] anesthesia, were compared to fentanyl[94] and morphine/(α-chloralose/urethane anesthesia.[95] Volatile anesthesia was found to result in better recovery of contractile function following a 15-minute coronary artery occlusion. Another variable that influences functional recovery of stunned myocardium is temperature.[42] Consequently, a rigorous control of body temperature is mandatory when studying myocardial stunning. Open-chest animal preparations are more susceptible to temperature variations than closed chest animals.

Most investigators have used a single coronary artery occlusion, which produces stunning, provided that its duration is sufficiently long,[96] but multiple occlusions have also been used. Of interest is that when a large number of occlusions is used there is a progressive loss in the relation between the degree of stunning and residual perfusion during ischemia,[97] a relation which is prominently present when a single 15-minute coronary occlusion is used.[91] Furthermore, partial coronary artery occlusions of sufficient duration,[98,99] or in the presence of exercise-induced ischemia[100] also produce stunning. Finally, rapid ventricular pacing for 24 to 48 hours leads to post-pacing dysfunction which has all the characteristics of global myocardial stunning,[101,102] although pacing did not lead to demonstrable ischemia. Global stunning can also be produced by cardioplegia and sequential regional ischemia. To this end, Heyndrickx et al.[103] applied one hour flow reductions of the left circumflex coronary artery and the left anterior descending coronary artery at 30 minute intervals.

Recovery of post-ischemic contractile function is sometimes also examined in models which employ periods of ischemia that are sufficiently long to produce a mixture of both reversible and irreversible myocardial injury.[104-106] In these studies regional post-ischemic function will not return to pre-ischemia values and functional data obtained during the first hours of reperfusion period do not reflect recovery of stunned myocardium and are therefore difficult to interpret.

Isolated perfused hearts

Even more so than the *in situ* anesthetized animal model, the isolated perfused heart model is limited by the relatively short period in which recovery of post-ischemic function can be studied. Because full recovery of post-ischemic function is rarely observed in isolated heart studies, one must include other pertinent features of stunning such as absence of irreversible injury, response to inotropic stimulation and the rebound in creatine phosphate to supranormal levels[107,108] to assure that one is dealing with models of pure stunning. With respect to this, it should be noted that durations of ischemia that do not result in myocardial necrosis under *in vivo* conditions, may already produce irreversible damage in isolated perfused hearts. For instance, Borgers et al.[109] observed that even a 15-minute period of global ischemia in isolated rabbit hearts irreversibly damaged a significant number of myocytes.

In isolated hearts, stunning has been produced by single or repeated brief periods of total global ischemia, by transient occlusion of a coronary artery producing regional ischemia,[110] and by pacing-induced tachycardia in the presence of a lowered coronary perfusion pressure thereby producing global demand ischemia.[111] In addition to hearts of rats, also hearts of rabbits,[109,112] ferrets[113] and pigs[76] have been used in isolated heart models to study stunning.

Limitations of functional parameters

Understanding the limitations of the parameters that are used to describe contractile function is essential for proper assessment of the degree of stunning. Because *in vitro* studies usually employ models of global myocardial ischemia, global cardiac function parameters such as left ventricular developed pressure (isovolumically beating hearts) and cardiac output (working hearts) are used to assess the degree of stunning. In *in vivo* experiments regional stunning is most commonly assessed by measurement of local contractile function, such as regional segment shortening or wall thickening. These parameters depend on pre- and afterload, which may be different from baseline during the post-ischemic period. In addition, the afterload-dependency is more pronounced in stunned compared to normal myocardium,[114] so that when the effect of an intervention on stunning is investigated, functional measurements should be made under identical or at least very similar loading conditions. One approach to correct for decreased afterload in *in vivo* preparations is by partially clamping the aorta or by inflation of a balloon positioned in the aorta. Alternatively, changes in left ventricular pressure can be included in the assessment of contractile performance by using parameters derived from the left ventricular pressure-volume (time varying elastance concept) or left ventricular pressure-segment length (or wall thickness) relations such as external work and end systolic elastance.[114-116]

Ischemic preconditioning

In 1985 Murry et al.[117] described that in normal dog myocardium the development of irreversible damage was delayed when a sustained coronary artery occlusion was preceded by brief periods of reversible myocardial ischemia, a phenomenon which was termed ischemic preconditioning. To date ischemic preconditioning is the most potent endogenous mechanism by which infarct size can be limited. Since the original description by Murry et al.,[117] numerous studies have been undertaken to elucidate the mechanism of ischemic preconditioning and to investigate whether the protection by ischemic preconditioning also applies to other endpoints than infarct size (e.g. arrhythmias and recovery of function following reversible ischemia) and to demonstrate its existence and relevance in the clinical setting.[118,119] When infarct is taken as endpoint, the clinical importance of ischemic preconditioning has yet to be convincingly demonstrated. The lack of the definite proof for the occurrence of ischemic preconditioning in man arises from the fact that in patients the accurate assessment of determinants of infarct size such as the area at risk, the residual (collateral) blood flow, the duration and completeness of the infarct producing coronary artery occlusion, and also the characteristics of the preceding ischemia that serves as preconditioning-stimulus, are extremely difficult.[120] Furthermore, patients have usually pre-existing lesions and a long history of angina pectoris before infarction develops, which gives rise to further caution in extrapolating data obtained in normal animal hearts. Two important but unanswered questions regarding the clinical implication of ischemic preconditioning therefore are: 'does recurrent angina lead to a preconditioned state?' and 'can the as yet incompletely known mechanism of preconditioning be mimicked pharmacologically?' Cohen et al.[121] have shown that awake rabbits become tolerant to multiple episodes of ischemic preconditioning. With respect to this it is important to investigate if tolerance develops when pharmacological agents, that mimic ischemic preconditioning, are administered chronically.

The choice of the experimental animal does not appear to matter because positive results have been obtained in each species investigated in which infarct size was chosen as endpoint. These species now include dogs[117], pigs[122], sheep[123], rabbits[11], rats[10], marmots[124] and ferrets.[125] There is some evidence that the mechanism underlying ischemic preconditioning may not be the same in all species.[126] For instance, the role of activation of protein kinase C is still a very much debated issue.[127,128] Similar controversies exist about the activation of ATP sensitive K^+ channels and adenosine. However, such data should not play a decisive role in the choice of the experimental species as long as it has not been established whether such a mechanism could be operative in man.

At variance with infarct size limitation, studies using other endpoints such as ventricular arrhythmias or attenuation of stunning have yielded less uniform results. Protection by ischemic preconditioning against arrhythmias has conclusively been reported in studies using rats, but its protective effect in large animals is controversial.[129,130] It also appears that in the

same species, the stimuli required to precondition the heart against necrosis and arrhythmias may be different.[131] Presently, it is unclear if these different results are due to differences in species or to differences in experimental protocols. The age of the animal might need attention as Tani et al.[132] reported that in middle aged rats (50 weeks) the beneficial effects of ischemic preconditioning against reperfusion ventricular fibrillation and the rate of sarcoplasmic reticular Ca^{2+} uptake were reversed compared to young rats (12 weeks). Although it has been well established that senescent animals (and also patients) are more vulnerable to the consequences of ischemia than young animals, it should be taken into account that the observations by Tani et al.[132] were made in isolated hearts, while using an endpoint of ischemic preconditioning that is still controversial. Attenuation of stunning by ischemic preconditioning is also still controversial. It now appears that classical preconditioning does not protect against stunning, but that tbe second window of protection, which occurs 24 to 72 hours after the preconditioning stimulus has been applied, offers protection in large animals.[133]

Principally, all models used in the study of myocardial ischemia are suited to study ischemic preconditioning. In order to mimic the clinical situation as closely as possible awake animals are preferred, but producing myocardial infarction in animals without anesthesia meets almost insurmountable resistance from many ethical committees on animal experimentation. As a matter of fact there are only very few studies in awake animals in which ischemic preconditioning with infarct size as endpoint has been investigated.[121,133]

In *in vivo* studies, a wide variety of anesthesia regimens have been used, quite often depending on the species. Because the precise mechanism of ischemic preconditioning is unknown, one should carefully describe the agents used to anesthetize animals as anesthetic agents may affect the magnitude of protection. This is supported by studies of Haessler et al.[134] who showed that using identical experimental protocols, protection in pentobarbital anesthetized rabbits was significantly greater than in isoflurane- or ketamine/xylazine-anesthetized rabbits, which was not caused by differences in area at risk, temperature, heart rate or arterial blood pressure. Infarct sizes of the control groups were reported to be similar, but there was a clear tendency for the isoflurane-treated animals to have smaller infarcts, an observation, which has later been firmly established by Cope et al.[135] A single 5-minute coronary artery occlusion, which was an effective preconditioning stimulus in pentobarbital anaesthetized dogs without premedication of the opioid analgesic butorphanol,[136,137] was not sufficient to protect the myocardium after premedication of dogs with butorphanol,[138] the threshold for preconditioning was increased as two episodes of 5-minute occlusions were required to provide an effective preconditioning stimulus.

Ytrehus et al.[139] have reported that infarct size development and the protection by ischemic preconditioning are very similar for *in situ* and isolated Krebs-Henseleit bicarbonate buffer-perfused rabbit heart. Downey and Yellon[140] have pointed out that this observation strongly supports the use of the isolated heart models, because in these models the

pharmacology of preconditioning can be better studied as administration of drugs can be precisely controlled and drugs that are not tolerated when administered systemically can be administered directly to the heart. However, of the rat heart, which is also used in *in situ* experiments and isolated heart preparations, it is unknown whether the degree of protection is similar in these two models.[141] Isolated cardiomyocytes[142,143] also eliminate the interaction with non-cardiac tissue. The major attraction of this model is that it is very inexpensive and therefore permits studies using agents and techniques, which cannot be afforded otherwise.

However, the lack of innervation and extracardiac factors in isolated hearts and myocytes may be a shortcoming as evidence is now accumulating that brief supply or demand ischemia in other organs (kidney, small intestine and skeletal muscle) is capable of delaying myocardial infarct development produced by a coronary artery occlusion.[144,145] Therefore, it appears that to fully appreciate the processes by which the myocardium can be protected, the intact animal is the most appropriate model.

Experimental design

The experimental protocol of an ischemic preconditioning study consists of five distinct phases (i) stabilization period after surgery, (ii) the preconditioning stimulus, (iii) a period of intermittent reperfusion, (iv) a prolonged period of test ischemia during which infarction develops and (v) a period of reperfusion at the end of which the effect of the preconditioning stimulus on infarct size is assessed. Each of these phases require some comment;

(i) Sandhu et al.[146] observed in anesthetized rabbits that a single 5-minute coronary artery occlusion limited infarct size development during a subsequent 30-minute occlusion when the preconditioning stimulus was given shortly (5 minutes) after the surgical procedures were completed, but was ineffective when the stabilization period was extended to 30 minutes. The reason for this observation is unknown but the authors speculated that the stress associated with surgery may have resulted in systemic release of catecholamines, a possible mechanism involved in ischemic preconditioning.[147] This observation may be important for the design of experimental protocols, in which different groups of animals are studied (for instance comparison of the protective effect of a single versus multiple brief occlusions). Some investigators avoid differences in the duration of the experimental protocol by adjusting the duration of the stabilization period. But they should thus be aware that this could blur their data.

(ii) In the vast majority of studies, the preconditioning stimulus consists of one or multiple brief periods of ischemia produced by abrupt occlusion and reperfusion of a coronary artery.[132] The preconditioning stimulus should produce no or only negligible necrosis. Depending on the severity of the flow reduction, partial coronary artery occlusions, alone[148] or in the presence of atrial pacing in order to produce demand ischemia,[149] can be used to precondition the myocardium.

(iii) Intermittent reperfusion between the preconditioning stimulus and the period of test ischemia is required when complete occlusions are used to precondition the myocardium, but may not be necessary when a partial occlusion is used to either precondition the myocardium[150,151] or during the test period of ischemia.[152] The duration of the intermittent reperfusion period is an important determinant for the outcome of preconditioning studies as, depending on the species, protection is lost after a few hours (classic preconditioning or first window of protection). However, in some species protection reappears after 24-48 hours (second window of protection), illustrating that ischemic preconditioning is, at least in some species, a biphasie phenomenon.[153,154]

(iv) As far as the duration of the infarct producing sustained coronary artery occlusion is concerned, one should take into account that infarct size development is different in the various species.[155] Ischemic preconditioning has been shown in animals with[117] and without[122] a collateral circulation, implying that the extent of the collateral circulation is not crucial for the occurrence of ischemic preconditioning. However, collateral blood flow is a major determinant of infarct size and in order to obtain a proper assessment of infarct size collateral blood flow should always be determined in species with a collateral circulation. Body temperature is another important determinant of infarct size development with the magnitude of the effect depending on the duration of the coronary artery occlusion.[156-158] The need for rigorous control of temperature also follows from the observation that protection by ischemic preconditioning is extended to longer occlusion durations when the experiments are performed at lower body temperature.[159]

Abrupt and complete reperfusion after the sustained occlusion has been a feature of all preconditioning studies except in the study by Kapadia et al.[160] The latter studied ischemic preconditioning in closed chest pigs by subjecting them to instrumentation with a percutaneous transluminal angioplasty catheter on which an artificial stenosis (82% diameter reduction and 30% flow reduction) was mounted just proximal to the balloon. Because the stenosis was left in place during the entire experimental protocol, reperfusion was incomplete. Although the stenosis was not severe enough to precondition the myocardium by itself, the presence of the stenosis did not prevent preconditioning by multiple balloon inflations. The presence of a stenosis during reperfusion has yielded conflicting reports on infarct size as no changes[161,162] as well as increases[163,164] have been reported. In trying to explain these differences, one must keep in mind that the presence of a stenosis during reperfusion could lead to both persistent endocardial ischemia as well as a limitation of the reperfusion-induced damage by preventing an excessive burst of oxygen-derived free radicals. Nevertheless, a nice feature of the model by Kapadia et al.[160] is that it resembles a clinical condition (patients with a coronary artery stenosis which becomes totally obstructed by thrombus and is treated with thrombolysis) more closely than any of the abrupt total occlusion - total reperfusion models.

(v) The vast majority of studies use the ratio of infarcted area (IA) and the area at risk (AR) to assess infarct size limitation by ischemic preconditioning. In the further analysis it is then assumed that this ratio is independent of the area at risk i.e. the relation between AR and IA is proportional. Several groups of investigators have however shown that this is true for rats,[165] but not for pigs[151] and rabbits[165] i.e. the linear regression line relating infarcted area and area at risk has a zero-intercept on the AR-axis. This implies that the mathematical description of this relationship is IA=a*AR + b or IA/AR=a + b/AR. Especially for small AR, IA/AR depends on AR. Thus unless AR exceeds 20% of the left ventricular mass, IA/AR should be used with care, in particular when studies are compared in which the areas at risk differ substantially.

Hibernation

While myocardial stunning and ischemic preconditioning were originally laboratory observations, the concept of myocardial hibernation originates from clinical observations that in some patients with coronary artery disease and chronic left ventricular dysfunction, wall function improved after coronary bypass surgery.[166] The decreased wall motion before surgery was therefore viewed as a chronic adaptive response to a reduced coronary blood flow, with the reduction in contractility being reversible upon reinstatement of normal myocardial perfusion. The clinical importance of hibernation is that viability of myocardium is preserved due to restoration of aerobic metabolism, in the presence of a chronic blood flow reduction.[167] In an earlier issue of this journal, Hearse[1] made therefore a distinction between physiological and biochemical ischemia. In the latter condition the myocardium is metabolically distressed and cell death will evolve when this condition is maintained for a prolonged period of time. Hibernating myocardium can therefore be biochemical ischemic for only brief periods, while its function is depressed chronically because of subnormal flow i.e. the myocardium is chronically physiologically ischemic.[167] The most difficult modeling problem in hibernation is therefore how to achieve a state of reduced flow (function), which does not cause infarction.

Hibernation should, by definition, be studied in chronic models, which implies the use of awake animals in which blood flow in a coronary artery is chronically and sufficiently reduced such that the reduced coronary blood flow leads to a permanent regional dysfunction (which can be assessed continuously by sonomicrometry) but not to cell death. Regional metabolism should also be monitored continuously in the distribution area of the obstructed coronary artery and preferentially also in the normal myocardium to determine the nature of the metabolic adaptation. Essential for these models is that the function of the hibernating myocardium must recover after blood flow is normalized. Finally, at the end of the study the post-hibernating myocardium should be examined for the absence of cell death. None of the current models have met all these requirements, although in several studies chronically instrumented animals (pigs and dogs) have been employed in which regional dysfunction was produced for several

days to months by partial narrowing of a coronary artery.[52,98,168-171] A further limitation of the existing models is that concentric stenoses have been applied, thereby excluding a potential role of the local intact endothelium as would have been possible if eccentric stenoses were used. Because of the lack of coronary collaterals the use of the pigs appears to be advantageous, when the flow restriction in the native coronary artery can be controlled. However, Millard et al.[28] have shown that coronary collaterals also develop in pigs with a fixed stenosis.

Based on the results of some studies in man[172,173] and in animals,[174] some investigators have hypothesized that chronic hibernation is nothing else than chronic stunning resulting from repetitive stress-induced ischemia, rather than a chronic downregulation of function secondary to a decreased coronary blood flow. If true, flow might be normal or almost normal under resting conditions and even be increased during exercise-induced ischemia, while function should return to normal after a sufficiently long stress-free period. To test this hypothesis continuous assessment of regional myocardial function in patients with hibernating myocardium is required. If confirmed, brief repetitive coronary artery occlusions in the presence and possibly even in the absence of a mild stenosis, could produce hibernation.

In several studies, hibernation has been produced by partially restricting coronary artery flow during periods ranging from only a few hours up to a few days.[175-179] In these 'short-term hibernation models' the essential characteristics of chronic hibernation cannot be studied. The usefulness of these short-term models is therefore limited, but they may provide some insight into the triggers leading to hibernation. However, for 'each short-term hibernation model' it should be shown that chronic hibernation develops if the model is used in chronic studies. Otherwise the putative triggers identified in the short-term hibernation models' cannot be related to the development of hibernation. Isolated heart models using sequences of no-flow and low-flow ischemia have also been employed to study metabolic adaptation as a possible trigger for hibernation.[180] This model is not only limited by the short time in which it can be studied, but has also the disadvantage that it produces global ischemia, and lacks the interaction with the other organs (neural reflexes) which may be vital in the development of hibernation. For these reasons isolated heart models and other less well advanced models such as isolated cardiomyocytes appear not to be very useful in the study of hibernation at the present time.

Future developments

Molecular biologists have provided physiologists in recent years with new animal models of cardiovascular disease by altering the mammalian genome through introduction or modification of genes. The mouse has become the animal of choice because their genome is well characterized, their cost is relatively low and a large series of animals can be obtained in a

short period of breeding.[181] Assessment of cardiac function in these transgenic mice (e.g. gene-targeted knock-out) is essential for understanding the functional consequences of the manipulation of the genome. Because in the past measurements of cardiovascular function in mice have been rare, the possibilities to determine cardiac function in this species are just developing.[182] In recent years, however, we have witnessed the development of miniaturized Langendorff preparations of isolated (work performing) mouse hearts for measurement of left ventricular contractility and relaxation,[183,184] *in situ* measurements of aortic and left ventricular pressure,[185,186] the use of microsphere and dilution techniques for the determination of blood flow and volumes,[187] the application of echocardiography for assessment of left ventricular dimensions, wall thickness, mass, circumferential fibre shortening and left ventricular wall stress-shortening relationships,[188-190] and the application of Doppler echocardiography to obtain tracings from the left ventricular outflow tract for estimating peak blood velocity.[189] Desai et al.,[191] employing techniques, previously used in large animals, have described the feasibility of chronic monitoring hemodynamic and metabolic parameters in conscious mice at rest and during exercise.

The purpose of the aforementioned studies was to improve the ability to examine the phenotypic changes after gene targeted manipulations rather than the study of myocardial ischemia and ischemic syndromes. These transgenic models will, however, become useful for the study of processes involved in myocardial ischemia and ischemic syndromes, once we have learned to apply our knowledge and techniques obtained in large animal studies.

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References

1. Hearse DJ. Myocardial ischaemia: can we agree on a definition for the 21st century? *Cardiovasc Res.* 1994;28:1737-1744.
2. Reimer KA, Jennings RB. Myocardial ischemia, hypoxia and infarction. In: Fozzard HA, Haber E, Jennings RB, Katz AM, Morgan HE, eds. *The Heart and Cardiovascular System.* New York: Raven Press; 1992:1875-1973.
3. Bustad LK, McClennan RO. *Swine in biomedical research.* Richland, WA: Pacific Northwest Laboratory; 1966.
4. Tumbleson ME. *Swine in biomedical research.* New York and London: Plenum Press; 1986.
5. Da Vinci L. Instituto Graphico de Agostini. New York: Reynal; 1956:383.
6. Buckley NM, Gootman PM, Yellin EL, Brazeau P. Age-related cardiovascular effects of catecholamines in anesthetized piglets. *Circ Res.* 1979;45:282-292.
7. Gootman PM, Gootman N, Buckley BJ. Maturation of central autonomic control of the circulation. *Fed Proc.* 1983;42:1648-1655.
8. Hanson LE. Fifty years progress in swine nutrition. *J Animal Sci.* 1958;17:1029-1057.
9. Selye H, Bajusz E, Grasso S, Mendel P. Simple techniques for the surgical occlusion of coronary vessels in the rats. *Angiology.* 1960;11:398-407.

10. Liu Y, Downey JM. Ischemic preconditioning protects against infarction in rat heart. *Am J Physiol.* 1992;263:H1107-H1112.
11. Cohen MV, 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.
12. Obata T, Hosokawa H, Yamanaka Y. *In vivo* monitoring of norepinephrine and .OH generation on myocardial ischemic injury by dialysis technique. *Am J Physiol.* 1994;266:H903-H908.
13. Pfeffer JM, Pfeffer MA, Fletcher PJ, Braunwald E. Progressive ventricular remodeling in rat with myocardial infarction. *Am J Physiol.* 1991;260:H1406-H1414.
14. Pfeffer JM, Pfeffer MA. Angiotensin converting enzyme inhibition and ventricular remodeling in heart failure. *Am J Med.* 1988;84:37-44.
15. Harken AH, Simson MB, Haselgrove J, Wetstein L, Harden 3rd WR, Barlow CH. Early ischemia after complete coronary ligation in the rabbit, dog, pig, and monkey. *Am J Physiol.* 1981;241:H202-H210.
16. Schaper W. *The collateral circulation of the heart.* In: Black DAK, editor, Clinical Studies. Amsterdam: North Holland Publishing Company; 1971.
17. Schaper W, Jageneau A, Xhonneux R. The development of collateral circulation in the pig and dog heart. *Cardiologia.* 1967;51:321-335.
18. Schaper W, Bernotat-Danielowski S, Nienaber C, Schaper J. Collateral circulation. In: Fozzard HA, Haber E, Jennings RB, Katz AM, eds. *The Heart and Cardiovascular System.* New York: Raven Press; 1992:1427-1464.
19. White FC, Bloor CM. Coronary collateral circulation in the pig: correlation of collateral flow with coronary bed size. *Basic Res Cardiol.* 1981;76:189-196.
20. Shen YT, Fallon JT, Iwase M, Vatner SF. Innate protection of baboon myocardium: effects of coronary artery occlusion and reperfusion. *Am J Physiol.* 1996;270:H1812-H1818.
21. Crozatier B, Ross Jr. J, Franklin D, Bloor CM, White FC, Tomoike H, McKown DP. Myocardial infarction in the baboon: regional function and the collateral circulation. *Am J Physiol.* 1978;235:H413-H421.
22. Winkler B, Sass S, Binz K, Schaper W. Myocardial blood flow and myocardial infarction in rats, guinea, pigs and rabbits (Abstract). *J Mol Cell Cardiol.* 1984;16:48.
23. Schaper W. Experimental infarcts and the microcirculation. In: Hease DJ, Yellon DM, eds. *Therapeutic Approaches to Myocardial Infarct Size Limitation.* New York: Raven Press; 1984:79-90.
24. Franklin D, McKown D, McKown M, Hartley J, Caldwell M. Development and regression of coronary collaterals induced by repeated , reversible ischemia in dogs (Abstract). *Fed Proc.* 1981;40:339.
25. Yamamoto H, Tomoike H, Shimokawa H, Nabeyama S, Nakamura M. Development of collateral function with repetitive coronary occlusion in a canine model reduces myocardial reactive hyperemia in the absence of significant coronary stenosis. *Circ Res.* 1984;55:623-632.
26. Kersten JR, McGough MF, Pagel PS, Tessmer JP, Warltier DC. Temporal dependence of coronary collateral development. *Cardiovasc Res.* 1997;34:306-312.
27. Litvak J, Siderides LE, Vineberg AM. The experimental production of coronary artery insufficiency and occlusion. *Am Heart J.* 1957;53:505-518.
28. Millard RW. Induction of functional coronary collaterals in the swine heart. *Basic Res Cardiol.* 1981;76:468-473.
29. Scheel KW, Ingram LA, Wilson JL. Effects of exercise on the coronary and collateral vasculature of beagles with and without coronary occlusion. *Circ Res.* 1981;48:523-530.
30. Sanders M, White FC, Peterson TM, Bloor CM. Effects of endurance exercise on coronary collateral blood flow in miniature swine. *Am J Physiol.* 1978;234:H614-H619.
31. Cohen MV, Yipintsoi T, Scheuer J. Coronary collateral stimulation by exercise in dogs with stenotic coronary arteries. *J Appl Physiol.* 1982;52:664-671.
32. Chilian WM, Mass HJ, Williams SE, Layne SM, Smith EE, Scheel KW. Microvascular occlusions promote coronary collateral growth. *Am J Physiol.* 1990;258:H1103-H1111.
33. Bloor CM, White FC, Lammers RJ. Cardiac ischemia and coronary flow in swine. In: Stanton HC, Mersmann HJ, eds. *Swine in cardiovascular research.* Boca Baton, Florida: C.R.C. Press Inc.; 1986:87-104.
34. Domenech RJ, Hoffman JI, Noble MI, Saunders KB, Henson JR, Subijanto S. Total and regional coronary blood flow measured by radioactive microspheres in conscious and anesthetized dogs. *Circ Res.* 1969;25:581-596.
35. Heymann MA, Payne BD, Hoffman JI, Rudolph AM. Blood flow measurements with radionuclide-labeled particles. *Prog Cardiovasc Dis.* 1977;20:55-79.
36. Kowallik P, Schulz R, Guth BD, Schade A, Paffhausen W, Gross R, Heusch G. Measurement of regional myocardial blood flow with multiple colored microspheres. *Circulation.* 1991;83:974-982.

37. Hale SL, Alker KJ, Kloner RA. Evaluation of nonradioactive, colored microspheres for measurement of regional myocardial blood flow in dogs. *Circulation.* 1988;78:428-434.
38. Gallagher KP, Matsuzaki M, Koziol JA, Kemper WS, Ross Jr. J. Regional myocardial perfusion and wall thickening during ischemia in conscious dogs. *Am J Physiol.* 1984;247:H727-H738.
39. Manders WT, Vatner SF. Effects of sodium pentobarbital anesthesia on left ventricular function and distribution of cardiac output in dogs, with particular reference to the mechanism for tachycardia. *Circ Res.* 1976;39:512-517.
40. Zimpfer M, Manders WT, Barger AC, Vatner SF. Pentobarbital alters compensatory neural and humoral mechanisms in response to hemorrhage. *Am J Physiol.* 1982;243:H713-H721.
41. Merin RG, Verdouw PD, de Jong JW. Myocardial functional and metabolic responses to ischemia in swine during halothane and fentanyl anesthesia. *Anesthesiology.* 1982;56:84-92.
42. Triana JF, Li XY, Jamaluddin U, Thornby JI, Bolli R. Postischemic myocardial "stunning". Identification of major differences between the open-chest and the conscious dog and evaluation of the oxygen radical hypothesis in the conscious dog. *Circ Res.* 1991;69:731-747.
43. Ihara T, Shannon RP, Komamura K, Pasipoularides A, Patrick T, Shen YT, Vatner SF. Effects of anaesthesia and recent surgery on diastolic function. *Cardiovasc Res.* 1994;28:325-336.
44. Corday E, Lang TW, Meerbaum S, Gold H, Hirose S, Rubins S, Dalmastro M. Closed chest model of intracoronary occlusion for study of regional cardiac function. *Am J Cardiol.* 1974;33:49-59.
45. Debley VG. Miniature hydraulic occluder for zero blood flow determination. *J Appl Physiol.* 1971;31:138-139.
46. 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.
47. Elzinga WE, Skinner DB. Hemodynamic characteristics of critical stenosis in canine coronary arteries. *J Thorac Cardiovasc Surg.* 1975;69:217-222.
48. Hosko MJ, Gross GJ, Warltier DC. Technique for precise, graded arterial stenosis and occlusion. *Basic Res Cardiol.* 1977;72:651-659.
49. Van der Meer JJ, Reneman RS. An improved technique to induce a standardized functional stenosis of a coronary artery. *Eur Surg Res.* 1972;4:407-418.
50. Liedtke AJ, DeMaison L, Eggleston AM, Cohen LM, Nellis SH. Changes in substrate metabolism and effects of excess fatty acids in reperfused myocardium. *Circ Res.* 1988;62:535-542.
51. Schulz R, Rose J, Martin C, Brodde OE, Heusch G. Development of short-term myocardial hibernation. Its limitation by the severity of ischemia and inotropic stimulation. *Circulation.* 1993;88:684-695.
52. 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.
53. Haase J, Di Mario C, Slager CJ, van der Giessen WJ, den Boer A, de Feyter PJ, Reiber JH, Verdouw PD, Serruys PW. In-vivo validation of on-line and off-line geometric coronary measurements using insertion of stenosis phantoms in porcine coronary arteries. *Cathet Cardiovasc Diagn.* 1992;27:16-27.
54. Ichikawa Y, Yokoyama M, Akita H, Fukuzaki H. Constriction of a large coronary artery contributes to serotonin-induced myocardial ischemia in the dog with pliable coronary stenosis. *J Am Coll Cardiol.* 1989;14:449-459.
55. Theroux P, Ross Jr. J, Franklin D, Kemper WS, Sasayama S. Coronary arterial reperfusion. III. Early and late effects on regional myocardial function and dimensions in conscious dogs. *Am J Cardiol.* 1976;38:599-606.
56. Theroux P, Ross J, Jr., Franklin D, Kemper WS, Sasyama S. Regional Myocardial function in the conscious dog during acute coronary occlusion and responses to morphine, propranolol, nitroglycerin, and lidocaine. *Circulation.* 1976;53:302-314.
57. Hoit BD, Lew WY. Functional consequences of acute anterior vs. posterior wall ischemia in canine left ventricles. *Am J Physiol.* 1988;254:H1065-H1073.
58. Sassen LMA, Duncker DJ, Gho BC, Diekmann HW, Verdouw PD. Haemodynamic profile of the potassium channel activator EMD 52692 in anaesthetized pigs. *Br J Pharmacol.* 1990;101:605-614.
59. Headrick JP, Emerson CS, Berr SS, Berne RM, Matherne GP. Interstitial adenosine and cellular metabolism during beta-adrenergic stimulation of the *in situ* rabbit heart. *Cardiovasc Res.* 1996;31:699-710.
60. Massie BM, Schwartz GG, Garcia J, Wisneski JA, Weiner MW, Owens T. Myocardial metabolism during increased work states in the porcine left ventricle *in vivo. Circ Res.* 1994;74:64-73.
61. McFalls EO, Baldwin D, Palmer B, Marx D, Jaimes D, Ward HB. Regional glucose uptake within hypoperfused swine myocardium as measured by positron emission tomography. *Am J Physiol.* 1997;272:H343-H349.

62. Bush LR, Shebuski RJ. *In vivo* models of arterial thrombosis and thrombolysis. *Faseb J.* 1990;4:3087-3098.
63. Langendorff O. Untersuchungen am uberlebenden Saugerthierherzen. *Pflugers Arch Ges Physiol.* 1895;61:291-332.
64. Döring HJ, Dehnert H. The isolated perfused heart. According to Langendorff. In: *BVM-Biomesstechnik*: V-Verlag March GmbH; 1988:D-7806.
65. Langendorff O. Zur Kenntniss des Blutlaufs in den Kranzgefässen des Herzens. *Pflugers Arch Ges Physiol.* 1899;78:423.
66. Gottlieb R, Magnus R. Digitalis und Herzarbeit. Nach Versuchen am überlebenden Warmblüterherzen. *Arch Exp Path Pharmakol.* 1904;51:30-63.
67. Heymans JF, Kochmann F. Une nouvelle méthode de circulation artificielle à travers le coeur isolé de mammifère. *Arch Int Pharmacodyn Thérap.* 1904;5:379-386.
68. Humphrey SM, Gavin JB, Herdson PB. The relationship of ischemic contracture of vascular reperfusion in the isolated rat heart. *J Mol Cell Cardiol.* 1980;12:1397-1406.
69. Humphrey SM, Thomson RW, Gavin JB. The effect of an isovolumic left ventricle on the coronary vascular competence during reflow after global ischemia in the rat heart. *Circ Res.* 1981;49:784-791.
70. Ganote CE, Humphrey SM. Effects of anoxic or oxygenated reperfusion in globally ischemic, isovolumic, perfused rat hearts. *Am J Pathol.* 1985;120:129-145.
71. De Leiris J, Harding DP, Pestre S. The isolated perfused rat heart: a model for studying myocardial hypoxia or ischaemia. *Basic Res Cardiol.* 1984;79:313-321.
72. Manning AS, Hearse DJ, Dennis SC, Bullock GR, Coltart DJ. Myocardial ischaemia: an isolated, globally perfused rat heart model for metabolic and pharmacological studies. *Eur J Cardiol.* 1980;11:1-21.
73. De Jong JW, Harmsen E, De Tombe PP, Keijzer E. Nifedipine reduces adenine nucleotide breakdown in ischemic rat heart. *Eur J Pharmacol.* 1982;81:89-96.
74. Duncker DJ, Hartog JM, Hugenholtz PG, Saxena PR, Verdouw PD. The effects of nisoldipine (Bay K 5552) on cardiovascular performance and regional blood flow in pentobarbital-anaesthetized pigs with or without beta-adrenoceptor blockade. *Br J Pharmacol.* 1986;88:9-18.
75. Neely JR, Liebermeister H, Battersby EJ, Morgan HE. Effect of pressure development on oxygen consumption by isolated rat heart. *Am J Physiol.* 1967;212:804-814.
76. Krams R, Janssen M, Van der Lee C, Van Meegen J, De Jong JW, Slager CJ, Verdouw PD. Loss of elastic recoil in post-ischemic myocardium induces a rightward shift of the systolic pressure-volume relationship. *Am J Physiol.* 1994;267:H1557-H1564.
77. Bayliss CE, Crawford FB, Nsafoah B. The interventricular septum: an isolated perfused model for assessing myocardial function. *Can J Physiol Pharmacol.* 1977;55:1358-1368.
78. Fan D, Soei LK, Stubenitsky R, Boersma E, Duncker DJ, Verdouw PD, Krams R. Contribution of asynchrony and nonuniformity to mechanical interaction in normal and stunned myocardium. *Am J Physiol.* 1997;273:H2146-H2154.
79. Stemmer P, Wisler PL, Watanabe AB. Isolated myocytes in experimental cardiology. In: Fozzard HA, Haber E, Jennings RB, Katz AM, eds. *The heart and vascular system.* New York: Raven press; 1992:387-404.
80. Haworth RA, Hunter DR, Berkoff HA, Moss RL. Metabolic cost of the stimulated beating of isolated adult rat heart cells in suspension. *Circ Res.* 1983;52:342-351.
81. Spieckermann PG, Piper HM. Oxygen demand of calcium-tolerant adult cardiac myocytes. *Basic Res Cardiol.* 1985;80(Suppl.2):71-74.
82. Niggli E. A laser diffraction system with improved sensitivity for long-time measurements of sarcomere dynamics in isolated cardiac myocytes. *Pflügers Arch.* 1988;411:462-468.
83. Tung L. An ultrasensitive transducer for measurement of isometric contractile force from single heart cells. *Pflugers Arch.* 1986;407:109-115.
84. Roos KP, Brady AJ. Individual sarcomere length determination from isolated cardiac cells using high-resolution optical microscopy and digital image processing. *Biophys J.* 1982;40:233-244.
85. Murphy MP, Hohl C, Brierley GP, Altschuld RA. Release of enzymes from adult rat heart myocytes. *Circ Res.* 1982;51:560-568.
86. Linden J, Patel A, Spanier AM, Weglicki WB. Rapid agonist-induced decrease of ^{125}I-pindolol binding to beta-adrenergic receptors. Relationship to desensitization of cyclic AMP accumulation in intact heart cells. *J Biol Chem.* 1984;259:15115-15122.
87. Heyndrickx GR, Millard RW, McRitchie RJ, Maroko PR, Vatner SF. Regional myocardial functional and electrophysiological alterations after brief coronary artery occlusion in conscious dogs. *J Clin Invest.* 1975;56:978-985.

88. Lerch R. Myocardial stunning: the role of oxidative substrate metabolism. In: Heusch G, Schulz R, eds. *New paradigms of coronary artery disease. Hibernation, stunning, ischemic preconditioning.* Darmstadt: Steinkopff Verlag; 1996:88-99.
89. Braunwald E, Kloner RA. The stunned myocardium: prolonged, postischemic ventricular dysfunction. *Circulation.* 1982;66:1146-1149.
90. Heyndrickx GR, Vatner SF. Myocardial stunning and hibernation experimental models. In: Heyndrickx GR, Vatner SF, Wijns W, eds. *Stunning, hibernation and preconditioning. Clinical pathopysiology of myocardial ischemia.* Philadelphia: Lippincott-Raven Publishers; 1997:177-193.
91. Bolli R, Zhu WX, Thornby JI, O'Neill PG, Roberts R. Time course and determinants of recovery of function after reversible ischemia in conscious dogs. *Am J Physiol.* 1988;254:H102-H114.
92. Wouters PF, Van de Velde M, Van Aken H, Flameng W. Ischemic event characteristics determine the extent of myocardial stunning in conscious dogs. *Basic Res Cardiol.* 1996;91:140-146.
93. Shen YT, Vatner SF. Differences in myocardial stunning following coronary artery occlusion in conscious dogs, pigs, and baboons. *Am J Physiol.* 1996;270:H1312-H1322.
94. White JL, Myers AK, Analouei A, Kim YD. Functional recovery of stunned myocardium is greater with halothane than fentanyl anaesthesia in dogs. *Br J Anaesth.* 1994;73:214-219.
95. Kanaya N, Fujita S. The effects of isoflurane on regional myocardial contractility and metabolism in "stunned" myocardium in acutely instrumented dogs. *Anesth Analg.* 1994;79:447-454.
96. Gaasch WH, Bernard SA. The effect of acute changes in coronary blood flow on left ventricular end-diastolic wall thickness. An echocardiographic study. *Circulation.* 1977;56:593-598.
97. Bolli R, Zughaib M, Li XY, Tang XL, Sun JZ, Triana JF, McCay PB. Recurrent ischemia in the canine heart causes recurrent bursts of free radical production that have a cumulative effect on contractile function. A pathophysiological basis for chronic myocardial "stunning". *J Clin Invest.* 1995;96:1066-1084.
98. Matsuzaki M, Gallagher KP, Kemper WS, White F, Ross Jr. J. Sustained regional dysfunction produced by prolonged coronary stenosis: gradual recovery after reperfusion. *Circulation.* 1983;68:170-182.
99. Heyndrickx GR, Wijns W, Vogelaers D, Degrieck Y, Bol A, Vandeplassche G, Melin JA. Recovery of regional contractile function and oxidative metabolism in stunned myocardium induced by 1-hour circumflex coronary artery stenosis in chronically instrumented dogs. *Circ Res.* 1993;72:901-913.
100. Homans DC, Laxson DD, Sublett E, Pavek T, Crampton M. Effect of exercise intensity and duration on regional function during and after exercise-induced ischemia. *Circulation.* 1991;83:2029-2037.
101. Komamura K, Shannon RP, Pasipoularides A, Ihara T, Lader AS, Patrick TA, Bishop SP, Vatner SF. Alterations in left ventricular diastolic function in conscious dogs with pacing-induced heart failure. *J Clin Invest.* 1992;89:1825-1838.
102. De Pauw M, Vincent A, Hodeige D, Heyndrickx GR. Ca^{2+}-responsiveness after 48 hrs of rapid pacing in conscious dogs (Abstract). *Circulation.* 1994;90 [suppl]:I58.
103. Heyndrickx GR, Pannier JL, Vogelaers D, Degrieck Y, Vantrimpont P. Exercise induced diastolic dysfunction after global myocardial stunning in conscious dogs (Abstract). *Circulation.* 1990;82 [suppl]:III606.
104. Mercier JC, Lando U, Kanmatsuse K, Ninomiya K, Meerbaum S, Fishbein MC, Swan HJ, Ganz W. Divergent effects of inotropic stimulation on the ischemic and severely depressed reperfused myocardium. *Circulation.* 1982;66:397-400.
105. Ellis SG, Wynne J, Braunwald E, Henschke CI, Sandor T, Kloner RA. Response of reperfusion-salvaged, stunned myocardium to inotropic stimulation. *Am Heart J.* 1984;107:13-19.
106. Arnold JM, Braunwald E, Sandor T, Kloner RA. Inotropic stimulation of reperfused myocardium with dopamine: effects on infarct size and myocardial function. *J Am Coll Cardiol.* 1985;6:1026-1034.
107. Taegtmeyer H, Roberts AF, Raine AE. Energy metabolism in reperfused heart muscle: metabolic correlates to return of function. *J Am Coll Cardiol.* 1985;6:864-870.
108. Nayler WG, Elz JS, Buckley DJ. The stunned myocardium: effect of electrical and mechanical arrest and osmolarity. *Am J Physiol.* 1988;255:H60-H69.
109. Borgers M, Shu LG, Xhonneux R, Thone F, Van Overloop P. Changes in ultrastructure and Ca^{2+} distribution in the isolated working rabbit heart after ischemia. A time-related study. *Am J Pathol.* 1987;126:92-102.
110. De Graeff PA, Van Gilst WH, De Langen CD, Kingma JH, Wesseling H. Concentration-dependent protection by captopril against ischemia- reperfusion injury in the isolated rat heart. *Arch Int Pharmacodyn Ther.* 1986;280:181-193.
111. Isoyama S, Apstein CS, Wexler LF, Grice WN, Lorell BH. Acute decrease in left ventricular diastolic chamber distensibility during simulated angina in isolated hearts. *Circ Res.* 1987;61:925-933.

112. Krause SM. Effect of global myocardial stunning on Ca^{2+}-sensitive myofibrillar ATPase activity and creatine kinase kinetics. *Am J Physiol.* 1990;259:H813-H819.
113. Kusuoka H, Porterfield JK, Weisman HF, Weisfeldt ML, Marban E. Pathophysiology and pathogenesis of stunned myocardium. Depressed Ca^{2+} activation of contraction as a consequence of reperfusion-induced cellular calcium overload in ferret hearts. *J Clin Invest.* 1987;79:950-961.
114. Fan D, Soei LK, Sassen LM, Krams R, Verdouw PD. Mechanical efficiency of stunned myocardium is modulated by increased afterload dependency. *Cardiovasc Res.* 1995;29:428-437.
115. Aversano T, Maughan WL, Hunter WC, Kass D, Becker LC. End-systolic measures of regional ventricular performance. *Circulation.* 1986;73:938-950.
116. Vinten-Johansen J, Gayheart PA, Johnston WE, Julian JS, Cordell AR. Regional function, blood flow, and oxygen utilization relations in repetitively occluded-reperfused canine myocardium. *Am J Physiol.* 1991;261:H538-H547.
117. Murry CE, Jennings RB, Reimer KA. Preconditioning with ischemia: a delay of lethal cell injury in ischemic myocardium. *Circulation.* 1986;74:1124-1136.
118. Lawson CS. Does ischaemic preconditioning occur in the human heart? *Cardiovasc Res.* 1994;28:1461-1466.
119. Verdouw PD, Gho BC, Duncker DJ. Ischaemic preconditioning: is it clinically relevant? *Eur Heart J.* 1995;16:1169-1176.
120. Verdouw PD, van den Doel MA, de Zeeuw S, Duncker DJ. On the relevance of ischemic preconditioning in humans. *Basic Res Cardiol.* 1997;92:51-53.
121. Cohen MV, Yang XM, Downey JM. Conscious rabbits become tolerant to multiple episodes of ischemic preconditioning. *Circ Res.* 1994;74:998-1004.
122. Schott RJ, Rohmann S, Braun ER, Schaper W. Ischemic preconditioning reduces infarct size in swine myocardium. *Circ Res.* 1990;66:1133-1142.
123. Bukhari EA, Krukenkamp IB, Burns PG, Gaudette GR, Schulman JJ, al-Fagih MR, Levitsky S. Does aprotinin increase the myocardial damage in the setting of ischemia and preconditioning? *Ann Thorac Surg.* 1995;60:307-310.
124. McKean T, Mendenhall W. Comparison of the responses to hypoxia, ischaemia and ischaemic preconditioning in wild marmot and laboratory rabbit hearts. *J Exp Biol.* 1996;199:693-697.
125. Gomoll AW. Cardioprotection associated with preconditioning in the anesthetized ferret. *Basic Res Cardiol.* 1996;91:433-443.
126. Lawson CS, Downey JM. Preconditioning: state of the art myocardial protection. *Cardiovasc Res.* 1993;27:542-550.
127. Brooks G, Hearse DJ. Role of protein kinase C in ischemic preconditioning: player or spectator? *Circ Res.* 1996;79:627-630.
128. Gho BC, Eskildsen-Helmond YEG, de Zeeuw S, Lamers JMJ, Verdouw PD. Does protein kinase C play a pivotal role in the mechanisms of ischemic preconditioning? *Cardiovasc Drugs Ther.* 1997;10:775-786.
129. Ovize M, Aupetit JF, Rioufol G, Loufoua J, André-Fouët X, Minaire Y, Faucon G. Preconditioning reduces infarct size but accelerates time to ventricular fibrillation in ischemic pig heart. *Am J Physiol.* 1995;269:H72-H79.
130. Grund F, Sommerschild HT, Kirkeboen KA, Ilebekk A. Proarrhythmic effects of ischemic preconditioning in anesthetized pigs. *Basic Res Cardiol.* 1997;92:417-425.
131. 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.
132. Tani M, Suganuma Y, Hasegawa H, Shinmura K, Hayashi Y, Guo X, Nakamura Y. Changes in ischemic tolerance and effects of ischemic preconditioning in middle-aged rat hearts. *Circulation.* 1997;95:2559-2566.
133. Qiu Y, Tang XL, Park SW, Sun JZ, Kalya A, Bolli R. The early and late phases of ischemic preconditioning: a comparative analysis of their effects on infarct size, myocardial stunning, and arrhythmias in conscious pigs undergoing a 40-minute coronary occlusion. *Circ Res.* 1997;80:730-742.
134. Haessler R, Kuzume K, Chien GL, Wolff RA, Davis RF, Van Winkle DM. Anaesthetics alter the magnitude of infarct limitation by ischaemic preconditioning. *Cardiovasc Res.* 1994;28:1574-1580.
135. Cope DK, Impastato WK, Cohen MV, Downey JM. Volatile anesthetics protect the ischemic rabbit myocardium from infarction. *Anesthesiology.* 1997;86:699-709.
136. Gross GJ, Auchampach JA. Blockade of ATP-sensitive potassium channels prevents myocardial preconditioning in dogs. *Circ Res.* 1992;70:223-233.

137. Grover GJ, Sleph PG, Dzwonczyk S. Role of myocardial ATP-sensitive potassium channels in mediating preconditioning in the dog heart and their possible interaction with adenosine A_1-receptors. *Circulation.* 1992;86:1310-1316.
138. Schwartz LM, Jennings RB, Reimer KA. Premedication with the opioid analgesic butorphanol raises the threshold for ischemic preconditioning in dogs. *Basic Res Cardiol.* 1997;92:106-114.
139. Ytrehus K, Liu Y, Tsuchida A, Miura T, Liu GS, Yang XM, Herbert D, Cohen MV, Downey JM. Rat and rabbit heart infarction: effects of anesthesia, perfusate, risk zone, and method of infarct sizing. *Am J Physiol.* 1994;267:H2383-H2390.
140. Downey JM, Yellon DM. The biology of preconditioning. In: Heyndrickx GR, Vatner SF, Wijns W, eds. *Stunning, hibernation and preconditioning. Clinical pathophysiology of myocardial ischemia.* Philadelphia: Lippincott-Raven Publishers; 1997:105-119.
141. Bugge E, Ytrehus K. Inhibition of sodium-hydrogen exchange reduces infarct size in the isolated rat heart-a protective additive to ischaemic preconditioning. *Cardiovasc Res.* 1995;29:269-274.
142. Gottlieb RA, Gruol DL, Zhu JY, Engler RL. Preconditioning rabbit cardiomyocytes: role of pH, vacuolar proton ATPase, and apoptosis. *J Clin Invest.* 1996;97:2391-2398.
143. Armstrong S, Downey JM, Ganote CE. Preconditioning of isolated rabbit cardiomyocytes: induction by metabolic stress and blockade by the adenosine antagonist SPT and calphostin C, a protein kinase C inhibitor. *Cardiovasc Res.* 1994;28:72-77.
144. Gho BC, Schoemaker RG, van den Doel MA, Duncker DJ, Verdouw PD. Myocardial protection by brief ischemia in noncardiac tissue. *Circulation.* 1996;94:2193-2200.
145. Birnbaum Y, Hale SL, Kloner RA. Ischemic preconditioning at a distance: reduction of myocardial infarct size by partial reduction of blood supply combined with rapid stimulation of the gastrocnemius muscle in the rabbit. *Circulation.* 1997;96:1641-1646.
146. Sandhu R, Diaz RJ, Mao GD, Wilson GJ. Ischemic preconditioning: differences in protection and susceptibility to blockade with single-cycle versus multicycle transient ischemia. *Circulation.* 1997;96:984-995.
147. Banerjee A, Locke-Winter C, Rogers KB, Mitchell MB, Brew EC, Cairns CB, Bensard DD, Harken AH. Preconditioning against myocardial dysfunction after ischemia and reperfusion by an alpha 1-adrenergic mechanism. *Circ Res.* 1993;73:656-670.
148. 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.
149. Iwamoto T, Bai XJ, Downey HF. Preconditioning with supply-demand imbalance limits infarct size in dog heart. *Cardiovasc Res.* 1993;27:2071-2076.
150. 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.
151. Koning MMG, Gho BC, 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.
152. Schulz R, Post H, 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. *Circ Res.* 1995;76:942-950.
153. Kuzuya T, Hoshida S, Yamashita N, Fuji H, Oe H, Hori M, Kamada T, Tada M. Delayed effects of sublethal ischemia on the acquisition of tolerance to ischemia. *Circ Res.* 1993;72:1293-1299.
154. Marber MS, Latchman DS, Walker JM, 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.
155. Nienaber C, 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;78:210-226.
156. Chien GL, Wolff RA, Davis RF, van Winkle DM. "Normothermic range" temperature affects myocardial infarct size. *Cardiovasc Res.* 1994;28:1014-1017.
157. Duncker DJ, Klassen CL, Ishibashi Y, Herrlinger SH, Pavek TJ, Bache RJ. Effect of temperature on myocardial infarction in swine. *Am J Physiol.* 1996;270:H1189-199.
158. Schwartz LM, Verbinski SG, Vander Heide RS, Reimer KA. Epicardial temperature is a major predictor of myocardial infarct size in dogs. *J Mol Cell Cardiol.* 1997;29:1577-1583.
159. Van den Doel MA, Gho BC, Duval SY, Schoemaker RG, Duncker DJ, Verdouw PD. Hypothermia extends the cardioprotection by ischaemic preconditioning to coronary artery occlusions of longer duration. *Cardiovasc Res.* 1998;37:76-81.

160. Kapadia SJ, Terlato JS, Most AS. Presence of a critical coronary artery stenosis does not abolish the protective effect of ischemic preconditioning. *Circulation.* 1997;95:1286-1292.
161. Lefkowitz CA, Pace DP, Gallagher KP, Buda AJ. The effects of a critical stenosis on myocardial blood flow, ventricular function, and infarct size after coronary reperfusion. *Circulation.* 1988;77:915-926.
162. Richard VJ, Brooks SE, Jennings RB, Reimer KA. Effect of a critical coronary stenosis on myocardial neutrophil accumulation during ischemia and early reperfusion in dogs. *Circulation.* 1989;80:1805-1815.
163. Schmidt SB, Varghese PJ, Bloom S, Yackee JM, Ross AM. The influence of residual coronary stenosis on size of infarction after reperfusion in a canine preparation. *Circulation.* 1986;73:1354-1359.
164. Myears DW, Nohara R, Abendschein DR, Saffitz JE, Sobel BE, Bergmann SR. Compromise of beneficial effects of reperfusion on myocardium supplied by vessels with critical residual stenosis. *J Am Coll Cardiol.* 1988;11:1078-1086.
165. Ytrehus K, Cohen MV, Downey J. Volume of risk zone influences infarct size in rabbits and may account for unexplained variability in infarction studies (Abstract). *Circulation.* 1993;88:I137.
166. Rahimtoola SH. Coronary bypass surgery for chronic angina--1981. A perspective. *Circulation.* 1982;65:225-241.
167. Heusch G, Ferrari R, Hearse DJ, Ruigrok TJ, Schulz R. 'Myocardial hibernation'--questions and controversies. *Cardiovasc Res.* 1997;36:301-309.
168. Duncker DJ, Heiligers JP, Saxena PR, Verdouw PD. Nisoldipine and perfusion of post-stenotic myocardium in conscious pigs with different degrees of concentric stenosis. *Br J Pharmacol.* 1988;94:219-227.
169. Canty Jr. JM, Klocke FJ. Reductions in regional myocardial function at rest in conscious dogs with chronically reduced regional coronary artery pressure. *Circ Res.* 1987;61:II107-II116.
170. Bolukoglu H, Liedtke AJ, Nellis SH, Eggleston AM, Subramanian R, Renstrom B. An animal model of chronic coronary stenosis resulting in hibernating myocardium. *Am J Physiol.* 1992;263:H20-H29.
171. Mills I, Fallon JT, Wrenn D, Sasken H, Gray W, Bier J, Levine D, Berman S, Gilson M, Gewirtz H. Adaptive responses of coronary circulation and myocardium to chronic reduction in perfusion pressure and flow. *Am J Physiol.* 1994;266:H447-H457.
172. Vanoverschelde JL, Wijns W, Depré C, Essamri B, Heyndrickx GR, Borgers M, Bol A, Melin JA. Mechanisms of chronic regional postischemic dysfunction in humans. New insights from the study of noninfarcted collateral-dependent myocardium. *Circulation.* 1993;87:1513-1523.
173. Marinho NV, Keogh BE, Costa DC, Lammerstma AA, Ell PJ, Camici PG. Pathophysiology of chronic left ventricular dysfunction. New insights from the measurement of absolute myocardial blood flow and glucose utilization. *Circulation.* 1996;93:737-744.
174. Shen YT, Vatner SF. Mechanism of impaired myocardial function during progressive coronary stenosis in conscious pigs. Hibernation versus stunning? *Circ Res.* 1995;76:479-488.
175. Liedtke AJ, Renstrom B, Nellis SH, Subramanian R. Myocardial function and metabolism in pig hearts after relief from chronic partial coronary stenosis. *Am J Physiol.* 1994;267:H1312-H1319.
176. Liedtke AJ, Renstrom B, Nellis SH, Hall JL, Stanley WC. Mechanical and metabolic functions in pig hearts after 4 days of chronic coronary stenosis. *J Am Coll Cardiol.* 1995;26:815-825.
177. Ito BR. Gradual onset of myocardial ischemia results in reduced myocardial infarction. Association with reduced contractile function and metabolic downregulation. *Circulation.* 1995;91:2058-2070.
178. Chen C, Chen L, Fallon JT, Ma L, Li L, Bow L, Knibbs D, McKay R, Gillam LD, Waters DD. Functional and structural alterations with 24-hour myocardial hibernation and recovery after reperfusion. A pig model of myocardial hibernation. *Circulation.* 1996;94:507-516.
179. Heusch G, Rose J, Skyschally A, Post H, Schulz R. Calcium responsiveness in regional myocardial short-term hibernation and stunning in the *in situ* porcine heart. Inotropic responses to postextrasystolic potentiation and intracoronary calcium. *Circulation.* 1996;93:1556-1566.
180. Ferrari R, Cargnoni A, Bernocchi P, Pasini E, Curello S, Ceconi C, Ruigrok TJ. Metabolic adaptation during a sequence of no-flow and low-flow ischemia. A possible trigger for hibernation. *Circulation.* 1996;94:2587-2596.
181. Field LJ. Transgenic mice in cardiovascular research. *Annu Rev Physiol.* 1993;55:97-114.
182. Doevendans PA, Daemen MJ, de Muinck ED, Smits JF. Cardiovascular phenotyping in mice. *Cardiovasc Res.* 1998;39:34-49.
183. Ganim JR, Luo W, Ponniah S, Grupp I, Kim HW, Ferguson DG, Kadambi V, Neumann JC, Doetschman T, Kranias EG. Mouse phospholamban gene expression during development *in vivo* and *in vitro*. *Circ Res.* 1992;71:1021-1030.

184. Grupp IL, Subramaniam A, Hewett TE, Robbins J, Grupp G. Comparison of normal, hypodynamic, and hyperdynamic mouse hearts using isolated work-performing heart preparations. *Am J Physiol.* 1993;265:H1401-H1410.
185. Hunter JJ, Tanaka N, Rockman HA, Ross J, Jr., Chien KR. Ventricular expression of a MLC-2v-ras fusion gene induces cardiac hypertrophy and selective diastolic dysfunction in transgenic mice. *J Biol Chem.* 1995;270:23173-23178.
186. Dyson E, Sucov HM, Kubalak SW, Schmid-Schonbein GW, DeLano FA, Evans RM, Ross J, Jr., Chien KR. Atrial-like phenotype is associated with embryonic ventricular failure in retinoid X receptor alpha -/- mice. *Proc Natl Acad Sci U S A.* 1995;92:7386-7390.
187. Barbee RW, Perry BD, Re RN, Murgo JP. Microsphere and dilution techniques for the determination of blood flows and volumes in conscious mice. *Am J Physiol.* 1992;263:R728-R733.
188. Hoit BD, Khoury SF, Kranias EG, Ball N, Walsh RA. *In vivo* echocardiographic detection of enhanced left ventricular function in gene-targeted mice with phospholamban deficiency. *Circ Res.* 1995;77:632-637.
189. Tanaka N, Dalton N, Mao L, Rockman HA, Peterson KL, Gottshall KR, Hunter JJ, Chien KR, Ross J, Jr. Transthoracic echocardiography in models of cardiac disease in the mouse. *Circulation.* 1996;94:1109-1117.
190. Hoit BD, Khan ZU, Pawloski-Dahm CM, Walsh RA. *In vivo* determination of left ventricular wall stress-shortening relationship in normal mice. *Am J Physiol.* 1997;272:H1047-H1052.
191. Desai KH, Sato R, Schauble E, Barsh GS, Kobilka BK, Bernstein D. Cardiovascular indexes in the mouse at rest and with exercise: new tools to study models of cardiac disease. *Am J Physiol.* 1997;272:H1053-H1061.

Ca^{2+} sensitization and diastolic function of normal and stunned porcine myocardium

3

Ca^{2+} sensitization and diastolic function of normal and stunned porcine myocardium

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Background: Ca^{2+} sensitizers prolong myofibrillar force development *in vitro* and might therefore aggravate relaxation abnormalities of stunned myocardium. This is the first *in vivo* study of the effects of the thiadiazinone derivative EMD 60263 ((+)-5-(l-(alpha-ethylimino-3,4-dimethoxybenzyl)-1,2,3,4-tetrahydroquinoline-6-yl)-6-methyl-3,6-dihydro-2H-1,3,4-thiadiazine-2-on), a Ca^{2+}-sensitizing agent with negligible phosphodiesterase III inhibitory activity, on diastolic function of regionally stunned myocardium.

Methods and Results: After producing stunning by two sequences of 10-min coronary artery occlusion and 30 min of reperfusion, anesthetized pigs received either saline (n=7) or 1.5 and 3.0 mg/kg of EMD 60263 (n=8) or its enantiomer EMD 60264 (n=6), which lacks the Ca^{2+}-sensitizing properties but shares the bradycardiac action via inhibition of the delayed inward rectifier K^+ current. In stunned myocardium, systolic shortening was reduced to 46±4% of baseline ($P<0.05$) and mean rate of half end-diastolic segment lengthening, an index for diastolic function, to 35±4%; systolic shortening and mean rate of half end-diastolic lengthening of remote normal myocardium remained unchanged. Saline did not affect these parameters in stunned or normal myocardium. EMD 60264 did not affect systolic shortening but decreased mean rate of half end-diastolic lengthening in normal myocardium to 61±8% and in stunned myocardium to 16±5% of baseline. During saline and EMD 60264, normal and stunned segments started to lengthen immediately after minimal segment length was reached ($\Delta T = 0$). Low dose EMD 60263 restored systolic shortening of the stunned region with no effect on ΔT. The high dose increased systolic shortening above baseline and ΔT to 210±30 ms in both regions. Consequently, mean rate of half end-diastolic lengthening increased to 66±11% in stunned, while decreasing to 55±3% in normal myocardium. After elimination of bradycardia, ΔT and hence mean rate of half end-diastolic lengthening recovered in stunned myocardium, but in normal myocardium the latter remained depressed because ΔT persisted.

Conclusions: In conclusion, both doses of EMD 60263 improved systolic as well as diastolic function of stunned myocardium. The high dose delayed relaxation of normal myocardium without adversely affecting systolic function. ***(Eur J Pharmacol. 1999;386:55-67)***

Introduction

In regionally stunned myocardium both systolic and diastolic function are depressed.[1-3] Since a decreased responsiveness of the myofilaments to Ca^{2+} is one of the plausible mechanisms underlying stunning[3], it is not surprising that the effects of Ca^{2+}-sensitizing agents on regional systolic function of stunned myocardium have been investigated. Indeed, several studies in *in vivo*[4,5] and *in vitro*[6-8] models have shown that this class of drugs is capable of completely restoring systolic function. However, since Ca^{2+}-sensitizing agents may prolong force development of the myofibrils[9,10], there is some concern that an increase in the myofibrillar responsiveness to Ca^{2+} might delay the onset of left ventricular relaxation, thereby impairing early left ventricular filling and ultimately systolic function. This may be of particular importance under conditions in which diastolic Ca^{2+} concentrations are elevated such as heart failure[11] and possibly myocardial stunning[12,13], although in the latter case this is not a ubiquitous finding.[14-18] Indeed, there are *in vitro* studies that reported a Ca^{2+} sensitization-induced impairment of relaxation in myocardium obtained from normal[19-21], stunned[8] and failing hearts.[21] However, in several studies no adverse effects of Ca^{2+}-sensitizing agents on diastolic function of normal myocardium *in vitro* [6,22-24] and *in vivo*[25-27] or stunned myocardium *in vitro*[6,7] could be demonstrated. Importantly, the agents used in these studies (EMD 53998, EMD 57033, CGP 48506, MCI-154 and levosimendan) do not only possess Ca^{2+}-sensitizing but also phosphodiesterase III inhibiting properties.[10] It can therefore not be excluded that a potentially adverse effect of increased Ca^{2+} sensitivity on diastolic function was blunted by the positive lusitropic effect of phosphodiesterase III inhibition.

Currently, there are no *in vivo* studies in which the effect of Ca^{2+}-sensitizing agents without phosphodiesterase inhibiting properties on diastolic function of regional stunned myocardium has been evaluated. In the present study, we therefore assessed the effect of the thiadiazinone derivative EMD 60263 ((+)-5- (l- (alpha-ethylimino-3,4-dimethoxybenzyl) - 1,2,3,4-tetrahydroquinoline-6-yl)-6-methyl-3,6-dihydro - 2H-1,3,4 -thiadiazine-2-on), a Ca^{2+}-sensitizing agent with negligible phosphodiesterase III inhibiting properties[9,10], on regional left ventricular diastolic function of stunned and adjacent normal myocardium. Since *in vitro* experiments suggest that the nature of the response of normal as well as stunned myocardium may be dose-dependent[8] two doses were chosen, one that is sufficient and one that is in excess of that required to restore systolic function.[4] As an index of regional left ventricular diastolic function the mean rate of half end-diastolic lengthening was employed.[1,28] Since EMD 60263 produces profound bradycardia[4] that potentially could mask untoward effects on diastolic function, we also studied the actions of EMD 60263 after its bradycardic action was abolished by atrial pacing. In addition, we compared the actions of EMD 60263 to those of its (-) enantiomer EMD 60264, a compound that lacks the Ca^{2+}-sensitizing properties of EMD 60263 but shares its inhibitory action on the delayed rectifier inward K^+ current, that is most likely responsible for the bradycardic action of EMD 60263.[10] EMD 60264 shows some weak phosphodiesterase III inhibitory activity with a concentration for half maximum inhibition of this enzyme (1.4 μM), which is approximately one twentieth of that of

EMD 60263 (see reference 10). Finally, disturbances in diastolic function are more likely to be aggravated and thereby to affect systolic function when the diastolic period is shortened. Therefore, we also studied whether adverse effects emerged or were aggravated after heart rate was increased above baseline levels in the presence of EMD 60263.

Methods

All experiments were performed in accordance with the "Guiding Principles in the Care and Use of Laboratory Animals" as approved by the Council of the American Physiological Society and with prior approval of the Animal Care Committee of the Erasmus University Rotterdam.

Surgical preparation

Cross-bred Landrace x Yorkshire pigs of either sex (n=21, 28-30 kg) were sedated with ketamine i.m. (20-30 mg/kg, Apharmo, Huizen, The Netherlands) and anesthetized with pentobarbital i.v. (20 mg/kg followed by 5-10 mg/kg per hour, Sanofi, Paris, France) before they were intubated and connected to a respirator for intermittent positive pressure ventilation with a mixture of oxygen and nitrogen; arterial blood gas values were kept within the normal range by adjusting respiratory rate and tidal volume. Haemaccel (Behringwerke A.G., Marburg, FRG) was administered via an intravenous infusion to maintain fluid balance. A micromanometer-tipped catheter (B. Braun Medical B.V., Uden, The Netherlands) was inserted into the left carotid artery and advanced into the left ventricular cavity for recording of left ventricular blood pressure and its first derivative (LVdP/dt), while a fluid-filled catheter was positioned in the descending aorta for monitoring arterial blood pressure.[4,29]

After administration of 4 mg pancuronium bromide (Organon Teknika, Oss, The Netherlands), a midsternal thoracotomy was performed and the heart was suspended in a pericardial cradle. An electromagnetic flow probe (Skalar, Delft, The Netherlands) was then positioned around the ascending aorta for measurement of aortic blood flow (cardiac output) and pacing leads were attached to the right atrial appendage and connected to a pacemaker. A proximal segment of the left anterior descending coronary artery was dissected free for placement of an atraumatic clamp, while the anterior interventricular vein draining the left anterior descending coronary artery perfusion territory was cannulated for collection of local coronary venous blood.[30]

One pair of ultrasonic crystals was inserted inside the distribution territory of the left anterior descending coronary artery and another pair inside the distribution territory of the left circumflex coronary artery for measurement of regional myocardial function by sonomicrometry (Triton Technology Inc., San Diego, CA, USA). Crystals were positioned in the midmyocardial layer approximately 10 mm apart and parallel to fibre direction.

Experimental protocol and groups

Baseline values of systemic hemodynamics and regional myocardial function were recorded

after hemodynamic variables had been stable for at least 30 min following completion of instrumentation. After arterial and coronary venous blood, samples were collected for determination of hemoglobin, oxygen saturation and blood gas values, a batch ($1\text{-}2 \times 10^6$) of radioactive microspheres (15±1 μm (S.D.), labeled with either ^{46}Sc, ^{95}Nb, ^{103}Ru, ^{113}Sn or ^{141}Ce (NEN Company; Dreieich, FRG), was injected into the left atrium, and a reference blood sample was withdrawn at a rate of 10 ml/min for determination of regional myocardial blood flow.[29] Subsequently, the distribution area of the left anterior descending coronary artery was stunned by two sequences of 10-min occlusion and 30 min of reperfusion.[31] At the end of the second 30-min reperfusion period, the animals received either two doses (1.5 and 3.0 mg/kg administered intravenously over 3 min) of EMD 60263 (n=8) or EMD 60264 (n=6) or two equivalent volumes of the vehicle (3 and 6 ml of saline, n=7), separated by 15 min. Since both EMD 60263 and EMD 60264 produce bradycardia, measurements after the second dose of these compounds were repeated after heart rates were restored to pre-drug levels observed following stunning (HR_{st}) and additionally after heart rate was raised to 30 beats/min above HR_{st} (HR_{st+30}). In the animals that received EMD 60264, regional myocardial blood flows were not determined. Furthermore, in these animals heart rate could not be raised to HR_{st+30} due to development of atrio-ventricular block during pacing.

At the end of each experiment, normal and stunned myocardium were identified by a left atrial injection of patent blue violet (Sigma, St. Louis, MO, USA) after the left anterior descending coronary artery had been reoccluded. The animals were then killed with an overdose of pentobarbital, the heart excised and the left ventricle handled as described earlier to obtain regional myocardial blood flow data.[29]

Data analysis

Myocardial oxygen consumption of the perfusion territory of the left anterior descending coronary artery was calculated as the product of local transmural myocardial blood flow (radioactive microsphere data) and the difference in the oxygen contents of the arterial and local coronary venous blood, while the area inside the left ventricular pressure-segment length loop was taken as an index of external work.[32,33] Mechanical efficiency was defined as the ratio of external work and myocardial oxygen consumption per beat. The area inside the left ventricular pressure-segment length loop reflects mechanical work but does not have the dimensions of work and changes in mechanical efficiency have therefore been expressed as percentage of baseline.[4] Mechanical efficiency data are only presented for the distribution area of the left anterior descending coronary artery, because myocardial oxygen consumption of the adjacent normal myocardium could not be calculated as local coronary venous blood was not sampled from that area.

Systolic shortening was computed as $100\% \cdot (\text{EDL-ESL})/\text{EDL}$, in which EDL (end-diastolic length) and ESL (end-systolic length) are the segment length at the start and the end of left

ventricular ejection, respectively. Post-systolic shortening was calculated as 100% ·(ESL-L_{min})/EDL, in which L_{min} is the minimum segment length after closure of the aortic valves. All segment length data were normalized to an EDL of 10 mm at baseline. Analogous to the studies by Tilton et al.[28] and Charlat et al.[1], we used as index for early diastolic function the mean rate of half-end diastolic lengthening, which was defined as the slope of the line drawn from ESL to L_{50}, which is the point midway between ESL and the following EDL ((L_{50}-ESL)/ T_{50}, Fig. 1). We also determined the time interval (ΔT) between L_{min} and the onset of segment lengthening (L_{min} + 0.1 mm) and calculated mean rate of half end-diastolic lengthening as the mean rate of segment lengthening during the time interval between L_{min} + 0.1 mm and L_{50}.

All data have been presented as means ± S.E.M.. Statistical significance of the changes produced by stunning were evaluated by paired t-test. The effects of the administered agents were assessed by two-way analysis of variance for repeated measures followed by paired t-test with Bonferroni adjustment. Statistical significance was accepted for P<0.05 (two-tailed).

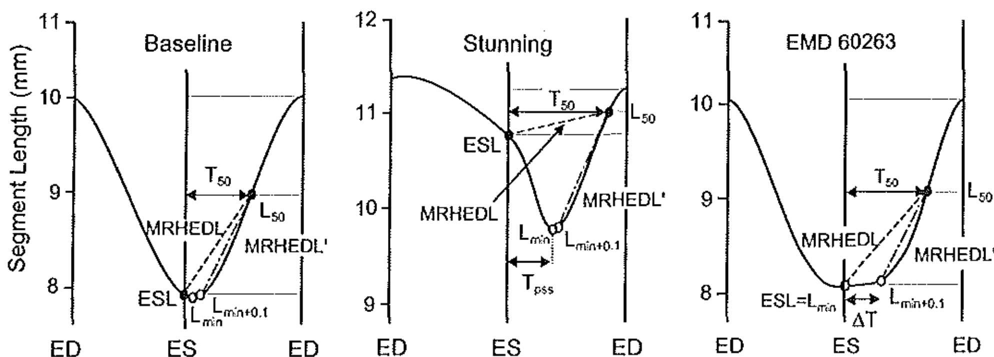

Figure 1 Definition of the mean rate of half end-diastolic lengthening (MRHEDL) during baseline conditions, stunning and after the higher dose of EMD 60263. Notice that at baseline and during stunning the segment started to lengthen immediately after L_{min} (o) was reached, but that the onset of lengthening was delayed by ΔT after the higher dose of EMD 60263 (see results). ΔT is the time interval in which the segment length increased from L_{min} to L_{min} + 0.1 mm (o). L_{50} is the point midway between ESL and the following EDL. MRHEDL (—) is the mean rate of lengthening between ESL and L_{50}, while MRHEDL' (-----) is defined as the mean rate of lengthening between L_{min} + 0.1 mm and L_{50}. EDL and ESL are the end-diastolic and end-systolic length, respectively; L_{min} = minimal segment length; T_{PSS} = duration of post-systolic shortening; T_{50} = the time interval between ESL and L_{50}.

Drugs

EMD 60263 and EMD 60264 (E. Merck Darmstadt, Germany) were dissolved in saline. Fresh solutions were prepared on the day of each experiment.

Results

Systemic hemodynamics

At the end of the second occlusion-reperfusion cycle, mean arterial blood pressure (6±3%), cardiac output (15±4%), stroke volume (11±6%), $LVdP/dt_{max}$ (21±5%) and $LVdP/dt_{min}$ (16±3%) had decreased from their respective baseline levels (all $P<0.05$, n=21), while heart rate, left ventricular systolic pressure and left ventricular end-diastolic pressure had remained unchanged (Table 1).

Infusion of saline did not produce any changes in global systemic hemodynamic variables of the control group, demonstrating the hemodynamic stability of the preparation. Increasing heart rate to HR_{st+30} via pacing decreased left ventricular end-diastolic pressure, stroke volume and $LVdP/dt_{min}$ (all $P<0.05$), while $LVdP/dt_{max}$ was not affected. Administration of EMD 60264 produced dose-dependent decreases in mean arterial blood pressure, heart rate, $LVdP/dt_{max}$ and $LVdP/dt_{min}$. A decrease in systemic vascular resistance, most likely secondary to the phosphodiesterase III inhibitory activity of EMD 60264, was responsible for the decrease in mean arterial blood pressure, as cardiac output was not altered. The lower dose of EMD 60264 had no effect on stroke volume, but the higher dose produced an increase (25±8%, $P<0.05$), which was a direct effect of the bradycardia as stroke volume returned to pre-EMD 60264 values after restoring heart rate to HR_{st}.

Administration of EMD 60263 lowered heart rate (up to 46±3%), mean arterial blood pressure (12±4%) and cardiac output (10±4%), but increased stroke volume (69±7%) and left ventricular end-diastolic pressure (from 10±1 to 14±1 mmHg) (all $P<0.05$). Despite the bradycardia, $LVdP/dt_{max}$ was maintained but $LVdP/dt_{min}$ showed a response similar to that observed with EMD 60264. After elevating heart rate to $HR_{st,}$, left ventricular end-diastolic pressure, mean arterial blood pressure and stroke volume returned to pre-EMD 60263 values. $LVdP/dt_{min}$ recovered partially, while the increase in $LVdP/dt_{max}$ did not reach levels of statistical significance. However, it is important that at corresponding time points and heart rates, EMD 60264 produced decreases in $LVdP/dt_{max}$. The further increase of heart rate to HR_{st+30} resulted in a decrease in cardiac output due to a further decrease in stroke volume from 25±2 to 17±2 ml/min, while left ventricular end-diastolic pressure increased from 10±1 to 13±2 mmHg ($P<0.05$ vs saline). $LVdP/dt_{max}$ as well as $LVdP/dt_{min}$ did not change.

Myocardial oxygen consumption per beat and mechanical efficiency

In stunned myocardium transmural blood flow was decreased by 23±3% (n=21) without affecting the endo-epi blood flow ratio. In the saline-treated animals no, changes were observed during infusion of saline or after heart rate was increased to HR_{st+30}. Because the difference in arterial and coronary venous oxygen contents remained unchanged, myocardial oxygen consumption of stunned myocardium decreased in parallel with transmural blood flow (Table 2). In normal myocardium transmural blood flow and the endo-epi flow ratio were not significantly

affected.

Administration of EMD 60263 had no effect on myocardial blood flow or the difference in arterial and coronary venous oxygen content of stunned myocardium. Consequently, myocardial oxygen consumption also remained unchanged. Raising heart rate to HR_{st+30} had no effect on transmural myocardial blood flow, the endo-epi blood flow ratio or arterio-coronary venous oxygen content difference. In normal myocardium, the endo-epi blood flow ratio decreased from 0.97±0.04 to 0.79±0.05 (P<0.05) due to a decrease in subendocardial blood flow, when the pacing rate was increased to HR_{st+30}.

Table 1 Systemic hemodynamics during intravenous infusion of EMD 60263 and its (-) enantiomer EMD 60264 in anesthetized pigs with stunned myocardium.

	Baseline	Stunning	D_1	D_2	HR_{st}	HR_{st+30}
CO ($l\ min^{-1}$)						
Saline	2.8±0.2	2.4±0.1^{a}	2.4±0.2	2.4±0.1	2.3±0.2	2.4±0.2
EMD 60264	2.6±0.2	2.1±0.2^{a}	1.9±0.1^{b}	1.9±0.2	1.8±0.2	
EMD 60263	3.0±0.2	2.7±0.2	2.5±0.2	2.4±0.2^{bc}	2.5±0.2	2.2±0.3^{e}
HR (beats min^{-1})						
Saline	107±2	104±7	105±6	102±6	106±4	138±6^{b}
EMD 60264	108±4	109±4	91±3^{bc}	80±5^{bc}	108±4	
EMD 60263	109±5	100±4	73±5^{bc}	54±5^{bcd}	100±4	130±5^{b}
LVSP (mmHg)						
Saline	111±3	104±4	104±3	108±3	109±5	104±3
EMD 60264	111±4	102±3	93±4	79±3^{bc}	74±5^{b}	
EMD 60263	113±2	108±3	106±3	109±4^{d}	103±8	6±6^{bc}
LVEDP (mmHg)						
Saline	9±1	9±1	9±1	10±1	8±1	7±1^{b}
EMD 60264	7±1	6±1	7±1	8±1	7±1	
EMD 60263	10±1	10±1	11±1	14±1^{bc}	10±1	13±2^{e}
$LVdP/dt_{max}$ (mmHg s^{-1})						
Saline	2110±190	1660±170^{a}	1670±160	1700±150	1660±140	1790±160
EMD 60264	2030±240	1560±120^{a}	1470±140	1210±100^{b}	1120±110^{b}	
EMD 60263	2130±130	1640±120^{a}	1620±130	1720±130	1870±120	1670±160
$LVdP/dt_{min}$ (mmHg s^{-1})						
Saline	-2120±150	-1780±210^{a}	-1730±150	-1930±300	-2010±300	-2230±260^{bc}
EMD 60264	-2450±350	-2060±310^{a}	-1240±140^{bc}	-870±70^{bc}	-870±90^{b}	
EMD 60263	-2110±90	-1740±80^{a}	-1140±120^{bc}	-890±110^{bc}	-1220±130^{bc}	-1320±160^{c}
MAP (mmHg)						
Saline	89±3	84±4	83±2	86±2	89±3	87±3
EMD 60264	92±5	85±3	75±4^{b}	61±4^{bc}	61±5^{b}	
EMD 60263	91±2	87±3	79±3^{b}	77±3^{bcd}	84±7	73±6^{b}
SV (ml)						
Saline	27±2	23±1	23±1	24±1	22±1	17±1^{b}
EMD 60264	24±1	19±2	21±1	24±1^{b}	17±2	
EMD 60263	27±1	27±1	34±2^{bc}	45±3^{bcd}	25±2	17±2^{be}
SVR (dynes•s cm^{-5})						
Saline	2640±240	2880±160	2800±160	2880±240	3200±400	3040±320
EMD 60264	2960±240	3360±160	3280±240	2640±240^{b}	2880±240	
EMD 60263	2560±160	2720±240	2720±240	2720±240	2880±320	2960±320

D_1 and D_2, 3 ml and 6 ml of saline for control and 1.5 mg/kg and 3.0 mg/kg for EMD 60264 and EMD 60263, respectively. HR_{st}, pacing at heart rate observed during stunning; HR_{st+30}, pacing at 30 beats/min above HR_{st}. [a]P<0.05 Stunning vs Baseline; [b]P<0.05 vs Stunning; [c]changes from Stunning are different (P<0.05) vs changes from Stunning in the Saline group; [d]changes from Stunning are different (P<0.05) vs changes from Stunning in the EMD 60264 group; [e]changes from HR_{st} are different (P<0.05) vs changes from HR_{st} in the Saline group (only for data at HR_{st+30}). Data are mean ± S.E.M.; n=7 (Saline); n=8 (EMD 60263)

External work (per beat) of the left anterior descending coronary artery perfusion territory had decreased by as much as 50% after production of stunning. While infusion of saline had no effect on external work, the latter recovered almost completely during administration of the lower dose of EMD 60263 and increased to 130% of baseline ($P<0.05$ vs baseline) following administration of the higher dose. In the left circumflex coronary artery perfusion territory, external work had decreased slightly at the end of the second ischemia-reperfusion cycle. External work was not affected by the lower dose of EMD 60263, but showed a similar increase as in the stunned myocardium during the higher dose. Raising heart rate to HR_{st+30} resulted in a significant fall of external work of both normal and stunned myocardium, which was accompanied by a smaller decrease in myocardial oxygen consumption per beat of stunned myocardium.

In view of the larger decrease in external work than in myocardial oxygen consumption per beat, it follows that mechanical efficiency had decreased (to approximately 60% of baseline) in stunned myocardium. This decrease remained unchanged during infusion of saline, but administration of EMD 60263 completely restored mechanical efficiency. When heart rate was raised to HR_{st+30} mechanical efficiency tended to decrease ($P=0.60$).

Segment shortening during systole and post-systole

At the end of the occlusion-reperfusion sequences, systolic shortening had decreased to 46±4% of baseline in stunned myocardium, while systolic shortening of normal myocardium remained unchanged. Infusion of saline had no effect on systolic shortening of either the stunned or normal myocardium, while the increase in heart rate to HR_{st+30} resulted in a decrease in systolic shortening of stunned myocardium ($P<0.05$) and tended to decrease systolic shortening of normal myocardium. Infusion of EMD 60264 and subsequent restoration of heart rate to HR_{st} had no effect on systolic shortening in either the normal or stunned myocardium (Table 3, Figure 2 and 3).

After infusion of 1.5 mg/kg of EMD 60263, systolic shortening of stunned myocardium increased from 9±1% to 15±1%, while systolic shortening of the normal myocardium increased from 14±1% to 17±1% (both $P<0.05$). Administration of 3.0 mg/kg of EMD 60263 increased systolic shortening of both the stunned and normal myocardium even further to 24±2% and 22±1%, respectively (both $P<0.05$ vs pre-stunning baseline). When heart rate was raised to HR_{st}, systolic shortening decreased to baseline values in both the stunned and normal myocardium and to levels below baseline when heart rate was further increased to HR_{st+30}.

Post-systolic shortening was negligible under baseline conditions, but became apparent during coronary artery occlusion (Figure 2) and persisted during reperfusion. Neither infusion of saline nor that of EMD 60264 affected post-systolic shortening. However, post-systolic shortening was markedly attenuated after infusion of the lower dose of EMD 60263 and was completely abolished after administration of the higher dose. Atrial pacing had no effect on post-systolic shortening in any of the three experimental groups.

Table 2 Myocardial perfusion and oxygen consumption of regionally stunned myocardium during intravenous infusion of EMD 60263 in anesthetized pigs with stunned myocardium.

	Baseline	Stunning	D_1	D_2	HR_{st+30}
Saline					
LADCA perfusion territory					
Transmural flow (ml/min $100g^{-1}$)	156±6	121±10[a]	111±10	123±8	119±7
Endo/Epi	1.14±0.05	1.18±0.08	1.17±0.04	1.19±0.05	1.13±0.11
Δ (aO_2 cont-cvO_2 cont) (μmol ml^{-1})	2.85±0.18	2.95±0.11	2.98±0.12	3.04±0.22	3.01±0.25
MVO_2 (μmol/min $100g^{-1}$)	441±26	353±26[a]	331±29	362±12	353±7
$MVO_{2\,beat}$ (μmol $100g^{-1}$)	4.1±0.3	3.4±0.2	3.2±0.3	3.6±0.2	2.6±0.1
EW_{beat} (mmHg•mm)	90±17	95±20[a]	100±18	108±23	63±18[b]
Mechanical efficiency (%)	100	57±9[a]	68±11	60±10	53±13
LCXCA perfusion territory					
Transmural flow (ml/min $100g^{-1}$)	168±7	160±15	143±15	148±8	146±9
Endo/Epi	1.05±0.05	0.98±0.06	0.94±0.07	0.95±0.06	0.91±0.04
EW_{beat} (mmHg•mm)	136±10	120±14	115±12	114±14	84±21[b]
EMD 60263					
LADCA perfusion territory					
Transmural flow (ml/min $100g^{-1}$)	167±5	122±8[a]	119±11	118±10	110±17
Endo/Epi	1.07±0.09	1.16±0.09[a]	1.13±0.09	1.17±0.08	1.07±0.07
Δ(aO_2 cont-cvO_2 cont) (μmol ml^{-1})	2.91±0.18	2.74±0.20	2.97±0.34	2.92±0.18	3.10±0.22
MVO_2 (μmol/min $100g^{-1}$)	484±27	328±19[a]	334±27	334±12	357±35
$MVO_{2\,beat}$ (μmol $100g^{-1}$)	4.5±0.3	3.3±0.2[a]	4.7±0.4[bc]	6.4±0.5[bc]	2.7±0.3[d]
EW_{beat} (mmHg•mm)	185±21	84±11[a]	159±1[bc]	243±21[bc]	89±9[d]
Mechanical efficiency (%)	100	64±9[a]	89±8[bc]	100±14[bc]	80±12
LCXCA perfusion territory					
Transmural flow (ml/min $100g^{-1}$)	180±7	164±10[a]	138±13	129±10[b]	116±17[b]
Endo/Epi	1.08±0.06	0.99±0.05[a]	0.99±0.04	0.97±0.04	0.79±0.05[b]
EW_{beat} (mmHg•mm)	161±16	145±17[a]	161±12	241±15[bc]	58±8[bd]

LADCA=left anterior descending coronary artery; LCXCA=left circumflex coronary artery; D_1 and D_2 are 3 ml and 6 ml of saline for the saline group and 1.5 mg/kg and 3.0 mg/kg for the EMD 60263 group. HR_{st+30}=pacing at 30 beats/min above the heart rate observed during stunning; μ(aO_2 cont-cvO_2 cont)=difference in the O_2 contents of the arterial and coronary venous blood; MVO_2= regional myocardial O_2 consumption; EW = external work; [a]$P<0.05$ Stunning vs Baseline; [b]$P<0.05$ vs Stunning; [c]changes from stunning are different ($P<0.05$) from changes in saline group; [d]changes from D_2 are different ($P<0.05$) vs changes from D_2 in saline group (only for data at HR_{st+30}). Data are expressed as means S.E.M.; n=7 (Saline); n=8 (EMD 60263) except for the measurements at HR_{st+30} which were for 6 and 7 animals, respectively.

Rate of segment lengthening during diastole

The stunning protocol reduced the mean rate of half end-diastolic lengthening in the territory perfused by the left anterior descending coronary artery to 35±4% of baseline (n = 21), but had no effect on the mean rate of half end-diastolic lengthening of the adjacent normal myocardium (96±7% of baseline). Infusion of saline and the subsequent increase in heart rate to 30 bpm above baseline did neither affect the mean rate of half end-diastolic lengthening of the stunned nor that of the normal myocardium. Administration of EMD 60264 caused a dose-dependent decrease in the mean rate of half end-diastolic lengthening of both the stunned and normal myocardium to 16±5% and 61±8% of baseline, respectively (both P<0.05 vs stunning). During pacing at HR_{st}, mean rate of half end-diastolic lengthening remained unchanged in both stunned and normal myocardium (Figure 4).

Table 3 Effects of EMD 60263 and its (-) enantiomer EMD 60264 on regional systolic shortening in anaesthetized pigs with stunned myocardium.

	Baseline	Stunning	D_1	D_2	HR_{st}	HR_{st+30}
LADCA perfusion territory						
End-diastolic length (mm)						
Saline	11.0±0.7	12.0±0.6^{a}	11.8±0.7^{b}	11.8±0.7	11.4±0.7^{b}	10.9±0.7^{b}
EMD 60264	9.8±0.9	10.7±1.1^{a}	10.8±1.1	10.7±1.1	10.4±1.1^{b}	nm
EMD 60263	12.0±1.2	13.0±1.4^{a}	12.8±1.3	12.6±1.3^{b}	11.7±1.2^{be}	10.8±1.1^{b}
Systolic shortening (%)						
Saline	17±1	8±1^{a}	8±1	9±2	9±2	6±2
EMD 60264	19±1	8±1^{a}	7±1	6±2	4±3	nm
EMD 60263	17±1	9±1^{a}	15±1^{bcd}	24±2^{bcd}	17±1^{bcde}	13±1
Post systolic shortening (%)						
Saline	3.1±0.9	7.2±0.8^{a}	6.2±0.8	5.6±1.1	5.1±0.7^{e}	4.9±0.6^{b}
EMD 60264	1.5±0.6	9.0±1.1^{a}	9.0±1.2	7.3±1.1	7.2±1.4	nm
EMD 60263	1.3±0.4	7.0±0.8^{a}	3.2±0.4^{bcd}	0.5±0.2^{bcd}	0.8±0.3^{bcd}	0.3±0.2^{bc}
LCXCA perfusion territory						
End-diastolic length (mm)						
Saline	9.9±0.8	10.1±0.7	10.0±0.7	10.1±0.8	9.5±0.6	9.2±0.6^{b}
EMD 60264	10.5±0.8	10.3±0.8	10.4±0.8	10.7±0.8	10.4±0.8	nm
EMD 60263	11.4±1.1	11.6±1.2	11.6±1.2	11.5±1.2	10.2±0.9^{b}	9.4±1.0^{bf}
Systolic shortening (%)						
Saline	12±1	11±2	11±2	11±1	11±2	9±2
EMD 60264	14±2	13±1	13±1	13±1	12±2	nm
EMD 60263	14±1	14±1	17±1^{bcd}	22±1^{bcd}	12±1^{e}	8±1^{bf}
Post systolic shortening (%)						
Saline	1.5±0.5	2.5±1.0	2.2±0.5	2.2±0.4	2.7±0.4	2.5±0.8
EMD 60264	0.4±0.2	0.6±0.3	0.6±0.3	1.0±0.4	0.4±0.3	nm
EMD 60263	2.1±0.5	1.5±0.7	0.6±0.4^{b}	0.5±0.3	0.8±0.4	0.6±0.2

D_1 and D_2 are 3 ml and 6 ml of saline for the saline group and 1.5 mg/kg and 3.0 mg/kg for the EMD 60264 and EMD 60263 groups, respectively. HR_{st}=pacing at heart rate observed during stunning; HR_{st+30}=pacing at 30 beats/min above HR_{st}. $^{a}P<0.05$ Stunning vs Baseline; $^{b}P<0.05$ vs Stunning; ^{c}changes from Stunning are different ($P<0.05$) vs changes from Stunning in saline group; ^{d}changes from Stunning are different ($P<0.05$) vs changes from Stunning in the EMD 60264 group; ^{e}changes from D_2 are different ($P<0.05$) vs changes from D_2 in Saline group; ^{f}changes from HR_{st} are different ($P<0.05$) vs changes from HR_{st} in Saline group, nm = not measured. Data are expressed as means ± S.E.M.; n=7 (Saline); n= 6 (EMD 60264); n=8 (EMD 60263).

Administration of EMD 60263 caused a dose-dependent increase in the mean rate of half end-diastolic lengthening of the stunned myocardium (Figure 4). However, at variance with systolic shortening, recovery of mean rate of half end-diastolic lengthening was only partial (to 66±11% of baseline; $P<0.05$ vs baseline). The major reason was that, while under baseline conditions the segment started to lengthen immediately after L_{min} had been reached ($\Delta T=0$), there was a delay ($\Delta T= 210\pm40$ ms) in the onset of segment lengthening during administration of the higher dose of EMD 60263. Once the segment started to lengthen, the rate of lengthening was not different from control. At HR_{st}, mean rate of half end-diastolic lengthening increased to 92±16% of baseline predominantly because of a reduction in ΔT to 60±20 ms. The further increase in heart rate to HR_{st+30} did not affect mean rate of half end-diastolic lengthening (93±18% of baseline). In contrast to the stunned myocardium, mean rate of half end-diastolic lengthening of the normal myocardium decreased dose-dependently to 55±3% of baseline ($P<0.05$), due to a doubling of T_{50}. The latter was again caused by an increase of ΔT to 210±40 ms. Increasing heart rate to HR_{st+30}

increased mean rate of half end-diastolic lengthening to 77±11% as ΔT decreased to 130±20 ms in the normal myocardium; this decrease in ΔT of the normal myocardium was, however, significantly less than in the stunned myocardium ($P<0.05$).

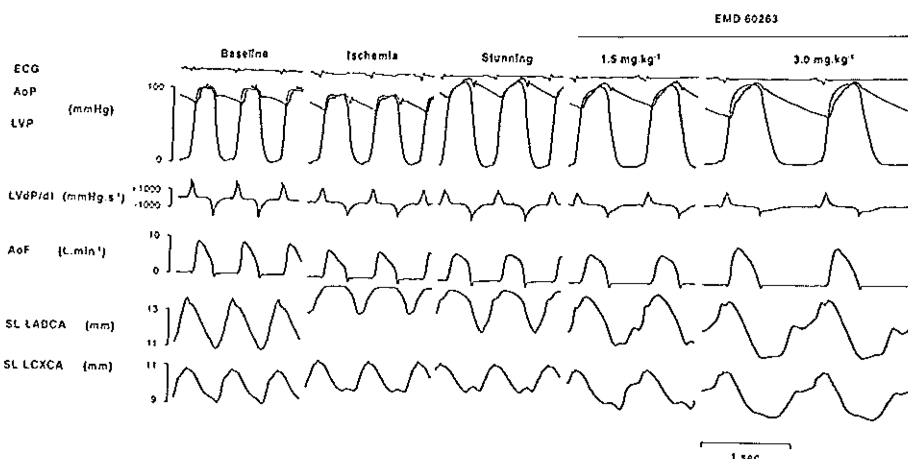

Figure 2 This recording of a representative experiment with EMD 60263 shows that systolic shortening already recovered during infusion the lower of dose of EMD 60263 and that in both stunned (LADCA) and normal (LCXCA) myocardium the onset of segment lengthening was delayed after administration of the higher dose. ECG, electrocardiogram; AoP, aortic pressure; LVP, left ventricular pressure; LVdP/dt, left ventricular dP/dt; AoF, aortic blood flow; SL, segment length, LADCA, left anterior descending coronary artery; LCXCA, left circumflex coronary artery.

Discussion

The use of Ca^{2+}-sensitizing agents in the treatment of cardiovascular conditions is still under debate. While there is a general consensus that Ca^{2+} sensitizers may be superior positive inotropic agents compared to beta sympathomimetic drugs as they can increase force production with no (or negligible) increments in energy cost[34-36], considerable concern has been expressed that the same characteristics could slow relaxation and elevate diastolic tension.[8,19-21] Several studies have examined the effects of Ca^{2+}-sensitizing agents on diastolic function of myocardium (Table 4). These studies differ with respect to species, myocardial tissue localization (right vs left ventricle), state of tissue (normal or diseased), type of agent used ("pure" Ca^{2+} sensitizer or mixed Ca^{2+} sensitizer / phosphodiesterase III inhibitor), doses used and the diastolic functional parameter that is studied. Studies in isolated intact or skinned muscle fibers obtained from right or left ventricle using EMD 53998, its (+) enantiomer EMD 57033, levosimendan, MCI-154 or CGP 48506 have yielded equivocal results with either no change or a reduction in the rate of relaxation (Table 4), while one study reported that the Ca^{2+}-sensitizing substance caffeine improved relaxation.[24] Moreover, studies in isolated or *in situ* hearts have shown that compounds such as EMD 57033, MCI-154 and levosimendan do not exert a negative effect on diastolic function of both normal and globally stunned myocardium, while MCI-154 enhanced relaxation in failing human[37,38] and

canine myocardium.[26] However, these agents also possess phosphodiesterase III inhibiting properties and it has been argued that the beneficial effect of phosphodiesterase III inhibition on diastolic function may have counterbalanced any adverse effect of the increased Ca^{2+} sensitivity. This argument cannot be dismissed as in none of the aforementioned studies the agents were administered in the presence of adrenergic blockade. This is also corroborated by the observation that when levosimendan was administered in the presence of the selective bradycardic agent zatebradine to prevent the tachycardia by levosimendan, the increase in the maximum rate of fall of LV pressure and the decrease in the relaxation time constant tau were no longer observed, indicating that the improved relaxation depended entirely on the tachycardia which is a feature of phosphodiesterase III inhibition.[39] In a recent study in awake pigs, we observed that EMD 57033 either in the absence or presence of ß-adrenoceptor blockade did not alter $LVdP/dt_{min}$ or LV end-diastolic pressure.[27] Those findings suggest that the lack of effect of EMD 57033 on diastolic function is not simply the result of simultaneous myocardial phosphodiesterase III inhibition. However, to allow the selective study of Ca^{2+}-sensitization on diastolic function in the present study we employed EMD 60263, a compound that is devoid of phosphodiesterase III inhibitory actions.[9,10]

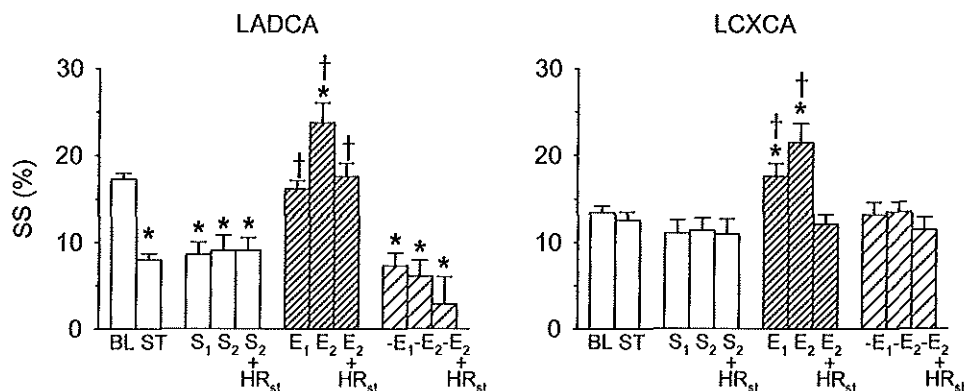

*Figure 3 Systolic shortening (SS) at baseline (BL), during stunning (ST) and after infusion of 2 volumes of saline (3 ml [S_1] and 6 ml [S_2]) or two doses of EMD 60263 (1.5 mg/kg [E_1] and 3.0 mg/kg [E_2]) or EMD 60264 (1.5 mg/kg [-E_1] and 3.0 mg/kg [-E_2]). After administration of the high dose the heart rate was increased to heart rate at stunning (HR_{st}) to eliminate the effect of bradycardia. *$P<0.05$ vs baseline; †$P<0.05$ vs stunning; LADCA = left anterior descending coronary artery distribution area; LCXCA = left circumflex coronary artery distribution area.*

In the present study, EMD 60263 produced a decrease in $LVdP/dt_{min}$ (i.e., became less negative), suggesting that it slowed the early diastolic relaxation of the left ventricle as a whole. However, this decrease was at least in part mediated by the bradycardic effect of EMD 60263, as it was partially restored when the hearts were paced at pre-drug levels. In the EMD 60264 group elevating heart rate to pre-drug levels did not increase $LVdP/dt_{min}$, furthermore suggesting an adverse effect of inhibition of delayed inward rectifier K^+current on early relaxation. It must

therefore be assumed that the EMD 60263-induced decrease in $LVdP/dt_{min}$ is at least in part due to the effect on the delayed inward rectifier K^+current.

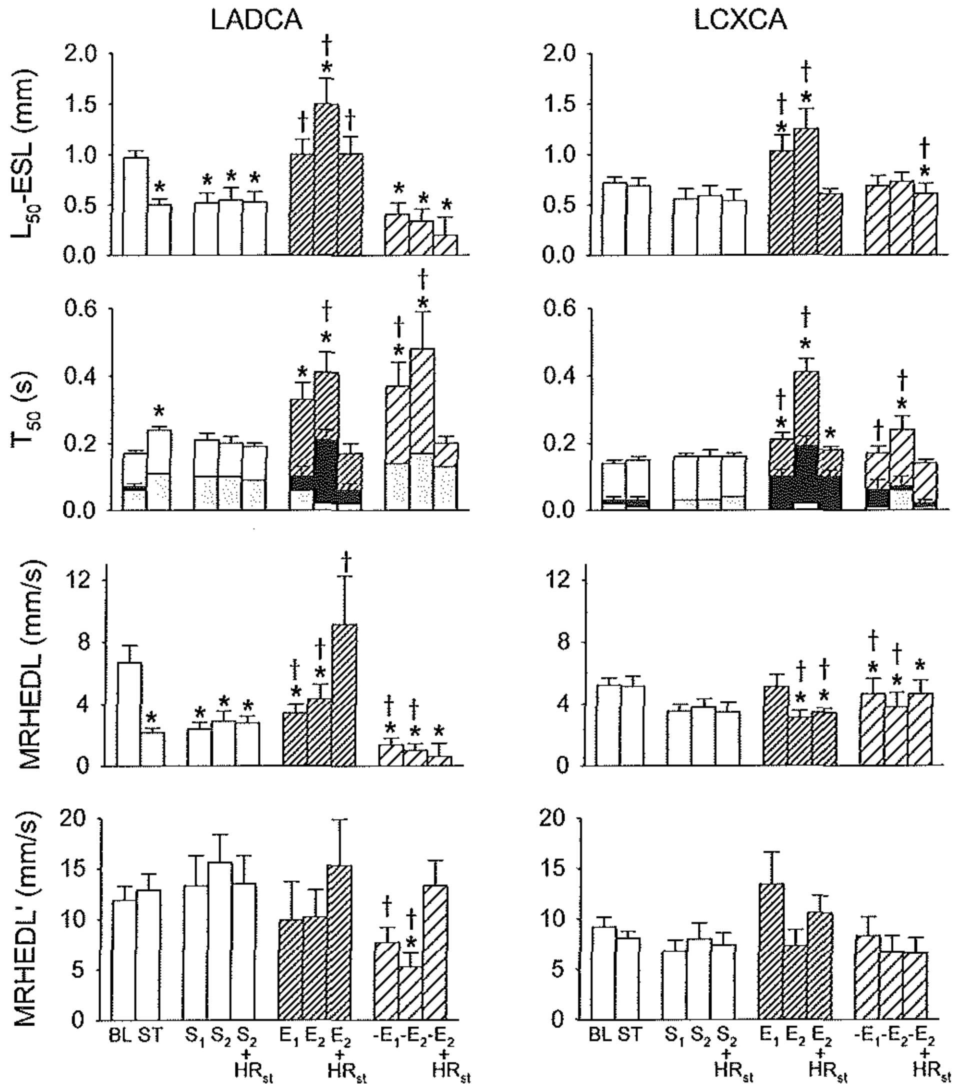

*Figure 4 From top to bottom are shown the effects of EMD 60263 on (L_{50}-ESL), T_{50}, mean rate of half end-diastolic lengthening (MRHEDL) and mean rate of half end-diastolic lengthening (MRHEDL') at baseline (BL), during stunning (ST) and after infusion of 2 volumes of saline (3 ml [S_1] and 6 ml [S_2]) or two doses of EMD 60263 (1.5 mg/kg [E_1] and 3.0 mg/kg [E_2]) or EMD 60264 (1.5 mg/kg [-E_1] and 3.0 mg/kg [-E_2]). After administration of the high dose the heart rate was increased to heart rate at stunning (HR_{st}) to eliminate the effect of bradycardia. Notice that T_{50} includes T_{PSS} (grey bars) and ΔT (solid bars) (see Figure 1). *$P<0.05$ vs baseline; †$P<0.05$ vs stunning; LADCA = left anterior descending coronary artery distribution area; LCXCA = left circumflex coronary artery distribution area.*

As index of "early" regional diastolic function we used analogous to Tilton et al.[28] and Charlat et al.[1] the mean rate of half end-diastolic lengthening. The latter group of authors[1] has shown that in conscious dogs this index of early diastolic function is as severely depressed as systolic function. Moreover, the time course of rate of recovery during the early hours of reperfusion was faster for systolic than for diastolic function, although complete recovery of diastolic function preceded that of systolic function. In the present study, the lower dose of EMD 60263 resulted in a complete recovery of systolic shortening, but in only a partial recovery of the mean rate of half end-diastolic lengthening. With the higher dose of EMD 60263, systolic shortening of the post-ischemic myocardium exceeded baseline values, but the mean rate of half end-diastolic lengthening remained depressed at 66±11% of baseline.

The depressed mean rate of half end-diastolic lengthening associated with the high dose was the result of a delay in the onset of segment lengthening ($\Delta T \neq 0$), because once the segment started to lengthen, the rate of lengthening was not different from baseline. To exclude that the effect of EMD 60263 on the delayed inward rectifier K^+current and on heart rate contributed to the increase in ΔT, we not only included a group of animals treated with EMD 60264, but we also studied the actions of EMD 60263 after eliminating its bradycardic action by atrial pacing. Since EMD 60264 did not cause an increase in ΔT, this suggests that the increase in ΔT with the higher dose of EMD 60263 was not the result of an effect on the delayed inward rectifier K^+current. In addition, retrospective inspection of diastolic function of normal and stunned myocardium during infusion of the selective bradycardic agent zatebradine, which reduced heart rate to 55 bpm[4], indicates that bradycardia per se can also not explain the delay in the onset of relaxation. Interestingly, pacing blunted the delay in onset of relaxation, but this effect was more pronounced in the stunned than in the normal myocardium.

The EMD 60263-induced increase in ΔT is consistent with *in vitro* reports that thiadiazinone derivatives prolong the duration of force development by enhancing the crossbridge interaction between actin and myosin.[9,10] This increase in ΔT may have an adverse effect on global left ventricular function, as it could impair left ventricular filling of which the largest fraction normally occurs during early diastole. Although after the higher dose of EMD 60263 left ventricular end-diastolic pressure increased, we failed to observe an impairment in left ventricular function as both the rate of segment lengthening and the end-diastolic segment length were not affected. Furthermore, systolic function was not impaired as stroke volume even increased to above baseline values. In a previous study using the same model, induction of bradycardia to similar heart rates with the specific bradycardic agent zatebradine increased left ventricular end-diastolic pressure to the same extent without affecting end-diastolic segment length (unpublished data). Similarly, zatebradine increased stroke volume although significantly less than EMD 60263.[4] These observations suggest that left ventricular filling and global systolic left ventricular function were not adversely affected by an increase in myofibrillar sensitivity to Ca^{2+} during the EMD 60263-induced bradycardia.

Table 4 Current studies of effects of Ca^{2+} sensitizers on diastolic function

Authors	Model	Myocardium	Ca^{2+} sensitizer	Dose	Relaxation-parameter	Remarks
White et al. (1993)	isolated ferret papillary muscle stimulated electrically	normal	EMD 57033	0.1-20 μM	$T_{1/2}$ ↑	at doses > 1 μM
Simnett et al. (1993)	skinned fibers of guinae pig right ventricle	normal	EMD 57033	10 μM	τ ↔	
Pagel et al. (1994)	anesthetized dogs	normal	levosimendan	0.5-4μg/kg min^{-1} iv	$LVdP/dt_{min}$ ↔, τ ↔	ANS blockade
	awake dogs	normal	levosimendan	0.5-4μg/kg min^{-1} iv	$LVdP/dt_{min}$ ↔, τ ↔	ANS blockade
Korbmacher et al. (1994)	isolated rabbit hearts	normal	EMD 57033	30 μM	$LVdP/dt_{min}$ ↑, LVEDP ↔	LVSP increased
		stunned	EMD 57033	30 μM	$LVdP/dt_{min}$ ↑, LVEDP ↔	LVSP increased
Pagel et al. (1995)	awake dogs	normal	levosimendan	0.5-4μg/kg min^{-1} iv	$LVdP/dt_{min}$ ↑, τ ↓	HR held constant by zatebradine
	awake dogs	normal	levosimendan	0.5-4μg/kg min^{-1} iv	$LVdP/dt_{min}$ ↔, τ ↔	
Mori et al. (1995)	awake humans	IHD	MCI-154	1.5μg/kg + 0.12 μg/kg min^{-1} iv	τ ↓, dV/dt_{max} ↔	
Korbmacher et al. (1995)	isolated rabbit hearts	stunned	EMD 57033	10-30 μM	LVEDP ↔, dP/dt_{min} ↑ LVSP↑	
Hgashiyama et al. (1995)	isolated ejecting rabbit hearts	normal	EMD 57033	5-5.8 μM	$LVdP/dt_{min}$ ↔, $T_{1/2}$ ↑, LVEDP ↑	
Haikala et al. (1995)	paced guinae pig papillary muscle	normal	levosimendan	0.03-3μM	"relaxation time" ↔	
			EMD 53998	0.1-3μM	"relaxation time" ↑	
Hajjar et al. (1997)	human LV trabeculae	normal failing	EMD 57033	1-50 μM	DF ↑ , $T_{80\%}$ ↑	effect greater in failing than in normal hearts
Palmer and Kentish (1997)	rat skinned RV trabeculae	normal	Caffeine	20 mM	τ ↓	
			CGP 48506	10 μM	τ ↔	
Takaoka et al. (1997)	humans	IHD	MCI-154	1.5-3μg/kg+ 16.6-33.2 μg/min iv	PCWP↓	
Korbmacher et al. (1997)	isolated rabbit hearts	normal and stunned	EMD 60263	3 μM	$LVdP/dt_{min}$ ↑	Detrimental effects less in stunned than normal hearts
				10 μM	$LVdP/dt_{min}$ ↔	
				30 μM	$LVdP/dt_{min}$ ↓	
Teramura et al. (1997)	open-chest dogs	normal	MCI-154	1μg/kg min^{-1} iv	$T_{1/2}$ ↔	
		failing	MCI-154	1μg/kg min^{-1} iv	$T_{1/2}$ ↓	
Stubenitsky et al. (1997)	awake swine	normal	EMD 57033	0.2-0.8mg/kg min^{-1} iv	$LVdP/dt_{min}$ ↔, LVEDP ↔	Similar response after β-blockade

IHD = ischemic heart disease; $T_{1/2}$ = time to 50% peak tension or peak pressure; $T_{80\%}$ = time to 80% peak tension; τ = time constant of tension or pressure decay; LV(SP) = left ventricular (systolic pressure); RV = right ventricular; DF: diastolic force; $LVdP/dt_{min}$ = maximum rate of fall of LV pressure; PCWP = pulmonary capillary wedge pressure; ANS = autonomic nervous system.

In the present study restoration of heart rate to pre-drug levels (HR_{st}) in the presence of EMD 60263, decreased systolic shortening in both stunned and normal myocardium, while the effects on regional diastolic segment dynamics differed between stunned and normal myocardium. The different response of the delay of relaxation to pacing in stunned and normal myocardium was unexpected, particularly in view of evidence that pacing can induce an increase in intracellular Ca^{2+} concentration[40,41] during both systole and diastole, which would tend to augment the EMD 60263-induced diastolic relaxation abnormalities. On the other hand, Kusuoka et al.[42] reported that pacing increases intracellular inorganic phosphate levels, which could reduce the myofibrillar sensitivity to Ca^{2+}. The latter observation may explain why despite an increased diastolic intracellular Ca^{2+} concentration pacing improved relaxation in the stunned myocardium (i.e., reduced the time delay to onset of segment lengthening). The persisting delay in segment lengthening in normal myocardium during pacing at a time when diastolic function of stunned myocardium was normal is supported by *in vitro* observations of Korbmacher et al.[8] in isolated rabbit hearts. This finding suggests that, despite the presence of EMD 60263, a difference in myofibrillar Ca^{2+} sensitivity remains between stunned and normal myocardium.

The prolonged development of force could be most detrimental when diastole is shortened, i.e., at higher heart rates. Thus, to study the physiological significance of the observed delay in diastolic lengthening, we paced the hearts at 30 beats/min above stunning level (HR_{st+30}). Pacing at HR_{st+30} decreased the duration of diastole to below baseline value which in the saline-treated group decreased end-diastolic segment length and systolic shortening of both stunned and normal myocardium. The delay in diastolic lengthening produced by EMD 60263 further shortened the period of diastolic lengthening and resulted in a decrease in end-diastolic length of both stunned and normal myocardium and an impairment of systolic shortening. However, only the changes in normal myocardium and not in stunned myocardium of the EMD 60263 treated group were statistically significant compared to the changes in the saline-treated group. This finding suggests that diastolic function of normal myocardium is more susceptible to increases in myofibrillar Ca^{2+} sensitivity than that of stunned myocardium, which is supported by observations in normal and post-ischemic isolated rabbit hearts.[8]

Another important point to consider in the assessment of the effect of Ca^{2+}-sensitizers on diastolic function is the dose required to normalize systolic function relative to the dose at which Ca^{2+}-sensitizers exert a negative effect on diastolic function. In the present study the higher dose (3.0 mg/kg, iv) is in excess of that needed to restore systolic shortening of stunned myocardium.[4] Indeed, in the present study, 1.5 mg/kg already normalized systolic segment shortening but had no adverse effect on the onset of relaxation. Similarly, previous studies have also indicated that the impairment of diastolic function by Ca^{2+}-sensitizers is likely a dose-related finding. Thus, Hajjar et al.[21] reported that EMD 57033 exerted its negative lusitropic effects in normal human myocardium at doses higher than 10 μM. Korbmacher et al.[8] observed, however, that at 3 μM EMD 57033 increased $LVdP/dt_{max}$ in post-ischemic isolated rabbit hearts by 50% while improving early

relaxation. A further increase to 10 μM had no additional effect on $LVdP/dt_{max}$ but tended to decrease the maximum rate of relaxation. Finally, at 30 μM and consistent with the report of Hajjar et al.[21], diastolic function was seriously impaired resulting in deterioration of systolic function.

In conclusion, in the present study, both doses of EMD 60263 improved systolic function (shortening and mechanical efficiency) as well as diastolic function (mean rate of half end-diastolic lengthening) of stunned myocardium. The high dose delayed relaxation of normal myocardium but without adversely affecting systolic function in the presence and absence of the EMD 60263 induced-bradycardia.

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References

1. Charlat ML, O'Neill PG, Hartley CJ, Roberts R, Bolli R. Prolonged abnormalities of left ventricular diastolic wall thinning in the "stunned" myocardium in conscious dogs: time course and relation to systolic function. *J Am Coll Cardiol.* 1989;13:185-194.
2. Ehring T, Schulz R, Schipke JD, Heusch G. Diastolic dysfunction of stunned myocardium. *Am J Cardiovasc Pathol.* 1993;4:358-366.
3. Bolli R, Marban E. Molecular and cellular mechanisms of myocardial stunning. *Physiol Rev.* 1999;79:609-34.
4. Soei LK, Sassen LM, 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.
5. Abe Y, Kitada Y, Narimatsu A. Effect of a calcium-sensitizing positive inotropic agent MCI-154 and its combined use with enalapril on postischemic contractile dysfunction of dog hearts. *J Cardiovasc Pharmacol.* 1995;26:653-659.
6. Korbmacher B, Sunderdiek U, Arnold G, Schulte HD, Schipke JD. Improved ventricular function by enhancing the Ca^{++} sensitivity in normal and stunned myocardium of isolated rabbit hearts. *Basic Res Cardiol.* 1994;89:549-562.
7. Korbmacher B, Sunderdiek U, Schulte HD, Arnold G, Schipke JD. Comparison between the effects of a novel Ca^{++} sensitizer and a phosphodiesterase inhibitor on stunned myocardium. *J Pharmacol Exp Ther.* 1995;275:1433-1441.
8. Korbmacher B, Sunderdiek U, Selcan G, Arnold G, Schipke JD. Different responses of non-ischemic and post-ischemic myocardium towards Ca^{2+} sensitization. *J Mol Cell Cardiol.* 1997;29:2053-2066.
9. Solaro RJ, Gambassi G, Warshaw DM, Keller MR, Spurgeon HA, Beier N, Lakatta EG. Stereoselective actions of thiadiazinones on canine cardiac myocytes and myofilaments. *Circ Res.* 1993;73:981-990.
10. Ravens U, Himmel HM, Fluss M, Davia K, Harding SE. Phosphodiesterase inhibition and Ca^{2+} sensitization. *Mol Cell Biochem.* 1996;157:245-249.
11. Hajjar RJ, Gwathmey JK. Calcium-sensitizing inotropic agents in the treatment of heart failure: a critical view. *Cardiovasc Drugs Ther.* 1991;5:961-965.
12. Kusuoka H, Koretsune Y, Chacko VP, Weisfeldt ML, Marban E. Excitation-contraction coupling in postischemic myocardium. Does failure of activator Ca^{2+} transients underlie stunning? *Circ Res.* 1990;66:1268-1276.
13. Gao WD, Atar D, Backx PH, Marban E. Relationship between intracellular calcium and contractile force in stunned myocardium. Direct evidence for decreased myofilament Ca^{2+} responsiveness and altered diastolic function in intact ventricular muscle. *Circ Res.* 1995;76:1036-1048.
14. Steenbergen C, Murphy E, Levy L, London RE. Elevation in cytosolic free calcium concentration early in myocardial ischemia in perfused rat heart. *Circ Res.* 1987;60:700-707.
15. Marban E, Kitakaze M, Koretsune Y, Yue DT, Chacko VP, Pike MM. Quantification of $[Ca^{2+}]_i$ in perfused hearts. Critical evaluation of the 5F-BAPTA and nuclear magnetic resonance method as applied to the study of ischemia and reperfusion. *Circ Res.* 1990;66:1255-1267.

16. Amende I, Bentivegna LA, Zeind AJ, Wenzlaff P, Grossman W, Morgan JP. Intracellular calcium and ventricular function. Effects of nisoldipine on global ischemia in the isovolumic, coronary-perfused heart. *J Clin Invest.* 1992;89:2060-2065.
17. Carrozza JP, Jr., Bentivegna LA, Williams CP, Kuntz RE, Grossman W, Morgan JP. Decreased myofilament responsiveness in myocardial stunning follows transient calcium overload during ischemia and reperfusion. *Circ Res.* 1992;71:1334-1340.
18. Harada K, Franklin A, Johnson RG, Grossman W, Morgan JP. Acidemia and hypernatremia enhance postischemic recovery of excitation- contraction coupling. *Circ Res.* 1994;74:1197-1209.
19. White J, Lee JA, Shah N, Orchard CH. Differential effects of the optical isomers of EMD 53998 on contraction and cytoplasmic Ca^{2+} in isolated ferret cardiac muscle. *Circ Res.* 1993;73:61-70.
20. Hgashiyama A, Watkins MW, Chen Z, LeWinter MM. Effects of EMD 57033 on contraction and relaxation in isolated rabbit hearts. *Circulation.* 1995;92:3094-3104.
21. Hajjar RJ, Schmidt U, Helm P, Gwathmey JK. Ca^{++} sensitizers impair cardiac relaxation in failing human myocardium. *J Pharmacol Exp Ther.* 1997;280:247-254.
22. Simnett SJ, Lipscomb S, Ashley CC, Mulligan IP. The effect of EMD 57033, a novel cardiotonic agent, on the relaxation of skinned cardiac and skeletal muscle produced by photolysis of diazo- 2, a caged calcium chelator. *Pflugers Arch.* 1993;425:175-177.
23. Haikala H, Nissinen E, Etemadzadeh E, Levijoki J, Linden IB. Troponin C-mediated calcium sensitization induced by levosimendan does not impair relaxation. *J Cardiovasc Pharmacol.* 1995;25:794-801.
24. Palmer S, Kentish JC. Differential effects of the Ca^{2+} sensitizers caffeine and CGP 48506 on the relaxation rate of rat skinned cardiac trabeculae. *Circ Res.* 1997;80:682-687.
25. Pagel PS, Harkin CP, Hettrick DA, Warltier DC. Levosimendan (OR-1259), a myofilament calcium sensitizer, enhances myocardial contractility but does not alter isovolumic relaxation in conscious and anesthetized dogs. *Anesthesiology.* 1994;81:974-987.
26. Teramura S, Yamakado T, Maeda M, Nakano T. Effects of MCI-154, a calcium sensitizer, on left ventricular systolic and diastolic function in pacing-induced heart failure in the dog. *Circulation.* 1997;95:732-739.
27. Stubenitsky R, van der Weerd RW, Haitsma DB, Verdouw PD, Duncker DJ. Cardiovascular effects of the novel Ca^{2+}-sensitiser EMD 57033 in pigs at rest and during treadmill exercise. *Br J Pharmacol.* 1997;122:1257-1270.
28. Tilton GD, Bush LR, Apprill PG, Buja LM, Willerson JT. Effect of diltiazem and propranolol on left ventricular segmental relaxation during temporary coronary arterial occlusion and one month reperfusion in conscious dogs. *Circulation.* 1985;71:165-175.
29. Sassen LM, Soei LK, Koning MM, Verdouw PD. The central and regional cardiovascular responses to intravenous and intracoronary administration of the phenyldihydropyridine elgodipine in anaesthetized pigs. *Br J Pharmacol.* 1990;99:355-363.
30. Bier J, Sharaf B, Gewirtz H. Origin of anterior interventricular vein blood in domestic swine. *Am J Physiol.* 1991;260:H1732-H1736.
31. Brand T, Sharma HS, Fleischmann KE, Duncker DJ, McFalls EO, Verdouw PD, Schaper W. Proto-oncogene expression in porcine myocardium subjected to ischemia and reperfusion. *Circ Res.* 1992;71:1351-1360.
32. Morris JJd, 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.
33. Vinten-Johansen J, Gayheart PA, Johnston WE, Julian JS, Cordell AR. Regional function, blood flow, and oxygen utilization relations in repetitively occluded-reperfused canine myocardium. *Am J Physiol.* 1991;261:H538-H547.
34. Gross T, Lues I, Daut J. A new cardiotonic drug reduces the energy cost of active tension in cardiac muscle. *J Mol Cell Cardiol.* 1993;25:239-244.
35. De Tombe PP, Burkhoff D, Hunter WC. Effects of calcium and EMD-53998 on oxygen consumption in isolated canine hearts. *Circulation.* 1992;86:1945-1954.
36. Hata K, Goto Y, Futaki S, Ohgoshi Y, Yaku H, Kawaguchi O, Takasago T, Saeki A, Taylor TW, Nishioka T, et al. Mechanoenergetic effects of pimobendan in canine left ventricles. Comparison with dobutamine. *Circulation.* 1992;86:1291-1301.
37. Mori M, Takeuchi M, Takaoka H, Yokoyama M. Lusitropic effects of a Ca^{2+} sensitization with a new cardiotonic agent, MCI-154, on diseased human hearts. *Cardiovasc Res.* 1995;30:915-922.
38. Takaoka H, Takeuchi M, Hata K, Hayashi Y, Mori M, Yamakawa H, Yamaguchi K, Yokoyama M. Beneficial effects of a Ca^{2+} sensitizer, MCI-154, on the myocardial oxygen consumption-cardiac output relation in patients with left ventricular dysfunction after myocardial infarction: comparison with dobutamine and phosphodiesterase inhibitor. *Am Heart J.* 1997;133:283-289.
39. Pagel PS, Harkin CP, Hettrick DA, Warltier DC. Zatebradine, a specific bradycardic agent, alters the hemodynamic and left ventricular mechanical actions of levosimendan, a new myofilament calcium sensitizer, in conscious dogs.

J Pharmacol Exp Ther. 1995;275:127-135.
40. Langer GA. Ion fluxes in cardiac excitation and contraction and their relation to myocardial contractility. *Physiol Rev*. 1968;48:708-757.
41. Frampton JE, Orchard CH, Boyett MR. Diastolic, systolic and sarcoplasmic reticulum [Ca^{2+}] during inotropic interventions in isolated rat myocytes. *J Physiol (Lond)*. 1991;437:351-375.
42. Kusuoka H, Weisfeldt ML, Zweier JL, Jacobus WE, Marban E. Mechanism of early contractile failure during hypoxia in intact ferret heart: evidence for modulation of maximal Ca^{2+}-activated force by inorganic phosphate. *Circ Res*. 1986;59:270-282.

Cardiovascular profile of the calcium sensitizer EMD 57033 in open-chest anesthetized pigs with regionally stunned myocardium

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Cardiovascular profile of the calcium sensitizer EMD 57033 in open-chest anesthetized pigs with regionally stunned myocardium

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Background Ca^{2+} sensitizers enhance systolic function, but impair relaxation *in vitro*; these effects may differ in stunned and normal myocardium. We therefore studied the effect of EMD 57033 on systolic and diastolic function of normal and stunned porcine myocardium *in vivo*.

Methods and Results Myocardial stunning by 15 min coronary occlusion and 30 min reperfusion, abolished systolic shortening (SS) (baseline 13±1 %) and decreased end-systolic elastance (E_{es}) from 67±7 to 47±5 mmHg mm^{-1} (both $P<0.05$). Maximum rate of fall of myocardial elastance (dE/dt_{min}) decreased from -850±100 to -320±30 mmHg/mm s^{-1}, while the time constant τ_e of the decay of elastance increased from 58±3 to 68±6 ms (both $P<0.05$). End-diastolic elastance (E_{ed}) was unchanged although the zero pressure intercept ($L_{0,ed}$) had increased. In the stunned region, EMD 57033 (0.2 mg/kg min^{-1} for 60 min, i.v., n=7) increased SS to 19±2 %, E_{es} to 287±40 mmHg mm^{-1}, dE/dt_{min} to -3630±640 mmHg/mm s^{-1} and decreased τ_e to 50±3 ms, while E_{ed} remained unchanged. In the normal region, EMD 57033 increased SS from 14±2 to 18±3 %, E_{es} from 59±4 to 263±23 mmHg mm^{-1}, dE/dt_{min} from -480±70 to -2280±700 mmHg mm^{-1} s^{-1} and decreased τ_e from 91±12 to 61±3 ms (all $P<0.05$), while E_{ed} remained unchanged. These responses were minimally affected by adrenoceptor blockade (n=7). Vehicle (n=7) had no effect on either region. EMD 57033 increased cardiac output (up to 27±8 %) and $LVdP/dt_{max}$ (86±19 %). Mean aortic pressure decreased (19±7 %) due to systemic vasodilation that was not amenable to blockade of adrenoceptors or NO synthesis.

Conclusions In conclusion, EMD 57033 restored systolic and diastolic function of stunned myocardium, and produced similar improvements in systolic and diastolic function in normal myocardium. ***(Br J Pharmacol. 2000;129:1413-1422)***

Introduction

Stunned myocardium is characterized by both a depressed systolic and diastolic function.[1-3] Since a decreased responsiveness of the myofilaments to calcium (Ca^{2+}) has been implicated in the mechanism underlying stunning, several groups of investigators have successfully employed "Ca^{2+}-sensitizing agents" to restore systolic function of stunned myocardium in a variety of *in vivo*[4,5] and *in vitro*[6-8] models. It has been suggested that the inotropic response to Ca^{2+}-sensitizers may differ between stunned and normal myocardium. For instance, we have shown that the EMD 60263-induced increase in regional systolic shortening in stunned myocardium of anesthetized pigs was much more pronounced than that in normal myocardium.[4] However, systolic shortening is strongly load-dependent, even more so in stunned myocardium, and does not necessarily reflect myocardial contractility.[9] That also the lusitropic response to Ca^{2+}-sensitizers in stunned and normal myocardium may differ was suggested by Korbmacher et al.[8], who showed that in response to high doses of EMD 60263 global diastolic function deteriorated more in normal than in globally stunned isolated rabbit hearts. However, data on diastolic functional responses of stunned myocardium to Ca^{2+}-sensitizing agents *in vivo* are currently lacking.

In view of these considerations, we studied the effects of EMD 57033 on regional systolic and diastolic function in an *in vivo* porcine model of stunned and normal myocardium. EMD 57033 was chosen because it is a thiadiazinone derivative, that lacks the inhibitory action on the delayed rectifier inward current that is exhibited by EMD 60263 and which may potentially modify systolic and diastolic responses by prolongation of the action potential duration.[10] However, EMD 57033 possesses phosphodiesterase III inhibiting properties (which EMD 60263 lacks) that could act to enhance systolic and diastolic function.[10] Therefore, we also studied EMD 57033 in the presence of α- and β-adrenergic receptor blockade. To circumvent the problem of load-dependency of systolic shortening we employed, in analogy to the time-varying elastance concept, regional LV end-systolic pressure-segment length relations to obtain a more load-independent measure of regional myocardial contractility.[11] Also based on the time-varying elastance concept we determined regional LV end-diastolic pressure-segment length relations to describe diastolic function. Since the latter is a measure of late diastolic function, we also determined the maximum rate of fall (dE/dt_{min}) and the time constant (τ_e) of the decay of myocardial elastance to describe early diastolic function.

Methods

Animal care

All experiments were performed in accordance with the "Guiding Principles in the Care and Use of Laboratory Animals" as approved by the American Physiological Society and with prior approval of the Animal Care Committee of the Erasmus University Rotterdam.

Animal preparation

Cross-bred Landrace x Yorkshire pigs of either sex (28-36 kg) were sedated with ketamine i.m. (20-30 mg kg^{-1}, Apharmo, Huizen, The Netherlands) and anesthetized with sodium pentobarbital i.v. (20 mg kg^{-1}, Sanofi, Paris, France), before they were intubated and connected to a respirator for intermittent positive pressure ventilation with a mixture (1:2 vol%) of oxygen and nitrogen. Arterial blood gas values were kept within the normal range (pH: 7.35-7.45; pCO_2: 35-45 mmHg; pO_2: 100-150 mmHg) by adjusting respiratory rate and tidal volume. Fluid-filled catheters were placed in the superior caval vein for administration of sodium pentobarbital (5-10 mg/kg h^{-1}) to keep a constant depth of anesthesia and for administration of Haemaccel (Behringwerke A.G., Marburg, Germany) to maintain fluid balance. A fluid-filled catheter was positioned in the descending aorta to monitor arterial blood pressure, while a micromanometer-tipped catheter (B. Braun Medical B.V., Uden, The Netherlands) was advanced into the left ventricle to measure LV blood pressure and its first derivative (LVdP/dt). In order to construct the LV pressure-segment length relations a balloon catheter was positioned in the inferior caval vein for varying LV preload.

After administration of pancuronium bromide (4 mg, Organon Teknika, Oss, The Netherlands) a midsternal thoracotomy was performed and the heart suspended in a pericardial cradle. Then, an electromagnetic flow probe (Skalar, Delft, The Netherlands) was placed around the ascending aorta to measure ascending aortic blood flow (cardiac output). A proximal segment of the left anterior descending coronary artery (LADCA) was dissected free for placement of a Doppler flow probe (Crystal Biotech, Northboro, MA, USA) to measure coronary blood velocity and for placement of an atraumatic clamp for occlusion of the artery. The vein accompanying the LADCA was cannulated for collection of blood samples for determination of coronary venous O_2 content.

Regional myocardial function was measured using sonomicrometry (Triton Technology Inc., San Diego, CA, USA) by placing one pair of ultrasonic crystals in the distribution area of the LADCA and one pair in the distribution area of the left circumflex coronary artery (LCXCA). Each pair was implanted in the midmyocardial layer approximately 10 mm apart and parallel to the fibre direction.

Experimental protocols

After a 30-45 min stabilization period, baseline data of systemic hemodynamics and regional myocardial function were recorded, while arterial and coronary venous blood samples were collected. Preload was transiently reduced (< 12 s)[11] by inflation of the balloon in the inferior caval vein for determination of the LV pressure-segment length relations. Subsequently, the LADCA was occluded for 15 min and after 15 min of reperfusion the 21 animals were randomly allocated to one of three groups. The first two groups received either intravenous 0.5 ml min^{-1} propylene glycol (n=7) or 0.2 mg/kg min^{-1} EMD 57033 (n=7) during

60 min. In 5 animals of the latter group blood samples were collected at 15 min intervals and the plasma stored at -25°C until determination of plasma levels of EMD 57033. Tissue samples were collected from various organs in two animals at the end of the experiment for determination of EMD 57033 levels. The third group (n=7) received EMD 57033 after adrenoceptor blockade to eliminate the putative dependency on adrenergic activity of the EMD 57033-induced changes. For this purpose, the α- and β-adrenoceptors were blocked after 15 min of reperfusion, i.e. 15 min before administration of EMD 57033 by intravenous infusion of 1 mg kg^{-1} phentolamine and 0.5 mg kg^{-1} propranolol (followed by 0.5 mg/kg h^{-1}), respectively. The adequacy of the doses of phentolamine and propranolol has been demonstrated previously.[12,13] Since we observed that the systemic vasodilation produced by EMD 57033 was unmitigated in the presence of combined α- and β-adrenoceptor blockade, we added a fourth group of pigs (n=4) in which EMD 57033 (0.2 mg/kg min^{-1} i.v.) was infused after 30 min of reperfusion in the presence of α- and β-adrenoceptor blockade and blockade of NO synthesis.[14] NO synthesis was inhibited with N^{ω}- nitro-L-arginine (20 mg kg^{-1}, i.v.).[15]

In the first three groups of animals, regional myocardial blood flows were determined by intra-atrial injection of 1-2x10^6 radioactive microspheres [15±1 μm (s.d.) in diameter] labelled with either ^{46}Sc, ^{95}Nb, ^{103}Ru, ^{113}Sn or ^{141}Ce (NEN Company, Dreieich, Germany), using the arterial reference sampling technique.[16] At the end of each experiment, the LADCA was ligated at the site of occlusion and the area perfused by the LADCA was identified by intracoronary injection of patent blue violet (Sigma Chemical Co., St. Louis, USA). Immediately thereafter, the animals were killed with an overdose of pentobarbital and the heart excised and handled as described earlier in order to obtain regional myocardial blood flow data in the LADCA and non-LADCA regions.[16,17]

Data acquisition and analysis

Systolic and post-systolic shortening. All segment length data were normalized to an end-diastolic length (EDL) of 10 mm at baseline to correct for variability in the implantation distance between the crystals. Systolic shortening (SS) was computed as 100% · (EDL-ESL)/EDL, in which EDL and ESL (end-systolic length) are the segment length at the onset of the rapid increase in LV pressure (LVdP/dt = 250 mmHg s^{-1}) and at the end of LV ejection, respectively. Post-systolic segment shortening (PSS) was calculated as 100% ·(ESL-L_{min})/EDL, in which L_{min} is the minimum segment length after closure of the aortic valves.

Regional myocardial elastance using the LV pressure-segment length relations. Regional myocardial end-systolic and end-diastolic elastance were determined from the LV pressure-segment length relations which were obtained by varying preload (Figure 1A). Using linear regression analysis end-systolic elastance (E_{es}) and the segment length at zero pressure intercept ($L_{0,es}$) were obtained applying the iterative method described by Van der Velde et

al.[18], while end-diastolic elastance (E_{ed}) and the segment length at zero pressure intercept ($L_{0,ed}$) were determined using the time point at which LVdP/dt had increased to 250 mmHg s^{-1}.

To describe early diastolic function in the LADCA and LCXCA regions we determined the time course of instantaneous regional elastance using the corresponding $L_{0,es}$ of the LADCA and LCXCA regions, obtained during the preload reduction (Figure 1B). From the time course of elastance (in analogy to the global LV indices of relaxation) we determined the time constant (τ_e) of the elastance decay between the occurrence of the maximum rate of fall of regional myocardial elastance (dE/dt_{min}) and the time point at which regional elastance had decreased to 20% of the peak systolic elastance at baseline. For this purpose the decay in elastance was fitted to $E(t) = E_0\ e^{-t/\tau e}$ where E(t) is the instantaneous elastance at time point t, and E_0 is the elastance at dE/dt_{min}. Finally, to study alterations in the onset of relaxation, we measured the duration of regional myocardial systole in the LADCA ($T_{systole,\ LADCA}$) and LCXCA ($T_{systole,\ LCXCA}$) perfused regions, which was defined as the time interval between the onset of the rapid increase in regional elastance and the occurrence of dE/dt_{min}, in analogy to the determination of the duration of global LV systole.

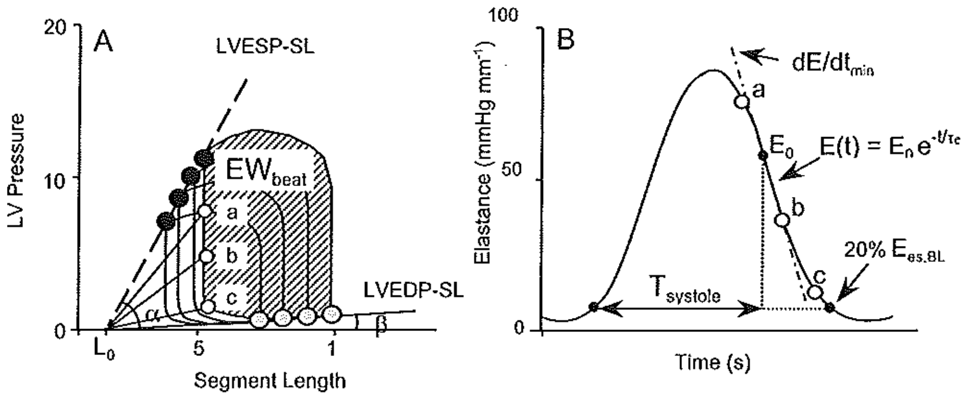

Figure 1 Left ventricular (LV) pressure-segment length relations during transient decrease of preload (A). External work (EW_{beat}) was determined by integrating the area enclosed by the pressure-segment length loop over a full cardiac cycle. Regional end-systolic (E_{es}) and end-diastolic (E_{ed}) elastance were defined as the slopes (tan α and tan β) of the LV end-systolic pressure-segment length (LVESP-SL) and LV end-diastolic pressure-segment length (LVEDP-SL) relations, respectively. From the time-elastance curve the duration of systole ($T_{systole}$) and the maximum rate of fall of elastance (dE/dt_{min}) were determined (B). The time constant (τ_e) of the elastance decay between the occurrence of the maximum rate of fall of regional myocardial elastance (dE/dt_{min}) and the time point at which regional elastance had decreased to 20% of the peak systolic elastance at baseline was determined by fitting the decay in elastance to $E(t) = E_0\ e^{-t/\tau e}$ where E is elastance, t is time, and E_0 is the elastance at dE/dt_{min}.

To complement these regional myocardial measurements we also determined the time constant of global LV pressure decay (τ_{LVP}) during the time-interval between the occurence of $LVdP/dt_{min}$ and the timepoint when LVP had reached 5 mmHg above LV end-diastolic pressure.[19] The duration of global LV systole ($T_{systole,\ LV}$) was defined as the time interval

between the onset of the rapid increase in LV pressure (LVdP/dt = 250 mmHg s^{-1}) and end-ejection.

Myocardial oxygen consumption, external work and mechanical efficiency. Myocardial oxygen consumption (MVO_2) of the perfusion territory of the LADCA was calculated as the product of local transmural myocardial blood flow and the difference in the oxygen contents of the arterial and local coronary venous blood. The area inside the LV pressure-segment length loop (Figure 1) was taken as an index of external work per beat (EW_{beat}),[20-22] while mechanical efficiency was defined as the ratio of EW_{beat} and $MVO_{2\ beat}$. Because EW reflects mechanical work but does not have the dimensions of work, the changes in mechanical efficiency have been expressed as percentage of baseline.

Determination of plasma and tissue concentrations of EMD 57033

To 600 µl of plasma or homogenized tissue 500 µl of water saturated ethylether was added and mixed. After the organic and aqueous phases were separated in an Eppendorff table centrifuge, the organic top layer was removed and collected in an Eppendorff vial. This extraction procedure was repeated five times and the ether phases were collected separately. Thereafter the ether was evaporated in a speed vac centrifuge and the residuals were resuspended and dissolved in 300 µl acetonitril. The amount of EMD 57033 in a given plasma or tissue sample was determined on a HPLC system. To this end 30 µl of the acetonitril solutions was injected on a LiChrosorb RP 8 (5µm) RT 125-4 column (Merck KGaA), which was equipped with a Hibar LiChroCart 4-4 precolumn (Merck KGaA). The column was equilibrated and developed in a buffer composed of 35% acetonitril and 65% 0.1 M sodium phosphate, pH 6.0 at a flow rate of 1 ml min^{-1}. The elution was monitored at a wavelength of 320 nm. The concentration of EMD 57033 was determined from the area of the peaks eluting at the appropriate time from the column by comparison with the values determined for identically treated standard samples. The plasma concentration of a given blood or tissue sample was determined by adding the peak areas of the ether extraction samples.

Statistical Analysis

All data have been presented as mean ± s.e. mean. Statistical significance ($P<0.05$, two-tailed) of the changes within each group was tested using one-way analysis of variance for repeated measures followed by Dunnet's test. Comparison between the changes produced by the different interventions was assessed by two-way analysis of variance for repeated measures.

Drugs

EMD 57033 (the (+) enantiomer of 5-[1-(3,4-dimethoxybenzoyl)-1,2,3,4-tetrahydro-6-quinolyl]-6-methyl-3,6-dihydro-2H-1,3,4-thiadiazin-2-one; E. Merck KGaA, Darmstadt, Germany) was dissolved in propylene glycol so that an infusion rate of 0.5 ml min^{-1} corresponded to 0.2 mg/kg min^{-1} EMD 57033. Propranolol hydrochloride (ICI-Pharma, Rotterdam, The Netherlands) and phentolamine-methanosulfonide (CIBA-Geigy, Basel, Switzerland) were dissolved in saline. N^{ω}- nitro-L-arginine (Sigma Chemical Co., St Louis, USA) was dissolved in deionized water. Fresh solutions were prepared on the day of each experiment.

Results

Systemic hemodynamics

During the 15 min LADCA occlusion, mean arterial pressure had decreased from 100±2 mmHg at baseline to 92±2 mmHg (n=21, P<0.05). Since the decrease in mean arterial pressure was accompanied by a similar fall in cardiac output it follows that systemic vascular resistance had remained unchanged. $LVdP/dt_{max}$ decreased by 14±3 %, but $T_{systole}$ was not affected. However, $LVdP/dt_{min}$ decreased from –2000±70 to –1590±70 mmHg s^{-1} (P<0.05), while τ_{LVP} and LV end-diastolic pressure increased (Table 1). After 15 min of reperfusion there was no recovery in any of the hemodynamic variables, except for LV end-diastolic pressure which had returned to baseline values. In the following 15 min the changes were negligible in the 14 animals which were left untreated, but in the other seven animals adrenergic blockade caused marked decreases in mean arterial pressure, heart rate, cardiac output, $LVdP/dt_{max}$ and $LVdP/dt_{min}$ (to –1340±140 mmHg s^{-1}, (P<0.05), and caused a further increase in τ_{LVP}, while $T_{systole}$ was maintained.

Infusion of the vehicle had minimal effects on systemic hemodynamics and global LV relaxation parameters (Table 1). In contrast, infusion of EMD 57033 caused a marked decrease in mean arterial pressure due to a decrease in systemic vascular resistance. LV systolic pressure did not change during the first 30 min (113±8 mmHg), but fell to 94±7 mmHg during the following 30 min. There was a gradual increase in $LVdP/dt_{max}$ up to 186±19 % of its stunning value (thereby exceeding the baseline value), which reflected an increase in global contractility as preload (LV end-diastolic pressure) and afterload (LV systolic pressure) both decreased. $LVdP/dt_{min}$ and τ_{LVP} remained unchanged initially, but after 60 min $LVdP/dt_{min}$ became less negative by 430±190 mmHg s^{-1} and τ_{LVP} had increased by 19±4% (P<0.05 vs vehicle) at a time when heart rate had increased and LV systolic pressure had decreased slightly. The increase in heart rate was also likely to be responsible for the decrease in $T_{systole}$. The effects of EMD 57033 did not depend on the activity of the adrenergic system, as the increases in heart rate and $LVdP/dt_{max}$ and the decrease in systemic vascular resistance were not affected when EMD 57033 was administered in the presence of adrenoceptor blockade.

$LVdP/dt_{min}$, however, became more negative initially by –430±110 mmHg s^{-1} (P<0.05), but had returned to stunning values at 60 min of infusion, possibly because LV systolic pressure was maintained during the infusion. Finally, τ_{LVP} remained unchanged.

Table 1 Effect of EMD 57033 on Systemic Hemodynamics in Anesthetized Pigs with Stunned Myocardium.

	Baseline (n=21)	15 min Occlusion (n=21)	15 min Reperfusion (n=21)	Adrenergic Blockade	30 min Reperfusion	Infusion	Δ_{abs} by Infusion 30 min	Δ_{abs} by Infusion 60 min
MAP mmHg	100±2	92±2*	91±3*	-	94±4	PG	5±3	1±3
						EMD	2±6	-18±7‡¶
				+	68±6†	EMD	11±3‡	-3±2
CO $l\ min^{-1}$	2.7±0.1	2.4±0.1*	2.5±0.1*	-	2.5±0.1	PG	0.2±0.1‡	0.2±0.1‡
						EMD	0.6±0.1‡¶	0.3±0.2‡
				+	2.0±0.1†	EMD	0.9±0.1‡¶	0.9±0.2‡¶§
SVR $mmHg\ min\ l^{-1}$	37±1	39±1	37±2	-	39±1	PG	1.1±1.5	-2.8±1.2
						EMD	-7.5±3.0‡	-11.6±3.0‡¶
				+	33±1	EMD	-5.9±0.8‡¶	-11.3±0.9‡¶
HR Bpm	117±4	21±4	124±5	-	119±6	PG	-7±2	-8±4
						EMD	6±4¶	16±8¶
				+	89±7†	EMD	11±2‡¶	19±4‡¶
LVSP mmHg	114±2	105±2*	104±3*	-	108±4	PG	7±3	3±3
						EMD	5±7	-14±6‡¶
				+	84±5	EMD	6±3‡¶	5±3§
$LVdP/dt_{max}$ $mmHg\ s^{-1}$	1850±90	1620±80*	1470±70*	-	1630±100	PG	-10±100	-130±90
						EMD	760±140‡¶	1230±200‡¶
				+	1060±80†	EMD	880±110‡¶	1380±210‡¶
$T_{systole}$ ms	306±7	308±8	305±9	-	316±11	PG	20±3‡	19±5‡
						EMD	-16±7‡¶	-27±14‡¶
				+	340±9†	EMD	-26±5‡¶	-35±9‡¶
τ_{LVP} ms	49±2	52±2*	50±2*	-	53±3	PG	2.3±0.7	2.3±1.4
						EMD	4.7±1.7	11.0±2.6‡¶
				+	57±4†	EMD	2.0±5.4	-6.3±5.1§
LVEDP mmHg	6.8±0.6	10.1±0.7*	7.6±0.7*	-	7.2±0.8	PG	.7±0.3	0.3±0.3
						EMD	-2.0±0.4‡¶	-1.9±0.7‡¶
				+	9.0±1.2	EMD	-2.5±0.6‡¶	-3.9±0.7‡¶

MAP, mean arterial pressure; HR, heart rate; CO, cardiac output; LVSP, left ventricular systolic pressure; $LVdP/dt_{max}$, maximal rate of rise in left ventricular pressure; LVEDP, left ventricular end-diastolic pressure; SVR, systemic vascular resistance; PG, propylene glycol (n=7); EMD, EMD 57033 (0.2 mg/kg min^{-1}, n=7 in each group); -, no α and β blockade; +, α and β blockade; Values are mean± s.e. mean; *P<0.05 vs Baseline (only for 15 min Occlusion and 15 min Reperfusion); †P<0.05 vs corresponding 15 min Rep (only for 30 min Reperfusion); ‡P<0.05 vs 30 min Reperfusion; ¶P<0.05 vs change in vehicle group; §P<0.05 vs EMD 57033-induced change in animals without adrenoceptor blockade.

Additional blockade of NO synthesis by N^{ω}-nitro-L-arginine, resulted in an elevated systemic vascular resistence (52±8 mmHg/min l^{-1}) compared to adrenoceptor blockade alone (33±1 mmHg/min l^{-1}, P<0.05). However, NO was not involved in the vasodilating actions of EMD 57033 as the decrease in systemic vascular resistance (-14±5 mmHg/min l^{-1}) was unmitigated at 60 min of infusion of EMD 57033. Similarly, additional blockade of NO also did not modify the responses of the other hemodynamic variables to EMD 57033 (not shown).

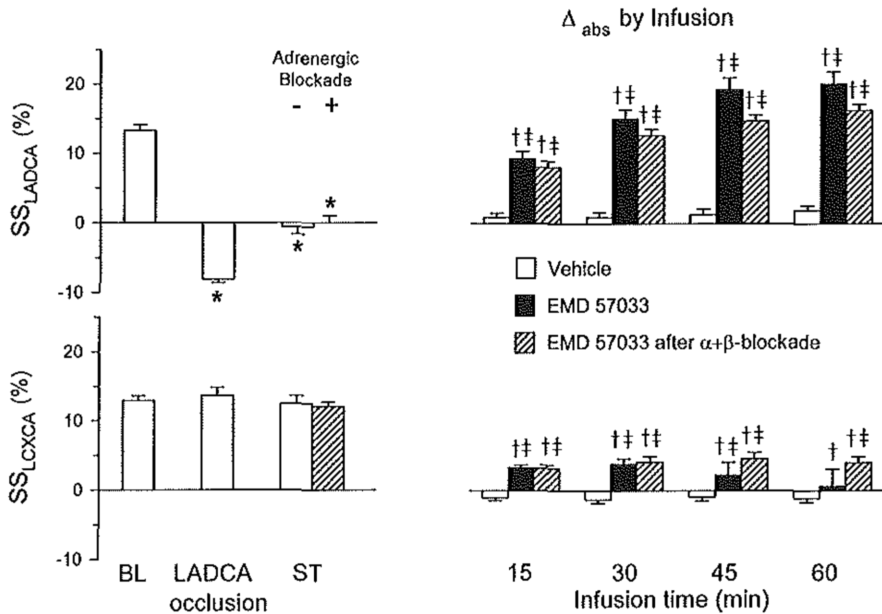

*Figure 2 Effect of EMD 57033 (0.2 mg/kg min^{-1}, i.v.) on regional systolic shortening (SS) in the area perfused by the LADCA and the area perfused by the LCXCA. Absolute values are shown at baseline (BL, n=21), during ischemia (after 15 min LADCA occlusion, n=21), and during stunning (ST) in the absence (n=14) and in the presence (n=7) of α+β-adrenoceptor blockade. The effects of the infusions of vehicle (n=7) and EMD 57033 (n=7 in both groups) have been presented as absolute changes (Δ_{abs}) from their respective stunning values. Data are mean ± s.e. mean; *P<0.05 stunning vs baseline, †P<0.05 vs stunning, ‡P<0.05 vs change in vehicle group, §P<0.05 vs EMD 57033-induced change in animals without adrenoceptor blockade.*

Regional systolic function

Systolic shortening After production of stunning, there was a complete loss of SS, and the appearance of a pronounced PSS (9.0±0.5 % vs 1.5±0.3 % at baseline) in the distribution area of the LADCA, while SS of the normal myocardium remained unchanged (Figure 2). Infusion of vehicle had a negligible effect on SS and PSS of both normal and stunned myocardium. During infusion of EMD 57033, SS in the stunned myocardium increased up to 19±2 %, while PSS disappeared. In the normal myocardium, SS increased from 14±2 % to 18±3 % (P<0.05). Adrenoceptor blockade did not modify the responses to EMD 57033 of regional wall motion in either stunned or normal myocardium.

End-systolic elastance Stunning produced a rightward shift of the LV end-systolic pressure-segment length relation in the LADCA area with an 18±7 % decrease in E_{es} (P<0.05, n=14; Figure 3; Table 2). Infusion of vehicle had no effect on this relation, but EMD 57033 produced a leftward shift with a progressive increase in E_{es} to four times above its stunning values (both P<0.05). Adrenoceptor blockade blunted the EMD 57033-induced increase in E_{es}.

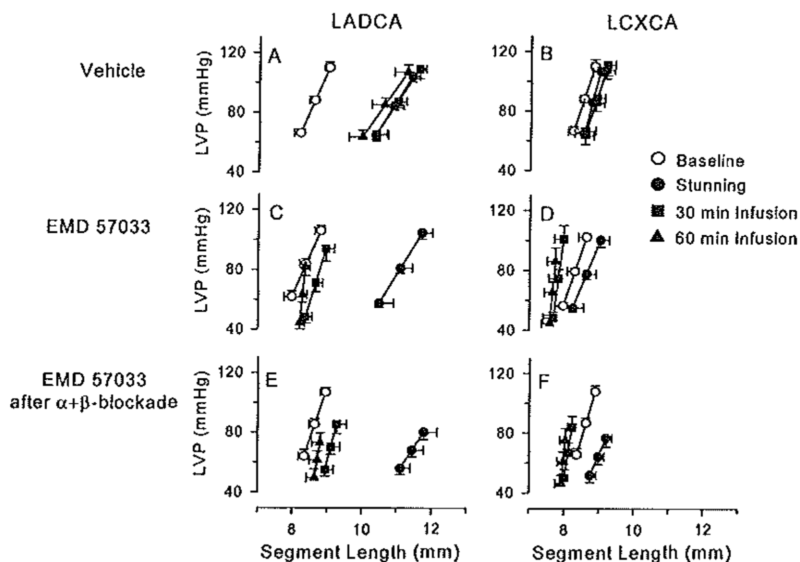

Figure 3 *LV end-systolic pressure-segment length relations in the LADCA (left panels) and LCXCA (right panels) perfusion territories at baseline, during stunning and after 30 min and 60 min of infusion of vehicle (A and B), or EMD 57033 (0.2 mg/kg min^{-1}, i.v.) in the absence (C and D) and in the presence (E and F) of adrenoceptor blockade. LVP = left ventricular pressure. For statistical analysis see Table 2.*

In the LCXCA area, stunning and the subsequent infusion of vehicle had no effect on the LV end-systolic pressure-segment length relation. Infusion of EMD 57033 caused a tripling of E_{es} without a change in $L_{0,es}$. Adrenoceptor blockade had no effect on the responses of E_{es} and $L_{0,es}$ to EMD 57033.

Regional diastolic function

Onset of relaxation Stunning did not alter the onset of relaxation as the duration of regional LV systole in either LADCA ($T_{systole,\ LADCA}$) or LCXCA ($T_{systole,\ LCXCA}$) perfused myocardium remained unchanged (Figure 4). The small decrease in heart rate that occurred during infusion of the vehicle was associated with small increases in $T_{systole,\ LADCA}$ and $T_{systole,\ LCXCA}$. Conversely, the EMD 57033-induced increase in heart rate resulted in reductions of $T_{systole,\ LADCA}$ and $T_{systole,\ LCXCA}$, that reached levels of statistical significance in the presence of adrenoceptor blockade.

Table 2 Effect of EMD 57033 on Regional Left Ventricular End-Systolic Elastance in Anaesthetised Pigs with Stunned Myocardium.

	Baseline	Reperfusion			Δ_{abs} by Infusion	
	(n=21)	Adrenergic blockade	30 min		30 min	60 min
LADCA perfusion territory						
E_{es}, mmHg mm^{-1}	67±7	-	46±5*	PG	-7±4	-8±4
				EMD	100±52	239±41†‡
		+	49±11	EMD	60±23†‡	126±29†‡¶
$L_{0,es}$, mm	7.0±0.2	-	8.9±0.4*	PG	-0.2±0.3	-0.7±0.3
				EMD	- 1.2±0.5†	-1.0±0.5
		+	9.7±0.3*	EMD	-1.3±0.4†	-1.4±0.4†
LCXCA perfusion territory						
E_{es}, mmHg mm^{-1}	80±7	-	76±7	PG	-18±7	-14±9
				EMD	165±49†‡	204±28†‡
		+	52±6*	EMD	119±26†‡	234±56†‡
$L_{0,es}$, mm	7.4±0.1	-	7.5±0.2*	PG	-0.2±0.1	-0.2±0.2
				EMD	0.1±0.1	0.1±0.2
		+	7.8±0.1	EMD	-0.1±0.1	0.1±0.1

LADCA, left anterior descending coronary artery; LCXCA, left circumflex coronary artery; E_{es}, end-systolic elastance; $L_{0,es}$, intercept at zero pressure of the LV end-systolic pressure-segment length relation; PG, propylene glycol (n=7); EMD, EMD 57033 (0.2 mg/kg min^{-1}, i.v., n=7 in each group); -, in the absence of α-and β-blockade; +, in the presence of α-and β-blockade; Values are mean± s.e. mean; *P<0.05 vs Baseline (only for 30 min Reperfusion); †P<0.05 vs 30 min Reperfusion; ‡P<0.05 vs change in vehicle group; ¶P<0.05 vs EMD 57033-induced in animals without adrenoceptor blockade.

Maximum rate of fall of elastance and time constant of decay of elastance Stunning resulted in a less negative dE/dt_{min} (from –850±100 mmHg/mm s^{-1} at baseline to –280±20 mmHg/mm s^{-1}) of the LADCA perfused area, but had no effect on dE/dt_{min} of the LCXCA perfused area (–890±80 mmHg/mm s^{-1} at baseline). Vehicle had no effect on dE/dt_{min} in either region but in the presence of EMD 57033, dE/dt_{min} became more negative by 3380±640 mmHg/mm s^{-1} and 1800±650 mmHg/mm s^{-1} in the LADCA and LCXCA areas, respectively. Although adrenoceptor blockade tended to blunt the effects of EMD 57033, this did not reach levels of statistical significance (not shown).

In view of the dependency of dE/dt_{min} on maximum elastance (E_{es}), we also determined the time constants of regional myocardial elastance decay (τ_e, Figure 5). Stunning increased τ_e in the LADCA area, but had no effect on τ_e in the LCXCA area. Vehicle had no effect on τ_e of either area, while EMD 57033 decreased τ_e in both areas (P<0.05). Adrenoceptor blockade blunted the EMD 57033-induced decrease in both stunned and normal myocardium.

End-diastolic elastance In the LADCA area stunning had no effect on E_{ed}, but increased $L_{0,ed}$ from 8.7±0.3 to 9.9±0.4 mm (Table 3, Figure 6). Vehicle had no effect on E_{ed} or $L_{0,ed}$. In contrast, EMD 57033, which had also no effect on E_{ed}, tended to decrease $L_{0,ed}$ (P=0.098). Adrenoceptor blockade had no significant effect on the responses of E_{ed} to EMD 57033, but

prevented the decrease in $L_{0,ed}$.

In the adjacent LCXCA area, the stunning protocol and subsequent infusion of vehicle had no effect on the LV end-diastolic pressure-segment length relation (Table 3, Figure 6). Infusion of EMD 57033 also had no effect on E_{ed}, but caused a leftward shift of the LV end-diastolic pressure-segment relation. Adrenoceptor blockade did not modify the responses of E_{ed} to EMD 57033, but blunted the decrease in $L_{0,ed}$.

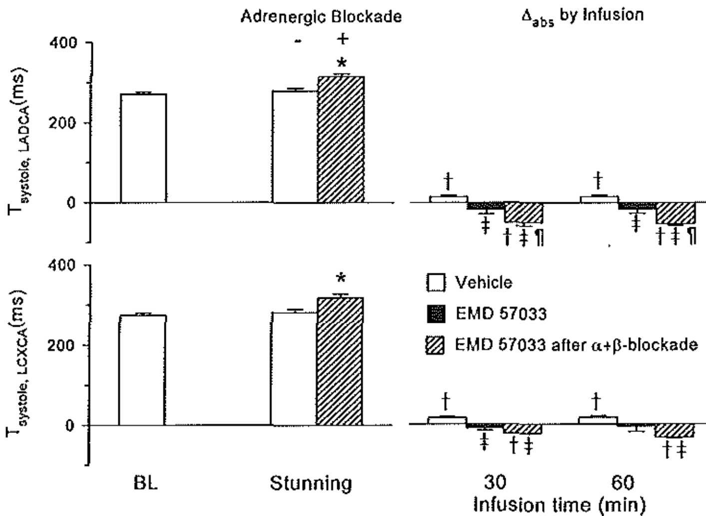

*Figure 4 The effect of EMD 57033 (0.2 mg/kg min⁻¹, i.v.) on the duration of systole ($T_{systole}$) in the LADCA (top) and the LCXCA (bottom) perfusion territories. Absolute values are shown at baseline (BL, n=21), and during stunning (ST) in the absence (n=14) and in the presence (n=7) of α+β-adrenoceptor blockade. The effects of the infusions of vehicle (n=7) and EMD 57033 (n=7 in both groups) have been presented as absolute changes (Δ_{abs}) from their respective stunning values. Data are mean ± s.e. mean; *P<0.05 stunning vs baseline, †P<0.05 vs stunning, ‡P<0.05 vs change in vehicle group, ¶P<0.05 vs EMD 57033-induced change in animals without adrenoceptor blockade.*

Mechanical efficiency of the stunned myocardium

Stunning caused a 68±5 % decrease (*P*<0.05) in EW_{beat} but only a 12±6 % decrease in the $MVO_{2\ beat}$ of the LADCA-perfused myocardium, so that mechanical efficiency ($EW_{beat}/MVO_{2\ beat}$) decreased by 66±6 % (Figure 7). Infusion of vehicle had no effect on mechanical efficiency as both EW_{beat} and $MVO_{2\ beat}$ remained unchanged. However, during infusion of EMD 57033 both EW_{beat} and $MVO_{2\ beat}$ returned to baseline values and consequently also mechanical efficiency was normalized. The increase in MVO_2 was accompanied by an equivalent increase in myocardial blood flow, so that myocardial O_2 extraction (62±6 % at stunning and 62±6 % at 60 min of infusion) and coronary venous PO_2 (27±3 mmHg at stunning and 28±3 mmHg at 60 min of infusion) remained unchanged. In the pigs in which EMD 57033 was infused in the presence of adrenoceptor blockade, the responses of EW_{beat}, $MVO_{2\ beat}$, mechanical efficiency and coronary venous PO_2 were not different from the esponses to EMD 57033 in the absence of adrenoceptor blockade.

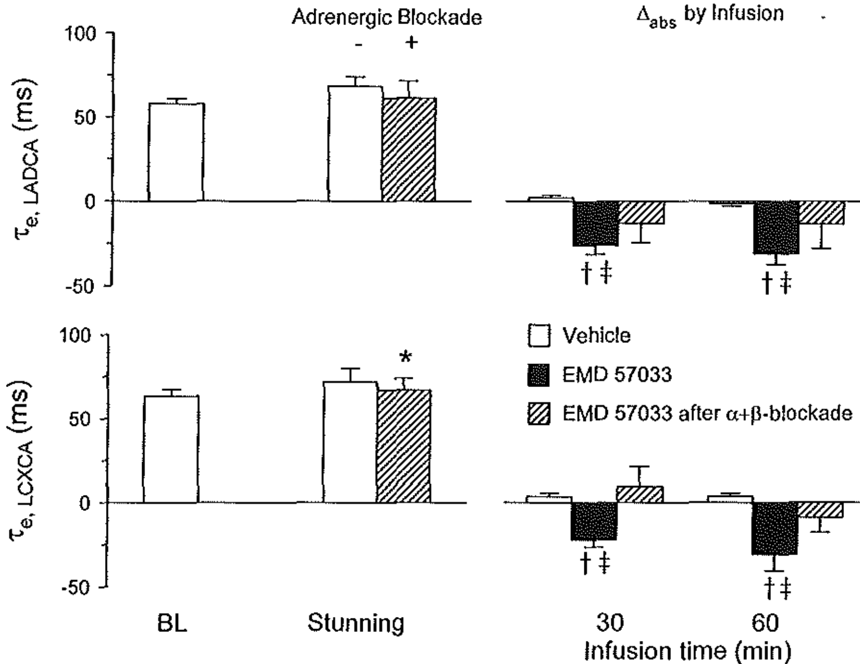

***Figure 5** From top to bottom are shown the time constants of decay of regional elastance (τ_e) during early diastole of the LADCA ($\tau_{e,\ LADCA}$) and LCXCA ($\tau_{e,\ LCXCA}$) perfusion territories. Absolute values are shown at baseline (BL, n=21) and during stunning in the absence (n=14) and in the presence (n=7) of α+β-adrenoceptor blockade. The effects of the infusions of vehicle (n=7) and EMD 57033 (0.2 mg/kg min^{-1}, i.v., n=7 in both groups) have been presented as absolute changes (Δ_{abs}) from their respective stunning values. Data are mean ± s.e. mean; *$P<0.05$ stunning vs baseline, †$P<0.05$ vs stunning, ‡$P<0.05$ vs change in vehicle group, §$P<0.05$ vs EMD 57033-induced change in animals without adrenoceptor blockade.*

Plasma and tissue levels of EMD 57033

EMD 57033 could not be detected in the pre-drug samples. During EMD 57033 infusion, the plasma levels increased time-dependently to 3.54±0.13, 5.03±0.63, 6.53±0.74 and 7.23±0.67 μg ml^{-1} at 15, 30, 45 and 60 min, respectively. In two of these experiments it was shown that at the end of the 60 min infusion EMD 57033 had accumulated in the left ventricle (26.6 and 29.5 μg per g of wet weight) and the liver (29.4 and 26.2 μg g^{-1}), while tissue levels in the stomach (6.7 and 6.6 μg g^{-1}) and skeletal muscle (13.5 and 5.3 μg g^{-1}) were in the range of the plasma concentrations. EMD 57033 concentrations in the cerebellum (4.3 and 4.2 μg g^{-1}) and cerebrum (4.4 and 5.3 μg g^{-1}) were below plasma levels, possibly due to the blood-brain barrier.

***Table 3** Effect of EMD 57033 on Regional Left Ventricular End-Diastolic Elastance in Anesthetized Pigs with Stunned Myocardium.*

	Baseline (n=21)	Reperfusion Adrenergic blockade	Reperfusion 30 min		Δ_{abs} by Infusion 30 min	Δ_{abs} by Infusion 60 min
LADCA perfusion territory						
E_{ed}, mmHg mm^{-1}			3.9±0.4	PG	-0.4±0.5	-0.7±0.5
	4.2±0.4	-		EMD	-0.7±0.6	-0.6±1.3
		+	4.4±0.4	EMD	-0.8±0.5	-1.0±0.9
$L_{0,ed}$, mm			9.9±0.4*	PG	0.0±0.1	-0.3±0.2
	8.7±0.3	-		EMD	-0.3±0.4	-1.9±1.4
		+	9.8±0.9*	EMD	-0.4±0.2	-0.6±0.3
LCXCA perfusion territory						
E_{ed}, mmHg mm^{-1}			4.6±0.4*	PG	0.4±0.4	0.5±0.5
	3.7±0.2	-		EMD	-0.6±1.3	1.9±2.1
		+	6.2±1.1	EMD	-1.9±0.8‡	-2.3±1.5
$L_{0,ed}$, mm			9.6±0.7*	PG	0.2±0.2	0.1±0.2
	9.1±0.4	-		EMD	-2.3±1.8†	-2.8±1.5†
		+	10.1±0.8*	EMD	-0.5±0.1†‡¶	-0.7±0.2†‡¶

*LADCA, left anterior descending coronary artery; LCXCA, left circumflex coronary artery; E_{ed}, end-diastolic elastance; $L_{0,ed}$, intercept at zero pressure of the LV end-diastolic pressure-segment length relation; PG, propylene glycol (n=7); EMD, EMD 57033 (0.2 mg/kg min^{-1}, i.v., n=7 in each group); -, in the absence of α-and β-blockade; +, in the presence of α-and β-blockade; Values are mean± s.e. mean; *P<0.05 vs Baseline (only for 30 min Reperfusion); †P<0.05 vs 30 min Reperfusion; ‡P<0.05 vs change in vehicle group; ¶P<0.05 vs EMD 57033-induced change in animals without adrenoceptor blockade.*

Discussion

The major findings in this *in vivo* porcine model of regional myocardial stunning are (*i*) EMD 57033 increased systolic shortening in stunned myocardium more than in the adjacent normal myocardium; (*ii*) EMD 57033 also increased end-systolic elastance in normal and stunned myocardium, but with similar responses in both regions; (*iii*) EMD 57033 did not delay the onset of relaxation, but improved the maximum rate of fall of regional myocardial elastance and decreased the time constant of early diastolic regional myocardial elastance decay; EMD 57033 had no effect on end-diastolic elastance in either normal and stunned myocardium; (*iv*) EMD 57033 restored mechanical efficiency of the stunned myocardium; (*v*) the effects on regional systolic and diastolic function were only slightly modified by pretreatment with α- and β-adrenoceptor blockade, indicating that at the dose used phosphodiesterase III inhibition contributes only minimally to the actions of EMD 57033; (*vi*) finally, the EMD-induced systemic vasodilation was not amenable to either adrenoceptor blockade or additional inhibition of NO synthesis.

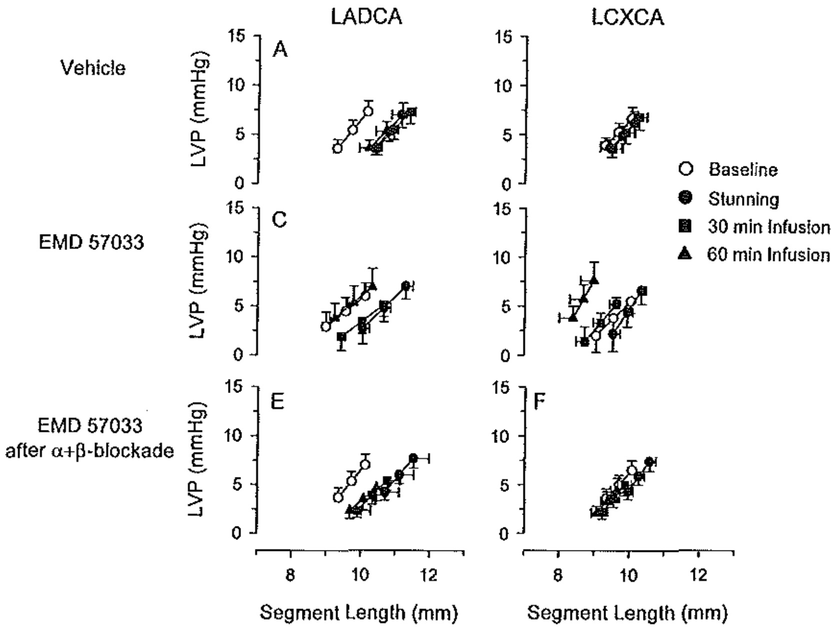

Figure 6 LV end-diastolic pressure-segment length relations in the LADCA (left panels) and LCXCA (right panels) perfusion territories at baseline, during stunning and after 30 min and 60 min of infusion of vehicle (A and B), or EMD 57033 (0.2 mg/kg min^{-1}, i.v.) in the absence (C and D) and in presence (E and F) of adrenoceptor blockade. LVP = left ventricular pressure. For statistical analysis see Table 3.

Systolic function

EMD 57033 resulted in a time-dependent (i.e. plasma concentration-dependent) restoration of SS in stunned myocardium, while producing only a small increase in SS in the remote normal region. This finding is in accordance with previous observations in our laboratory with EMD 60263 in a similar model of myocardial stunning in the *in vivo* pig heart.[4] These studies could be interpreted to suggest a relatively preferential effect of Ca^{2+}-sensitizing drugs on systolic function of stunned myocardium. However, SS is a load-sensitive index of systolic function and does not necessarily reflect the contractile state of the myocardium[22], particularly under conditions of stunning when its load-dependency is even greater.[9] Indeed, EMD 57033 produced similar increments in E_{es} in stunned and normal myocardium. This suggests that the greater increase in SS in stunned myocardium resulted principally from a greater sensitivity of regions with a lower E_{es} for positive inotropic interventions. This is supported by the observation that the β-adrenoceptor agonist dobutamine also produces a preferential increase in systolic shortening of stunned myocardium, whereas E_{es} responses were similar in normal and stunned region.[9,23] Similar to the present study, Korbmacher *et al.*[8] observed that EMD 60263 at an optimal dose of 3 μM produced similar

increments in $LVdP/dt_{max}$ in normal (from 1415 to 1885 mmHg s^{-1}) and globally stunned (from 845 to 1300 mmHg s^{-1}) isolated rabbit hearts in which afterload was held constant. Also with EMD 57033, these authors reported similar increments in LV systolic pressure in stunned and normal isolated rabbit hearts at constant afterload. Interestingly, we observed that the increase in E_{es} in the stunned myocardium was time-dependent whereas the E_{es} in normal myocardium at 30 min of infusion of EMD 57033 had not increased further after 60 min of infusion. In view of the progressive increase in plasma levels over time, this observation suggests that the maximum effect in normal myocardium was reached at a lower dose than that in stunned myocardium. Indeed, Korbmacher *et al.*[8] observed in their *in vitro* study that a further increase in dose of EMD 57033 to 10 μM did not further increase $LVdP/dt_{max}$ in stunned hearts, while it slightly decreased $LVdP/dt_{max}$ in normal myocardium. These findings suggest that the normal myocardium is more sensitive to adverse effects on systolic function than stunned myocardium in which the Ca^{2+} responsiveness is lower.[3]

It has been proposed that *in vitro* EMD 57033 exerts, besides its Ca^{2+}-sensitizing effects, phosphodiesterase inhibiting actions.[10,24] In the present study, blockade of α- and β-adrenoceptors did not modulate the inotropic responses to EMD 57033 in normal myocardium *in vivo*. Thus, the increase in E_{es} produced by EMD 57033 in normal myocardium was not altered by adrenoceptor blockade, while the index of global LV contractility $LVdP/dt_{max}$ was also unmitigated in the presence of adrenoceptor blockade. Hence the EMD-57033-induced actions appeared to be primarily the result of an increase in Ca^{2+} responsiveness which is in accordance with previous studies in awake pigs from our laboratory.[25] However, there was a slight blunting of the increase in E_{es} in stunned myocardium, which is difficult to explain by a phosphodiesterase III inhibitory action, as it seems unlikely that a pharmacological property such as phosphodiesterase III inhibition could differ in the stunned from normal myocardium. Moreover, the duration of global LV systole, and $T_{systole, LADCA}$ and $T_{systole, LCXCA}$ which were not altered by EMD 57033 in the presence of intact adrenoceptor activity, were not increased but were slightly shortened by EMD 57033 in the presence of adrenoceptor blockade. Taken together the present study suggests that the EMD-57033-induced systolic actions *in vivo* are not the result of phosphodiesterase III inhibition.

Stunning reduced mechanical efficiency likely due to a decrease in E_{es} which not only decreases external work but also increases potential work, resulting in a relatively high MVO_2.[22] EMD 57033 restored external work while producing only a small increase in MVO_2, thereby restoring efficiency of stunned myocardium. This favourable effect is most likely the result of positive inotropism, rather than a unique feature of EMD 57033, because we previously observed a similar restoration of mechanical efficiency of stunned myocardium during infusion of the β-adrenoceptor agonist dobutamine.[23]

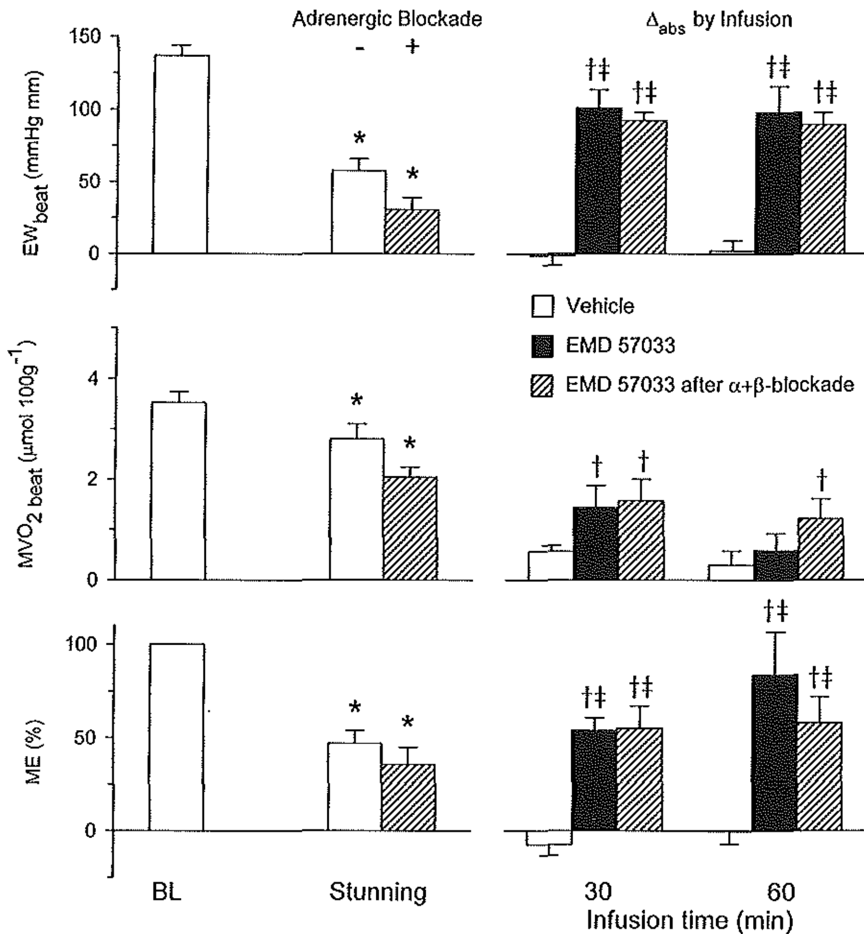

*Figure 7 EW_{beat}, $MVO_{2\,beat}$ and ME of the LADCA perfused area. Absolute values are shown at baseline (BL, n= 21), and during stunning in the absence (n=14) and in the presence (n=7) of α- and β-adrenoceptor blockade. The effects of vehicle (n=7) and EMD 57033 (0.2 mg/kg min^{-1}, i.v.) in the absence (n=7) or presence (n=7) of adrenoceptor blockade have been presented as absolute changes (Δ_{abs}) from their respective stunning values. Data are mean ± s.e. mean; *P<0.05 stunning vs baseline, †P<0.05 vs stunning, ‡P<0.05 vs change in vehicle group, §P<0.05 vs EMD 57033-induced change in animals without adrenoceptor blockade.*

Diastolic function

It has been suggested that increased Ca^{2+} responsiveness can lead to maintained contraction at Ca^{2+} levels at which normally relaxation occurs.[26] In rat skinned right ventricular trabeculae these authors have shown that EMD 57033 can even cause contraction in the absence of Ca^{2+}. Consequently, contraction could be prolonged thereby delaying the onset of relaxation[10,24], the rate of relaxation could be attenuated [8,27] and end-diastolic stiffness could be increased[27,28] even when Ca^{2+} concentrations are normal.

Early diastolic function appeared to be well preserved during EMD 57033 infusion in the present study. $T_{systole}$ slightly decreased in both normal and stunned myocardium and in the left ventricle as a whole, possibly due to the small increase in heart rate. Thus, the onset of relaxation was not delayed by EMD 57033 either in the presence or absence of intact adrenoceptor activity, indicating that phosphodiesterase III inhbition did not mask the potential contraction prolongation produced by the Ca^{2+}-sensitizing actions of EMD 57033. $LVdP/dt_{min}$ is often used as an index of global early LV relaxation, despite its dependency on, in particular, LV systolic pressure.[29,30] In the present study, $LVdP/dt_{min}$ improved slightly during the first 30 min of EMD 57033 infusion but fell to 75% of the value at stunning during the following 30 min. The decrease in $LVdP/dt_{min}$ may have been due to the 20% reduction of LV systolic pressure, and does not necessarily reflect an impairment of global LV relaxation. This is even more so, as in the adrenoceptor blocked animals, both $LVdP/dt_{min}$ and LV systolic pressure were not affected by EMD 57033. This strongly suggests that phosphodiesterase III inhibition did not contribute significantly to the maintenance of $LVdP/dt_{min}$. Inspection of the less load-sensitive time constant τ_{LVP} supports this notion. Nevertheless, both indexes are global variables and do not discriminate between the effects of EMD 57033 on normal and stunned myocardium, which is also why regional indexes of ventricular relaxation were employed. In both stunned and normal myocardial regions, the maximum rate of fall of regional myocardial elastance (dE/dt_{min}) increased, which is not surprising in view of the marked increments in E_{es}. However, even after correction for the load-dependency of dE/dt_{min}, i.e. by calculating the time constants of elastance decay τ_e, the data indicated no untoward effects of EMD 57033 on early diastolic function. Importantly, even in the presence of adrenoceptor blockade, EMD 57033 did not exert detrimental actions on dE/dt_{min} or the relaxation time constants, suggesting that the lack of adverse effects were not the result of masking of untoward effects of Ca^{2+}-sensitization by concomitant phosphodiesterase III inhibitory effects of the compound. In the present study we also failed to find evidence of a negative effect of EMD 57033 on late diastolic function, as E_{ed} was maintained during EMD 57033 infusion, even in the presence of adrenoceptor blockade.

The importance of abnormalities in diastolic function is that these could compromise systolic pump function via reduced LV filling and via impairment of myocardial perfusion by reducing the effective diastolic perfusion time of the coronary bed. However, in the present study EMD 57033, either with or without adrenoceptor blockade, restored regional systolic shortening, external work and mechanical efficiency, while myocardial oxygen supply was unimpeded.

Conclusions

The Ca^{2+}-sensitizing agent EMD 57033, at a dose of 0.2 mg/kg min^{-1}, restored regional systolic and diastolic function of stunned myocardium in the *in vivo* porcine heart. In normal

myocardium, quantitatively similar improvements in systolic and diastolic function were observed. Phosphodiesterase III inhibition contributed minimally to these actions. The results of the present study suggest that Ca^{2+}-sensitizing agents are prime candidates for complementing the current inodilator therapeutic arsenal in the clinical setting for acute states of heart failure, because they are powerful enhancers of systolic performance *in vivo* at doses that do not appear to exert adverse effects on diastolic function.

Acknowledgements

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References

1. Charlat ML, O'Neill PG, Hartley CJ, Roberts R, Bolli R. Prolonged abnormalities of left ventricular diastolic wall thinning in the "stunned" myocardium in conscious dogs: time course and relation to systolic function. *J Am Coll Cardiol.* 1989;13:185-194.
2. Ehring T, Schulz R, Schipke JD, Heusch G. Diastolic dysfunction of stunned myocardium. *Am J Cardiovasc Pathol.* 1993;4:358-366.
3. Bolli R, Marban E. Molecular and cellular mechanisms of myocardial stunning. *Physiol Rev.* 1999;79:609-634.
4. Soei LK, Sassen LM, 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.
5. Abe Y, Kitada Y, Narimatsu A. Effect of a calcium-sensitizing positive inotropic agent MCI-154 and its combined use with enalapril on postischemic contractile dysfunction of dog hearts. *J Cardiovasc Pharmacol.* 1995;26:653-659.
6. Korbmacher B, Sunderdiek U, Arnold G, Schulte HD, Schipke JD. Improved ventricular function by enhancing the Ca^{++} sensitivity in normal and stunned myocardium of isolated rabbit hearts. *Basic Res Cardiol.* 1994;89:549-562.
7. Korbmacher B, Sunderdiek U, Schulte HD, Arnold G, Schipke JD. Comparison between the effects of a novel Ca^{++} sensitizer and a phosphodiesterase inhibitor on stunned myocardium. *J Pharmacol Exp Ther.* 1995;275:1433-1441.
8. Korbmacher B, Sunderdiek U, Selcan G, Arnold G, Schipke JD. Different responses of non-ischemic and post-ischemic myocardium towards Ca^{2+} sensitization. *J Mol Cell Cardiol.* 1997;29:2053-2066.
9. Fan D, Soei LK, Sassen LM, Krams R, Verdouw PD. Mechanical efficiency of stunned myocardium is modulated by increased afterload dependency. *Cardiovasc Res.* 1995;29:428-437.
10. Ravens U, Himmel HM, Fluss M, Davia K, Harding SE. Phosphodiesterase inhibition and Ca^{2+} sensitization. *Mol Cell Biochem.* 1996;157:245-249.
11. Aversano T, Maughan WL, Hunter WC, Kass D, Becker LC. End-systolic measures of regional ventricular performance. *Circulation.* 1986;73:938-950.
12. Verdouw PD, Duncker DJ, Saxena PR. Poor vasoconstrictor response to adrenergic stimulation in the arteriovenous anastomoses present in the carotid vascular bed of young Yorkshire pigs. *Arch Int Pharmacodyn Ther.* 1984;272:56-70.
13. Duncker DJ, Saxena PR, Verdouw PD. Systemic haemodynamic and beta-adrenoceptor antagonistic effects of bisoprolol in conscious pigs: a comparison with propranolol. *Arch Int Pharmacodyn Ther.* 1987;290:54-63.
14. Nankervis R, Lues I, Brown L. Calcium sensitization as a positive inotropic mechanism in diseased rat and human heart. *J Cardiovasc Pharmacol.* 1994;24:612-617.
15. Duncker D, Stubenitsky R, Verdouw P. Endogenous nitric oxide contributes to coronary vasodilation but does not modify myocardial O2 consumption in awake swine at rest and during treadmill exercise. *Circulation.* 1997;96:I-73.
16. Duncker DJ, Heiligers J, Mylecharane EJ, Saxena PR, Verdouw PD. Nimodipine-induced changes in the

distribution of carotid blood flow and cardiac output in pentobarbitone-anaesthetized pigs. *Br J Pharmacol.* 1986;89:35-46.

17. Sassen LM, Soei LK, Koning MM, Verdouw PD. The central and regional cardiovascular responses to intravenous and intracoronary administration of the phenyldihydropyridine elgodipine in anaesthetized pigs. *Br J Pharmacol.* 1990;99:355-363.
18. van der Velde ET, Burkhoff D, Steendijk P, Karsdon J, Sagawa K, Baan J. Nonlinearity and load sensitivity of end-systolic pressure-volume relation of canine left ventricle in vivo. *Circulation.* 1991;83:315-327.
19. Harkin CP, Pagel PS, Tessmer JP, Warltier DC. Systemic and coronary hemodynamic actions and left ventricular functional effects of levosimendan in conscious dogs. *J Cardiovasc Pharmacol.* 1995;26:179-188.
20. Morris JJd, 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.
21. Vinten-Johansen J, Gayheart PA, Johnston WE, Julian JS, Cordell AR. Regional function, blood flow, and oxygen utilization relations in repetitively occluded-reperfused canine myocardium. *Am J Physiol.* 1991;261:H538-H547.
22. Krams R, Duncker DJ, McFalls EO, Hogendoorn A, Verdouw PD. Dobutamine restores the reduced efficiency of energy transfer from total mechanical work to external mechanical work in stunned porcine myocardium. *Cardiovasc Res.* 1993;27:740-747.
23. McFalls EO, Duncker DJ, Krams R, Sassen LM, Hoogendoorn A, Verdouw PD. Recruitment of myocardial work and metabolism in regionally stunned porcine myocardium. *Am J Physiol.* 1992;263:H1724-H1731.
24. Solaro RJ, Gambassi G, Warshaw DM, Keller MR, Spurgeon HA, Beier N, Lakatta EG. Stereoselective actions of thiadiazinones on canine cardiac myocytes and myofilaments. *Circ Res.* 1993;73:981-990.
25. Stubenitsky R, van der Weerd RW, Haitsma DB, Verdouw PD, Duncker DJ. Cardiovascular effects of the novel Ca^{2+}-sensitiser EMD 57033 in pigs at rest and during treadmill exercise. *Br J Pharmacol.* 1997;122:1257-1270.
26. Palmer S, Kentish JC. Differential effects of the Ca^{2+} sensitizers caffeine and CGP 48506 on the relaxation rate of rat skinned cardiac trabeculae. *Circ Res.* 1997;80:682-687.
27. Hajjar RJ, Schmidt U, Helm P, Gwathmey JK. Ca^{++} sensitizers impair cardiac relaxation in failing human myocardium. *J Pharmacol Exp Ther.* 1997;280:247-254.
28. Hgashiyama A, Watkins MW, Chen Z, LeWinter MM. Effects of EMD 57033 on contraction and relaxation in isolated rabbit hearts. *Circulation.* 1995;92:3094-3104.
29. Weisfeldt ML, Scully HE, Frederiksen J, Rubenstein JJ, Pohost GM, Beierholm E, Bello AG, Daggett WM. Hemodynamic determinants of maximum negative dP-dt and periods of diastole. *Am J Physiol.* 1974;227:613-621.
30. Gaasch WH, Carroll JD, Blaustein AS, Bing OH. Myocardial relaxation: effects of preload on the time course of isovolumetric relaxation. *Circulation.* 1986;73:1037-1041.

In vivo evidence that EMD 57033 restores myocardial responsiveness to intracoronary Ca^{2+} in stunned myocardium

5

In vivo evidence that EMD 57033 restores myocardial responsiveness to intracoronary Ca^{2+} in stunned myocardium

Sandra de Zeeuw, Serge AIP Trines, Rob Krams, Dirk J Duncker, Pieter D Verdouw

Background Despite ample *in vitro* evidence that myofilament Ca^{2+}-responsiveness of stunned myocardium is decreased, *in vivo* data are inconclusive. Conversely, while Ca^{2+}-sensitizing agents increase myofilament Ca^{2+}-responsiveness *in vitro*, it has been questioned whether this also occurs *in vivo*. We therefore tested in open-chest anesthetized pigs whether EMD 57033 (the (+) enantiomer of 5-[1-(3,4-dimethoxybenzoyl)-1,2,3,4-tetrahydro-6-quinolyl]-6-methyl-3,6-dihydro-2H-1,3,4-thiadiazin-2-one) increases responsiveness to Ca^{2+} of non-stunned myocardium and restores function of stunned myocardium by normalizing the responsiveness to Ca^{2+}.

Methods and Results Studies were performed under β-adrenoceptor blockade to minimize the contribution of the phosphodiesterase-III inhibitory actions of EMD 57033. Consecutive intracoronary Ca^{2+} infusions were used to evaluate the contractile response (assessed by the left ventricular end-systolic elastance, E_{es}) to added Ca^{2+} of non-stunned myocardium and myocardium stunned by 15 min coronary artery occlusion and 30 min reperfusion. In non-stunned propranolol-treated myocardium, the Ca^{2+} infusions doubled E_{es} (baseline 6.9±0.9 mmHg mm^{-2}, n=8). Following Ca^{2+}-washout, subsequent EMD 57033 infusion (0.1 mg/kg·min^{-1}, i.v.) tripled E_{es} ($P<0.05$) and potentiated the Ca^{2+}-induced increase in E_{es} to 55.7±10.0 mmHg mm^{-2} ($P<0.05$). Stunning (n=7) decreased E_{es} to 5.3±0.6 mmHg mm^{-2} ($P>0.10$) and attenuated the Ca^{2+}-induced increase in E_{es} ($P<0.05$). Subsequent infusion of EMD 57033, increased E_{es} to 6.8±1.8 mmHg mm^{-2} ($P<0.05$) and restored responsiveness to added Ca^{2+}.

Conclusions These *in vivo* findings are consistent with the *in vitro* observations that myofilament Ca^{2+}-responsiveness of stunned myocardium is reduced and that EMD 57033 increases contractility by enhancing myofilament Ca^{2+}-responsiveness. ***(Eur J Pharmacol. 2000;403:99-109)***

Introduction

It has been shown in *in vitro* models that a reduced responsiveness of the myofilaments to Ca^{2+} underlies the mechanism of myocardial stunning,[1] but evidence that this mechanism is also operative *in vivo* is lacking (see reference 2 and 25). For instance, Ito et al.[3] observed that in an *in vivo* canine model of regional myocardial stunning maximum systolic shortening attainable with intracoronary Ca^{2+} infusions was not different for stunned and normal myocardium. In addition, Heusch et al.[4] also failed to find evidence for a decreased Ca^{2+} responsiveness as assessed by the response of a regional work index to intracoronary Ca^{2+} in *in vivo* porcine myocardium, stunned by a 90-min flow reduction that decreased the local myocardial work index by 60%, a protocol that produced stunning without necrosis. A complicating factor in explaining the discrepancies between the *in vitro* and the *in vivo* results, is that in the *in vivo* studies contractile function was estimated using indices that display considerable load-dependency and of which it is known that their load-dependency increases with stunning.[5] Thus, Hofmann et al.[6] showed under well controlled *in vitro* conditions that pCa for half maximal activation of tension was decreased, compared to baseline, in single cell-sized preparations from porcine myocardium stunned *in vivo* by a 45-min period of 60% coronary flow reduction and 30 min of reperfusion. We have shown earlier that EMD 60263, a thiadiazinone derivative, which has been demonstrated to possess Ca^{2+}-sensitizing properties *in vitro*,[7,8] is capable of restoring systolic shortening of regionally stunned myocardium *in vivo*.[9] The beneficial effects of a number of Ca^{2+}-sensitizing agents on systolic function of stunned myocardium have now been confirmed in several isolated and intact heart studies,[10-12] but in none of these studies it was actually shown that during stunning, the responsiveness to Ca^{2+} was altered and that subsequent administration of the Ca^{2+}-sensitizing agents restored the responsiveness to Ca^{2+}.

Therefore, the aims of the present *in vivo* study were to determine whether administration of a Ca^{2+}-sensitizing agent alters the myocardial responsiveness to added Ca^{2+} in non-stunned myocardium and whether this agent restores function of stunned myocardium by normalization of the myocardial responsiveness to Ca^{2+}. As a Ca^{2+}-sensitizing agent we used EMD 57033 (the (+) enantiomer of 5-[1-(3,4-dimethoxybenzoyl)-1,2,3,4-tetrahydro-6-quinolyl]-6-methyl-3,6-dihydro-2H-1,3,4-thiadiazin-2-one), because we have shown that this agent, in a dose of 0.2 $mg/kg\cdot min^{-1}$, is capable of restoring systolic function of regionally stunned myocardium, without adversely affecting diastolic function,[12] a potential concern with this class of agents.[13,14] Because EMD 57033 possesses minor phosphodiesterase-III (PDE-III) inhibitory properties,[7,15] the effect of EMD 57033 was evaluated in the presence of the ß-adrenoceptor antagonist propranolol to minimize tonic and stimulated cAMP production, thereby keeping the contribution of PDE-III inhibition to the actions of EMD 57033 to a minimum. The myocardial responsiveness to added Ca^{2+} was determined using intracoronary infusions to prevent that changes in systemic hemodynamics act as confounding factors. Finally, to further

minimize the influence of changes in loading conditions, we used, in analogy to the left ventricular end-systolic pressure (LVESP)-volume[16] and LVESP-segment length[5,17] relations, the LVESP-segment area relation to evaluate the contractile response to Ca^{2+}.

Methods

Experiments were performed in accordance with the "Guide for the Care and Use of Laboratory Animals" of the Council of the American Physiological Society and under the regulations of the Erasmus University Rotterdam.

Animal preparation

Fifteen with ketamine (20-30 $mg \cdot kg^{-1}$ i.m.) sedated cross-bred Landrace-Yorkshire pigs (25-35 kg) were anesthetized with sodium pentobarbital (20 $mg \cdot kg^{-1}$, i.v.), intubated and ventilated with oxygen-enriched air.[12,14] Fluid-filled catheters were inserted for intravenous administration of sodium pentobarbital (5-10 $mg/kg \cdot h^{-1}$) and fluids, and for measurement of arterial blood pressure. A micromanometer-tipped catheter (B. Braun Medical) was inserted for monitoring left ventricular blood pressure. A balloon catheter was positioned in the inferior caval vein to transiently decrease left ventricular preload. After administration of pancuronium bromide (4 mg, i.v.), a midsternal thoracotomy was performed and an electromagnetic flow probe (Skalar) was placed around the ascending aorta, while a Doppler flow probe (Triton Technology) was placed proximally on the left anterior descending coronary artery (LADCA). Distal to the flow probe, the LADCA was dissected free for placement of an atraumatic clamp and cannulated for local infusion of Ca^{2+}. Segment area was measured using sonomicrometry (Triton Technology) by placing ultrasonic crystals in the midmyocardial layer approximately 10 mm apart in the distribution areas of the LADCA and the left circumflex coronary artery (LCXCA). To minimize the influence of malalignment of a single crystal pair with the fiber direction, two pairs of crystals were implanted in each region: one pair parallel and another pair perpendicular to the myocardial fiber direction.

Experimental protocols

Propranolol-treated pigs with non-stunned myocardium

To evaluate the effect of EMD 57033 on the responsiveness to intracoronary Ca^{2+} of non-stunned myocardium, 8 pigs received propranolol (0.5 $mg \cdot kg^{-1}$ + 0.5 $mg/kg \cdot h^{-1}$, a dose that inhibits the isoproterenol-induced increases in heart rate and left ventricular dP/dt_{max} by more than 90% in pigs[18]), after which hemodynamic variables were recorded and LVESP-segment area relations were constructed by transiently reducing preload.[12] Then, 3 consecutive 5-min infusions of Ca^{2+} ($CaCl_2 \cdot 2H_2O$ dissolved in saline) were administered into the LADCA at rates of 0.25, 0.50 and 0.75 ml/min (corresponding to 18, 36 and 54 $\mu mol \cdot min^{-1}$, respectively). At the end of each infusion step measurements were repeated (Figure 1). Following Ca^{2+}-washout,

the EMD 57033 infusion (0.1 mg/kg·min^{-1}, i.v.) was started and after 30 min the Ca^{2+} infusions were repeated.

Pigs with stunned myocardium

To evaluate the effect of EMD 57033 on the myocardial responsiveness to intracoronary Ca^{2+} during stunning, seven pigs received the intracoronary Ca^{2+} infusions before (baseline) and 30 min after a 15-min LADCA occlusion (stunning; Figure 1). Subsequently, the β-adrenoceptors were blocked by propranolol and after repeating the Ca^{2+} infusions (stunning + propranolol), the EMD 57033 infusion was started and 30 min later, the myocardial responsiveness to intracoronary Ca^{2+} was again determined (stunning + propranolol + EMD 57033).

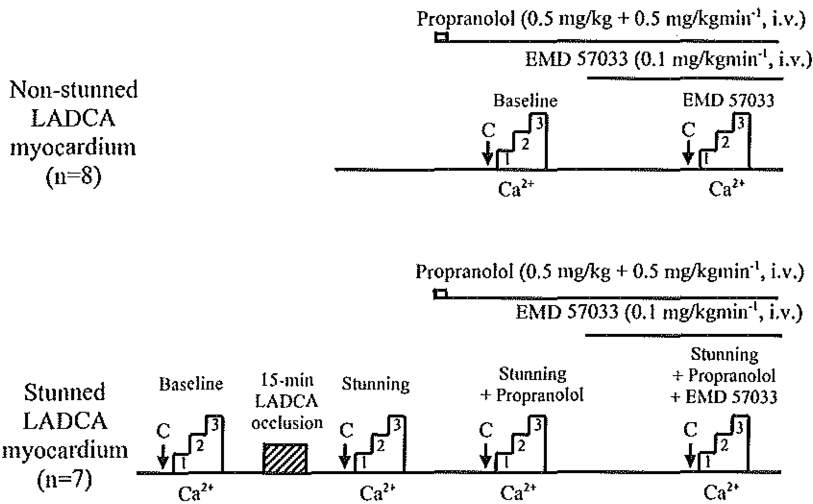

Figure 1 Experimental protocols for propranolol-treated pigs with non-stunned myocardium and pigs with regionally stunned myocardium. Stunning was produced by a 15-min left anterior descending coronary artery occlusion (LADCA) and 30 min of reperfusion. Three consecutive Ca^{2+} infusions into the LADCA were administered for 5 min each at rates of 18, 36 and 54 μmol/min, respectively. The arrows indicate the Control (C) measurements before the start of the Ca^{2+} infusions (Tables 1 and 2).

Data analysis

All data were digitized and stored for off-line analysis.[12] Segment length data were normalized to an end-diastolic length of 10 mm at baseline to correct for variability in the implantation distance between the various crystal pairs in the different animals. Regional left ventricular end-systolic elastance (E_{es}) was assessed using LVESP-segment area relations obtained from the relation between left ventricular pressure and the segment area encompassed by the two pairs of crystals. The slope of the LVESP-segment area relations (end-systolic elastance, E_{es}) and the zero pressure-area intercept (A_0) were determined via linear regression

analysis of the LVESP-segment area data points (Figure 2) which were obtained using an iterative method.[19] Data are mean±S.E.M. Statistical significance ($P<0.05$, two-tailed) of changes was determined using one-way or two-way analysis of variance. Post-hoc testing was performed using Dunnett's test.

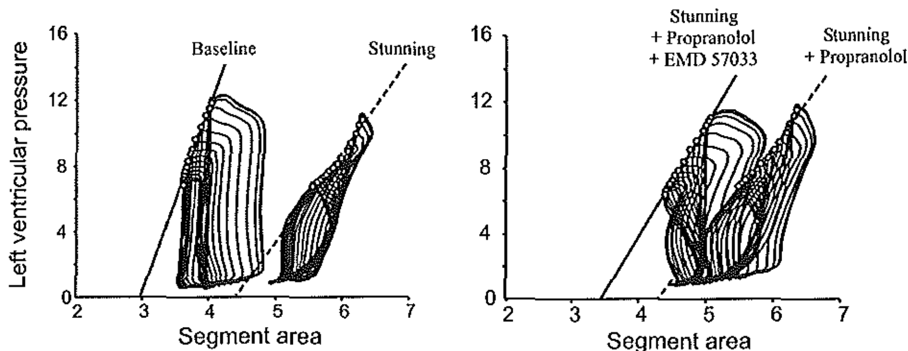

Figure 2 Example of LVESP-segment area relations during Baseline, Stunning, Stunning + Propranolol and Stunning + Propranolol + EMD 57033 in an individual pig in which the myocardium was stunned by a 15-min occlusion of the LADCA.

Results

Hemodynamics

Propranolol-treated pigs with non-stunned myocardium

In the propranolol-treated pigs there were no changes in any of the hemodynamic parameters during the intracoronary Ca^{2+} infusions (not shown), except for a dose-dependent increase in left ventricular dP/dt_{max} up to 17±4% ($P<0.05$). The infusion of EMD 57033, which was started after recovery from the Ca^{2+} infusions, increased left ventricular dP/dt_{max} by 28±8% and heart rate by 7±3% (both $P<0.05$; Table 1). The subsequent intracoronary Ca^{2+} infusions had again no hemodynamic effects (not shown), except for the increase in left ventricular dP/dt_{max} by 27±5% ($P<0.05$).

Pigs with stunned myocardium

When the β-adrenoceptors were still unblocked, the intracoronary Ca^{2+} infusions only increased left ventricular dP/dt_{max} (up to 17±4%, $P<0.05$). Production of myocardial stunning after recovery from the Ca^{2+} infusions, was accompanied by decreases in mean arterial blood pressure (9±3%), cardiac output (13±5%) and left ventricular dP/dt_{max} (18±5%), while heart rate and systemic vascular resistance remained unchanged (Table 1). Similar to the non-stunned myocardium, the intracoronary Ca^{2+} infusions increased left ventricular dP/dt_{max} dose-dependently by up to 13±5%, while the other parameters were not affected. In the presence of propranolol, infusion of Ca^{2+} into the stunned myocardium again affected only left ventricular dP/dt_{max} (24±5%). The subsequent infusion of EMD 57033 increased heart rate (4±2%),

cardiac output (21±8%) and left ventricular dP/dt_{max} (30±10%), while left ventricular end-diastolic pressure decreased slightly (all P<0.05). In the presence of EMD 57033 the only effects of the intracoronary Ca^{2+} infusions on hemodynamics were an increase in left ventricular dP/dt_{max} (27±3%, P<0.05) and a small decrease (12±2%, P<0.05) in mean arterial pressure.

Table 1 Effect of EMD 57033 on control values of global hemodynamics of propranolol-treated pigs with non-stunned myocardium and of pigs with stunned myocardium

	Propranolol-treated pigs with non-stunned myocardium (n=8)		Pigs with stunned myocardium (n=7)			
	Baseline	EMD 57033	Baseline	Stunning	Stunning + Propranolol	Stunning + Propranolol + EMD 57033
HR (bpm)	101±7	107±6[a]	106±6	109±5	91±5[c]	94±4[d]
MAP (mmHg)	86±4	83±6	94±4	85±4[b]	70±5[c]	76±4
CO (L/min)	2.7±0.2	2.9±0.3	3.3±0.3	2.8±0.3[b]	2.6±0.3	3.1±0.3[d]
SVR (mmHg/Lmin)	33±3	31±3	30±3	32±3	28±3[c]	25±2
$LVdP/dt_{max}$ (mmHg/s)	1360±80	1720±100[a]	1610±80	1320±70[b]	1010±100[c]	1270±90[d]
LVEDP (mmHg)	3.3±0.8	2.1±0.8	4.4±1.3	6.9±1.4[b]	7.3±1.5	5.0±1.5[d]
CBF (mL/min)	28±4	32±3	22±2	20±3	20±3	36±6[d]

HR, heart rate; MAP, mean arterial blood pressure; CO, cardiac output; SVR, systemic vascular resistance; $LVdP/dt_{max}$ maximal rate of rise in left ventricular pressure; LVEDP, left ventricular end diastolic pressure; CBF, coronary blood flow. Values are mean±S.E.M..[a]P<0.05 EMD 57033 vs Baseline; [b]P<0.05 Stunning vs Baseline; [c]P<0.05 Stunning + Propranolol vs Stunning; [d]P<0.05 Stunning + Propranolol + EMD 57033 vs Stunning + Propranolol.

Left ventricular end-systolic pressure-segment area relations

Propranolol-treated pigs with non-stunned myocardium

Infusion of Ca^{2+} caused a dose-dependent counter clockwise rotation of the LVESP-segment area relation of the myocardium perfused by the LADCA (Figure 3A), thereby almost doubling E_{es} (Figure 4A), without affecting A_o (27±3 and 29±2 mm^2 during control and during the highest Ca^{2+} infusion rate, respectively). After recovery from the Ca^{2+} infusions, the subsequent infusion of EMD 57033 more than doubled E_{es} (Table 2) and enhanced the Ca^{2+}-induced increments in E_{es} (Figures 3A and 4A) without affecting A_o (30±1 and 31±1 mm^2 during control and during the highest Ca^{2+} infusion rate, respectively).

The intravenous administration of EMD 57033 also caused a doubling of E_{es} in the myocardium perfused by the LCXCA (Table 2). However, in this segment the Ca^{2+} infusions into the LADCA before or during the EMD 57033 infusions altered neither E_{es} (P= 0.28 and P= 0.09; Figure 4B) nor A_o (P= 0.47 and P= 0.79, not shown). These results indicate that the Ca^{2+} infusions into the LADCA did not lead to a spillover of Ca^{2+} in the distribution area of the LCXCA and that the ongoing infusion of EMD 57033 itself did not further increase contractility during the Ca^{2+} infusions.

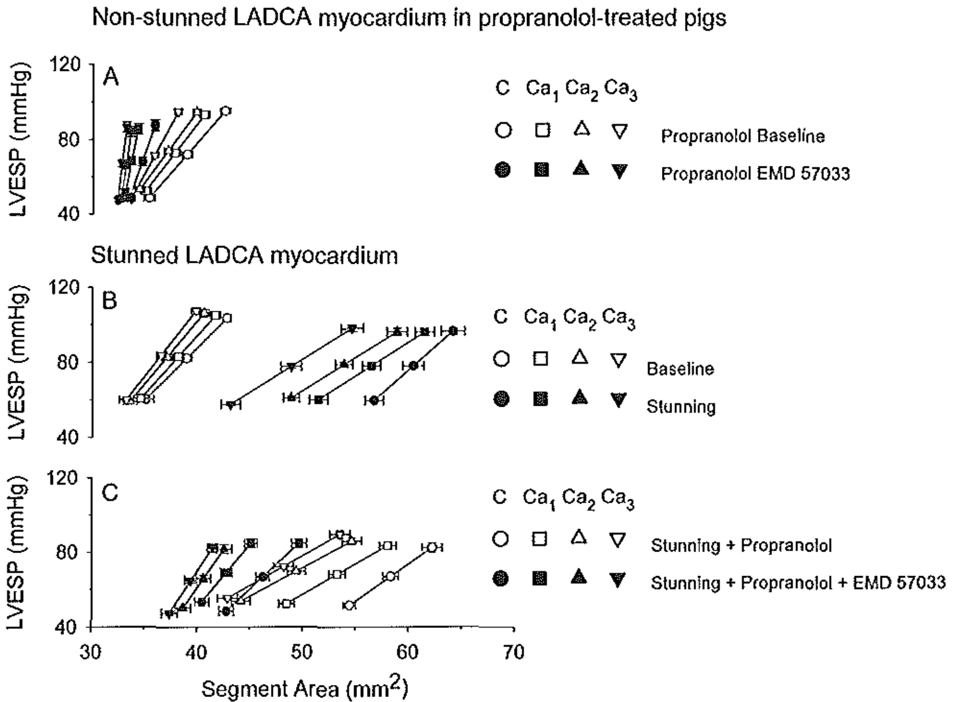

Figure 3. Effect of EMD 57033 (0.1 mg/kg•min⁻¹ i.v.) on the response to 3 Ca^{2+} infusions into the LADCA of the LVESP-segment area relation in the distribution area of the LADCA of non-stunned (panel A) and stunned (panels B and C) in vivo porcine myocardium. The 3 data points in each curve were derived from the linear LVESP-segment area relation by computing left ventricular end-systolic pressure at 3 segment areas within the range of actual measurements (minimum, maximum and [minimum + maximum] /2) for each animal. C = Control; Ca_1, Ca_2 and Ca_3 refer to the 3 Ca^{2+} infusion rates of 18, 36 and 54 µmol/min, respectively.

Pigs with stunned myocardium

Infusion of Ca^{2+} during baseline also caused a counter clockwise rotation of the LVESP-segment area relation (Figure 3B), thereby more than doubling E_{es} of the myocardium perfused by the LADCA (Figure 4C) without affecting A_0 (not shown). Myocardial stunning lowered E_{es} from 6.6±1.2 to 5.3±0.6 mmHg mm^{-2} (P>0.10), and caused a rightward shift of the LVESP-segment area relation reflected by the increase in A_0 from 24±4 to 44±2 mm^2 (P<0.05; Table 2). The Ca^{2+} infusions in the stunned myocardium caused a leftward shift of the LVESP-segment area relation (Figure 3C) reflected by a recovery of A_0 to 26±4 mm^2 (P<0.05). E_{es} was not significantly affected in the tested dose range of Ca^{2+}. The response of the LVESP-segment area relation of the stunned myocardium to the Ca^{2+} infusions was not modified by propranolol (Figures 3C and 4C). During the subsequent infusion of EMD 57033, E_{es} returned to baseline values, while A_0 recovered partly (Table 2). Importantly, the response of E_{es} to the Ca^{2+} infusions was restored to control levels (Figure 4C).

In the adjacent myocardium perfused by the LCXCA, neither E_{es} nor A_0 responded to the Ca^{2+} infusions into the LADCA before or after stunning the perfusion territory of the LADCA (Figure 4D), while E_{es} almost doubled during intravenous infusion of EMD 57033 (Table 2).

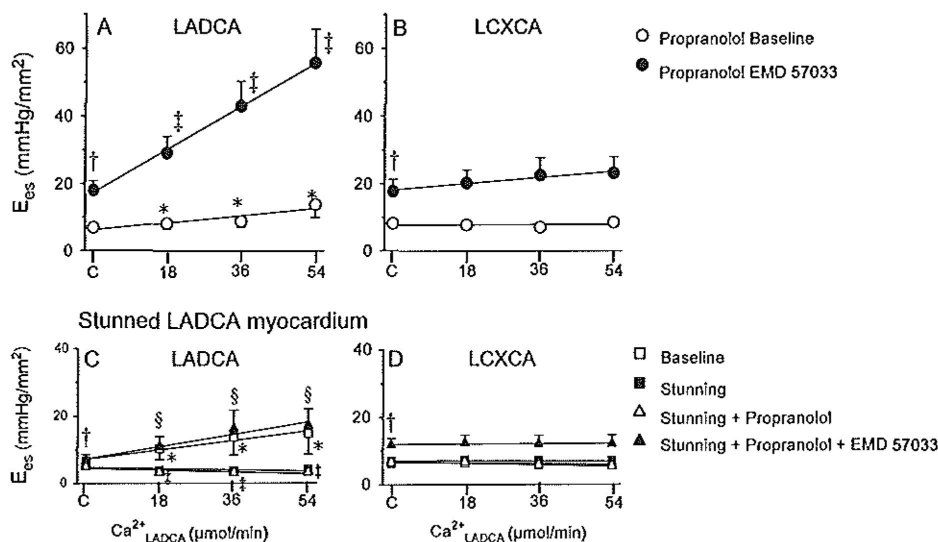

*Figure 4 Effect of EMD 57033 (0.1 mg/kg•min⁻¹ i.v.) on the response to 3 Ca^{2+} infusions into the LADCA of E_{es} in the distribution areas of the LADCA and the LCXCA of non-stunned (top panels) and stunned (bottom panels) in vivo porcine myocardium. C = Control; *$P<0.05$ Ca^{2+} vs Control (only for Baseline); †$P<0.05$ EMD 57033 vs Baseline (only for Control) or Stunning + Propranolol + EMD 57033 vs Stunning + Propranolol (only for Control); ‡$P<0.05$ Ca^{2+}-response significantly different from Ca^{2+}-response during Baseline; §$P<0.05$ Ca^{2+}-response significantly different from Ca^{2+}-response during Stunning + Propranolol.*

Discussion

In vivo assessment of myocardial responsiveness to Ca^{2+}

In analogy to the time varying elastance concept,[16] we employed the slope E_{es} of the LVESP-segment area relation to obtain a load-insensitive index of regional contractile function. A basic assumption for the determination of E_{es} is that during the preload reduction myocardial contractility (end-systolic stiffness of the myocardial tissue) does not change. For this reason, the preload reductions can only be studied during a period lasting no longer than 6-7 s[17] to avoid hypotension-induced reflex-mediated autonomic nervous system activity alterations. Consequently, the range over which the LVESP-segment area can be determined is limited. At a given level of contractility, the LVESP-segment area relation reflects that particular contractile state independent of whether the relation is constructed via alterations in

pre- or afterload. In other words, LVESP and end-systolic area are not independent but interrelated and are determined by the contractile state of the myocardium. Although alterations in diastolic function can modulate the diastolic elastance (and therefore, the left ventricular end-diastolic pressure-segment area relation), this should not affect the position of the LVESP-segment area relation, provided contractility is unchanged. However, we have previously noted that alterations in diastolic function (i.e. decrease in end-diastolic segment length) can produce translations in the LVESP-segment length relation. Thus, atrial pacing (which produces a decrease in end-diastolic segment length) also produces a leftward translation of the LVESP-segment length relation with no change in E_{es}.[20] Similarly, the Ca^{2+}-infusions in the present study also produced a leftward translation of the LVESP-segment area relation. These observations are difficult to explain but may be due to methodological limitations of the LVESP-regional segment area relations, which do not take into account the local left ventricular curvature (and hence, the radius) and wall thickness. For instance, at smaller segment areas, the wall thickness increases and radius decreases, which causes a decrease in regional wall stress that is larger than the decrease in left ventricular pressure. Consequently, it is possible that stress-strain relations would not show such a leftward shift.

Table 2 Effect of EMD 57033 on control values of E_{es} and A_0 of propranolol-treated pigs with non-stunned myocardium and of pigs with stunned myocardium.

	Propranolol-treated pigs with non-stunned myocardium (n=8)		Pigs with stunned myocardium (n=7)			
	Baseline	EMD 57033	Baseline	Stunning	Stunning + Propranolol	Stunning + Propranolol + EMD 57033
$E_{es, LADCA}$ (mmHg/mm^2)	6.9±0.9	18.3±2.8^a	6.6±1.2	5.3±0.6	5.0±0.9	6.8±1.8^d
$A_{0, LADCA}$ (mm^2)	27±3	30±1	24±4	44±2^b	41±3	33±3^d
$E_{es, LCXCA}$ (mmHg/mm^2)	8.1±1.2	18.0±3.5^a	6.5±0.8	7.1±1.2	6.6±1.4	11.8±2.0^d
$A_{0, LCXCA}$ (mm^2)	30±3	32±2	27±3	28±2	30±2	32±1

E_{es}, end-systolic elastance; A_0, area intercept at zero pressure of the LVESP-segment area relation. Values are mean±S.E.M.. ^{a}*P*<0.05 EMD 57033 vs Baseline; ^{b}*P*<0.05 Stunning vs Baseline; ^{c}*P*<0.05 Stunning + Propranolol vs Stunning; ^{d}*P*<0.05 Stunning + Propranolol + EMD 57033 vs Stunning + Propranolol.

Intracoronary Ca^{2+} infusions were employed to evaluate the myocardial responsiveness to added Ca^{2+}. As pointed out by Heusch et al.,[4] in *in vivo* experiments, the intracoronary infusion rates cannot be directly translated into myocardial intracellular Ca^{2+} concentrations. We chose to present the response of E_{es} as a function of Ca^{2+} infusion rates rather than added Ca^{2+} concentrations in the blood. Using added blood concentrations would have yielded identical conclusions, because firstly, coronary blood flows were very similar under control conditions and following stunning, while in the presence of EMD 57033, blood flows were higher. These higher blood flows would result in lower Ca^{2+} concentrations and, hence, the effect of EMD 57033 on Ca^{2+}-responsiveness may have been slightly underestimated. Another potential

concern is that intracoronary Ca^{2+} infusions may recruit different amounts of cytosolic Ca^{2+} (Ca^{2+}-induced Ca^{2+} release) in non-stunned myocardium under control conditions and in the presence of EMD 57033 or in stunned myocardium. However, *in vitro* studies have shown that EMD 57033 increases contractile force[21] without an effect on Ca^{2+} transients, indicating that the compound does not modulate activator Ca^{2+}, sarcoplasmic reticulum function or Ca^{2+}-induced Ca^{2+} release.[15,22] Also, several groups of authors have shown that the capacity of the sarcoplasmic reticulum for Ca^{2+} uptake and Ca^{2+} release is preserved in stunned myocardium,[2,23-25] and that, consequently, the Ca^{2+}-transients in stunned myocardium are not different from those in normal trabeculae.[1,26] Moreover, increases in the $[Ca^{2+}]_{out}$ produced identical increases in peak-systolic $[Ca^{2+}]_{in}$ in stunned and normal myocardial trabeculae,[1] lending further support to the concept that Ca^{2+}-induced Ca^{2+} release and sarcoplasmic reticulum function are unperturbed in stunned myocardium. Similar to other *in vivo* studies,[4,27,28] maximal Ca^{2+}-activated force could not be determined, because of the occurrence of arrhythmias and contracture-like phenomena (evidenced by marked reductions in end-diastolic segment length) and post-Ca^{2+}-infusion loss of regional contractile function at higher concentrations of intracoronary Ca^{2+}, which we observed in pilot experiments. In the present study, we used slightly lower Ca^{2+} concentrations as compared to previous studies[3,4,27,28] to facilitate detection of the putative Ca^{2+}-sensitizing properties of EMD 57033. Nevertheless, we observed in the normal myocardium that the intracoronary Ca^{2+}-infusions, in the dose-range tested, elicited relative increases in left ventricular dP/dt_{max} (10-20%) and segment shortening (13%, not presented) that were similar to the 15% increases in wall thickening,[3,27] external work[4] or segment shortening[28] and the 10-15% increases in left ventricular dP/dt_{max}[3,4,27,28] found in earlier studies.

Rationale for the dose of EMD 57033

To allow evaluation of the effect of EMD 57033 on the contractile response to the three 5-min intracoronary Ca^{2+} infusions, a steady state of hemodynamics and contractile function is mandatory during infusion of EMD 57033. In a previous study,[12] we observed that systolic shortening, as well as E_{es}, continued to increase throughout the 60 min infusion period of EMD 57033 at a rate of 0.2 $mg/kg{\cdot}min^{-1}$. Consequently, we selected a lower dose (0.1 $mg/kg{\cdot}min^{-1}$) and observed in the initial experiments that this dose was still effective as E_{es} in the myocardium perfused by the LCXCA increased from 8.1±1.2 to 18.0±3.5 mmHg mm^{-2} after 30 min of infusion, but did not further increase during the subsequent Ca^{2+} infusions into the LADCA (Figure 4), while the intravenous EMD 57033 infusion was continued. The latter observation precludes the necessity of a control group, in which Ca^{2+} infusions are replaced by vehicle to exclude the possibility that increases in E_{es} during the intracoronary Ca^{2+} infusions were caused by a progressive increase in contractility secondary to the continuous infusion of EMD 57033.

Effect of EMD 57033 on Ca^{2+}-responsiveness of non-stunned myocardium

Calcium-sensitizing agents are a heterogeneous class of drugs.[29-31] Thus, several of these agents have been shown to increase Ca^{2+}-sensitivity of the myofilaments *in vitro* by modifying the interaction between Ca^{2+} and Troponin C (e.g. sulmazole, levosimendan, pimobendan, MCI-154 and EMD 60263), by modifying the interaction between the various components of the thin filaments (e.g pimobendan, and MCI 154) or by altering actin-myosin crossbridge kinetics (EMD 57033). In addition to the Ca^{2+}-sensitizing properties, most of these agents also produce considerable PDE-III inhibitory activity (sulmazole, pimobendan, levosimendan and MCI-154). The exact mechanism by which EMD 57033 increases the Ca^{2+} sensitivity is still debated.[32] Solaro et al.[33] proposed that EMD 57033 principally acts at the actin-myosin site, where the compound reverses the inhibition of actin-myosin interactions by troponin-tropomyosin and may also promote transition of crossbridges from weak to strong force-generating states. On the other hand, Pan and Johnson[34] observed in an *in vitro* model of pure recombinant human cardiac TnC that EMD 57033 binds to the Ca^{2+}/Mg^{2+} sites of TnC.

In the present study, 0.1 mg/kg·min^{-1} EMD 57033 not only increased E_{es} but also enhanced the myocardial response to added Ca^{2+}. Thus, while E_{es} doubled (from 6.9±0.9 to 13.8±3.8 mmHg mm^{-2}) during the intracoronary Ca^{2+} infusions in the absence of EMD 57033, E_{es} tripled (from 18.3±2.8 to 55.7±10.0 mmHg mm^{-2}) during the Ca^{2+} infusions in the presence of EMD 57033, despite the higher control value during infusion of EMD 57033.

Several lines of evidence suggest that in the present *in vivo* study, the EMD 57033-induced systolic actions are principally the result of Ca^{2+}-sensitization, with a negligible contribution of PDE-III inhibition. In *in vitro* studies, varying dosages of EMD 57033 have been used. For instance, Grandis et al.[35] found in Langendorff perfused rat hearts that a dose of only 2 μM could already increase contractility without a significant change in MVO_2. Furthermore, Korbmacher et al.[10] used 30 μM EMD 57033 in isolated rabbit hearts and showed that at that concentration, EMD 57033 exerts its effect by both Ca^{2+}-sensitizing and PDE-III inhibitory properties. On the other hand, White et al.,[15] who used EMD 57033 in a dose range of 0.1-20 μM in isolated ferret cardiac muscle, reported that EMD 57033 acts predominantly by increasing myofilament Ca^{2+} sensitivity. Taken together, these *in vitro* studies suggest that PDE-III inhibition occurs principally at concentrations of EMD 57033 in excess of 20 μM. In our previous study [12] with EMD 57033 (0.2 mg/kg·min^{-1}), plasma levels increased time-dependently to 8, 12, 15 and 17 μM at 15, 30, 45 and 60 min infusion, respectively, which is below the *in vitro* PDE-III inhibiting threshold concentration of 20 μM. Since in the present study we infused 0.1 mg/kg·min^{-1}, this dose would therefore also not be expected to produce PDE-III inhibition. This is supported by the finding that the increases in both E_{es} and left ventricular dP/dt_{max} produced by 0.2 mg/kg·min^{-1} EMD 57033 (maximal plasma levels 17 μM) in normal myocardium were not altered by propranolol, suggesting minimal contribution of PDE-III inhibition to the positive inotropic actions of EMD 57033. In

contrast, we have previously shown that the same dose of propranolol virtually abolished the inotropic actions of the phosphodiesterase inhibitor / Ca^{2+}-sensitizer pimobendan.[36] Moreover, the duration of both global and regional left ventricular systole were not altered by EMD 57033 in the study by De Zeeuw et al.,[12] independent of the presence propranolol, indicating that a positive lusitropic effect of PDE-III inhibition was also unlikely. Taken together, the *in vivo* observation of an enhancement by EMD 57033 of the Ca^{2+}-induced increase in E_{es} is highly consistent with the *in vitro* observation that EMD 57033 increases myocardial contractile force via an increase in myofilament Ca^{2+} responsiveness.

Effect of EMD 57033 on Ca^{2+}-responsiveness of stunned myocardium

Myocardial stunning was characterized by a trend towards a decrease in E_{es} and a marked rightward shift of the LVESP-segment area relation. At first glance, this appears to be a surprising finding as most studies (including some from our own laboratory) have reported both a decrease in E_{es} and a rightward shift, although some studies have also reported a rightward shift as the most prominent feature of stunning.[37,38] The reason for the differences is unclear, but may be related to the range of pressures over which the LVESP-segment area relation was constructed. It is well known that the LVESP-segment length relation may be curvilinear and that the curvilinearity increases at higher end-systolic pressures that are obtained when increases in afterload are used.[39] For this reason, we used preload reductions to yield a pressure range of 40 mmHg over which good linearity was observed. A rightward shift of the LVESP-segment area relation is compatible with a decrease of elastic-restoring forces, probably induced by alterations in structural non-contractile elements, such as the extracellular collagen matrix and/or the cytoskeleton. However, as outlined under section "*In vivo assessment of myocardial responsiveness to Ca^{2+}*", the use of LVESP-segment area relations, rather than of left ventricular end-systolic stress-strain relations, may also have contributed to the marked rightward shift.

It is now generally accepted that the mechanism underlying myocardial stunning does not involve a decreased Ca^{2+} availability,[1,2,23,24] but a decreased responsiveness of the myofilaments to Ca^{2+}.[1,2] However, experimental support for this hypothesis is derived from studies in isolated muscle preparations, whereas evidence obtained in *in vivo* experiments is lacking. Thus, several groups of investigators have shown that in stunned myocardium, the response of systolic wall thickening[3], segment shortening,[27] or external work[4] to intracoronary Ca^{2+} infusions is not impaired. In two of these studies, Ca^{2+} infusions were used that produced maximal levels of systolic shortening[27] and external work[4] during control conditions, but whether these concentrations also resulted in maximum responses in stunned myocardium was not determined. Only Ito et al.[3] used Ca^{2+} doses that resulted in maximum responses of systolic shortening in both normal and stunned myocardium, and demonstrated an unperturbed maximum wall thickening in response to Ca^{2+} following stunning. Consistent with previous

studies, we observed a similar Ca^{2+}-induced increase in area reduction in normal and stunned myocardium. In contrast, however, in the dose-range tested, the Ca^{2+}-induced increases in E_{es} were depressed following stunning. These findings can be explained by a rightward shift of the [Ca^{2+}]-contractile force relations, i.e. a decrease in Ca^{2+} sensitivity. A decrease in Ca^{2+} sensitivity is supported by the study of Hofmann et al.[6] who showed a rightward shift of the [Ca^{2+}]-force relation with a maintained maximum Ca^{2+}-activated force. In addition, a decrease in maximum Ca^{2+}-activated force may also have contributed to the decreased Ca^{2+} responsiveness.[1]

An interesting observation in the present study was that the increase in E_{es} produced by EMD 57033 in stunned myocardium was less than in non-stunned myocardium. In our previous study, we also observed that at lower concentrations (12 μM), the effect of EMD 57033 on E_{es} was more pronounced in non-stunned than in stunned myocardium,[12] while at higher concentrations (17 μM) the E_{es} of non-stunned and stunned myocardium were no longer different. However, interpretation of these observations is difficult, because stunned myocardium perfused by the LADCA was compared to non-stunned myocardium perfused by the LCXCA, and hence, regional differences in contractile responses cannot be excluded. In the present study, we directly compared stunned myocardium to non-stunned myocardium of the same distribution area, which points, indeed, towards a reduced sensitivity of stunned myocardium to the actions of EMD 57033. This is also supported by observations by Korbmacher et al.[40] who found that non-stunned isolated rabbit hearts were also more sensitive to the Ca^{2+}-sensitizing effects of EMD 60263 than stunned hearts.

Both the response of E_{es} to EMD 57033 as well as the response of E_{es} to the Ca^{2+} infusions in the presence of EMD 57033, were less in stunned than in non-stunned LADCA perfused myocardium. However, infusion of 0.1 mg/kg·min^{-1} of EMD 57033 not only increased E_{es} of stunned myocardium (from 5.0±0.9 vs 6.9±0.9 mmHg mm^{-2}), but also restored its response to the Ca^{2+} infusions to baseline levels. These findings are consistent with a decreased myofilament Ca^{2+} responsiveness of stunned myocardium, and a restoration of myofilament Ca^{2+} responsiveness by EMD 57033. It is very well possible that the use of a higher dose of EMD 57033 in stunned myocardium that would have resulted in an increase in E_{es} comparable to the level of E_{es} produced by 0.1 mg/kg·min^{-1} in non-stunned myocardium (i.e. 18.3±2.8 mmHg mm^{-2}) would also result in comparable responses to intracoronary Ca^{2+}.

Conclusions

EMD 57033 enhanced the myocardial responsiveness to intracoronary added Ca^{2+} in non-stunned and stunned myocardium which supports the concept, based on *in vitro* observations, that EMD 57033 increases myocardial contractility via an increase in myofilament Ca^{2+}-responsiveness, and that myofilament Ca^{2+}-responsiveness of stunned myocardium is decreased, but can be restored by EMD 57033.

Acknowledgements

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References

1. Gao WD, Atar D, Backx PH, Marban E. Relationship between intracellular calcium and contractile force in stunned myocardium. Direct evidence for decreased myofilament Ca^{2+} responsiveness and altered diastolic function in intact ventricular muscle. *Circ Res.* 1995;76:1036-1048.
2. Bolli R, Marban E. Molecular and cellular mechanisms of myocardial stunning. *Physiol Rev.* 1999;79:609-634.
3. Ito BR, Tate H, Kobayashi M, Schaper W. Reversibly injured, postischemic canine myocardium retains normal contractile reserve. *Circ Res.* 1987;61:834-846.
4. Heusch G, Rose J, Skyschally A, Post H, Schulz R. Calcium responsiveness in regional myocardial short-term hibernation and stunning in the *in situ* porcine heart. Inotropic responses to postextrasystolic potentiation and intracoronary calcium. *Circulation.* 1996;93:1556-1566.
5. Fan D, Soei LK, Sassen LM, Krams R, Verdouw PD. Mechanical efficiency of stunned myocardium is modulated by increased afterload dependency. *Cardiovasc Res.* 1995;29:428-437.
6. Hofmann PA, Miller WP, Moss RL. Altered calcium sensitivity of isometric tension in myocyte-sized preparations of porcine postischemic stunned myocardium. *Circ Res.* 1993;72:50-56.
7. Ravens U, Himmel HM, Fluss M, Davia K, Harding SE. Phosphodiesterase inhibition and Ca^{2+} sensitization. *Mol Cell Biochem.* 1996;157:245-249.
8. Bezstarosti K, Soei LK, Verdouw PD, Lamers JM. Phosphorylation by protein kinase C and the responsiveness of Mg^{2+}- ATPase to Ca^{2+} of myofibrils isolated from stunned and non-stunned porcine myocardium. *Mol Cell Biochem.* 1997;176:211-218.
9. Soei LK, Sassen LM, 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.
10. Korbmacher B, Sunderdiek U, Arnold G, Schulte HD, Schipke JD. Improved ventricular function by enhancing the Ca^{++} sensitivity in normal and stunned myocardium of isolated rabbit hearts. *Basic Res Cardiol.* 1994;89:549-562.
11. Abe Y, Kitada Y, Narimatsu A. Effect of a calcium-sensitizing positive inotropic agent MCI-154 and its combined use with enalapril on postischemic contractile dysfunction of dog hearts. *J Cardiovasc Pharmacol.* 1995;26:653-659.
12. de Zeeuw S, Trines SA, Krams R, Verdouw PD, Duncker DJ. Cardiovascular profile of the calcium sensitizer EMD 57033 in open- chest anaesthetized pigs with regionally stunned myocardium. *Br J Pharmacol.* 2000;129:1413-1422.
13. Hajjar RJ, Gwathmey JK. Calcium-sensitizing inotropic agents in the treatment of heart failure: a critical view. *Cardiovasc Drugs Ther.* 1991;5:961-965.
14. Soei LK, de Zeeuw S, Krams R, Duncker DJ, Verdouw PD. Ca^{2+} sensitization and diastolic function of normal and stunned porcine myocardium. *Eur J Pharmacol.* 1999;386:55-67.
15. White J, Lee JA, Shah N, Orchard CH. Differential effects of the optical isomers of EMD 53998 on contraction and cytoplasmic Ca^{2+} in isolated ferret cardiac muscle. *Circ Res.* 1993;73:61-70.
16. Suga H. Ventricular energetics. *Physiol Rev.* 1990;70:247-277.
17. Aversano T, Maughan WL, Hunter WC, Kass D, Becker LC. End-systolic measures of regional ventricular performance. *Circulation.* 1986;73:938-950.
18. Wolffenbuttel BH, Verdouw PD. Nifedipine and myocardial performance in the presence and absence of beta-blockade with propranolol. *Arch Int Pharmacodyn Ther.* 1983;266:83-92.
19. van der Velde ET, Burkhoff D, Steendijk P, Karsdon J, Sagawa K, Baan J. Nonlinearity and load sensitivity of end-systolic pressure-volume relation of canine left ventricle *in vivo. Circulation.* 1991;83:315-327.
20. Krams R, Duncker DJ, McFalls EO, Hogendoorn A, Verdouw PD. Dobutamine restores the reduced efficiency of energy transfer from total mechanical work to external mechanical work in stunned porcine myocardium. *Cardiovasc Res.* 1993;27:740-747.
21. Lues I, Beier N, Jonas R, Klockow M, Haeusler G. The two mechanisms of action of racemic cardiotonic

EMD 53998, calcium sensitization and phosphodiesterase inhibition, reside in different enantiomers. *J Cardiovasc Pharmacol.* 1993;21:883-892.

22. Ferroni C, Hano O, Ventura C, Lakatta EG, Klockow M, Spurgeon H, Capogrossi MC. A novel positive inotropic substance enhances contractility without increasing the Ca^{2+} transient in rat myocardium. *J Mol Cell Cardiol.* 1991;23:325-331.
23. Kaplan P, Hendrikx M, Mattheussen M, Mubagwa K, Flameng W. Effect of ischemia and reperfusion on sarcoplasmic reticulum calcium uptake. *Circ Res.* 1992;71:1123-1130.
24. Lamers JM, Duncker DJ, Bezstarosti K, McFalls EO, Sassen LM, Verdouw PD. Increased activity of the sarcoplasmic reticular calcium pump in porcine stunned myocardium. *Cardiovasc Res.* 1993;27:520-524.
25. Duncker DJ, Schulz R, Ferrari R, Garcia-Dorado D, Guarnieri C, Heusch G, Verdouw PD. "Myocardial stunning" remaining questions. *Cardiovasc Res.* 1998;38:549-558.
26. Carrozza JP, Jr., Bentivegna LA, Williams CP, Kuntz RE, Grossman W, Morgan JP. Decreased myofilament responsiveness in myocardial stunning follows transient calcium overload during ischemia and reperfusion. *Circ Res.* 1992;71:1334-1340.
27. Buffington CW, Rothfield KP. Effects of intracoronary calcium chloride on the postischemic heart in pigs. *Ann Thorac Surg.* 1995;59:1448-1455.
28. Crystal GJ, Zhou X. Nitric oxide does not modulate the increases in blood flow, O_2 consumption, or contractility during $CaCl_2$ administration in canine hearts. *Cardiovasc Res.* 1999;42:232-239.
29. Haikala H, Linden IB. Mechanisms of action of calcium-sensitizing drugs. *J Cardiovasc Pharmacol.* 1995;26:S10-S19.
30. Endoh M. Changes in intracellular Ca^{2+} mobilization and Ca^{2+} sensitization as mechanisms of action of physiological interventions and inotropic agents in intact myocardial cells. *Jpn Heart J.* 1998;39:1-44.
31. Teramura S, Yamakado T. Calcium sensitizers in chronic heart failure: inotropic interventions- reservation to preservation. *Cardiologia.* 1998;43:375-385.
32. Lee JA, Allen DG. Calcium sensitisers: mechanisms of action and potential usefulness as inotropes. *Cardiovasc Res.* 1997;36:10-20.
33. Solaro RJ, Gambassi G, Warshaw DM, Keller MR, Spurgeon HA, Beier N, Lakatta EG. Stereoselective actions of thiadiazinones on canine cardiac myocytes and myofilaments. *Circ Res.* 1993;73:981-990.
34. Pan BS, Johnson RG, Jr. Interaction of cardiotonic thiadiazinone derivatives with cardiac troponin C. *J Biol Chem.* 1996;271:817-823.
35. Grandis DJ, DelNido PJ, Koretsky AP. Functional and energetic effects of the inotropic agents EMD-57033 and BAPTA on the isolated rat heart. *Am J Physiol.* 1995;269:C472-C479.
36. Duncker DJ, Hartog JM, Levinsky L, Verdouw PD. Systemic haemodynamic actions of pimobendan (UD-CG 115 BS) and its O- demethylmetabolite UD-CG 212 Cl in the conscious pig. *Br J Pharmacol.* 1987;91:609-615.
37. Stahl LD, Aversano TR, Becker LC. Selective enhancement of function of stunned myocardium by increased flow. *Circulation.* 1986;74:843-851.
38. Krams R, Janssen M, Van der Lee C, Van Meegen J, De Jong JW, Slager CJ, Verdouw PD. Loss of elastic recoil in postischemic myocardium induces rightward shift of the systolic pressure-volume relationship. *Am J Physiol.* 1994;267:H1557-H1564.
39. Miller WP. Effect of altered contractility on the linearity of regional left ventricular end-systolic relations in intact hearts. *Am Heart J.* 1994;128:114-123.
40. Korbmacher B, Sunderdiek U, Selcan G, Arnold G, Schipke JD. Different responses of non-ischemic and post-ischemic myocardium towards Ca^{2+} sensitization. *J Mol Cell Cardiol.* 1997;29:2053-2066.

New Insights into cardioprotection by ischemic preconditioning and other forms of stress

6

New insights into cardioprotection by ischemic preconditioning and other forms of stress

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Ischemic preconditioning has not only received wide attention in heart research, but has also been a topic of extensive studies involving other organs. In several of these studies, it has been shown that in spite of differences in the endpoints used to assess protection, the same mediators as in myocardial ischemic preconditioning may be involved. However, several of the putative mediators do not require ischemia to become activated. This has guided us and others to investigate whether the myocardium can also be protected by brief ischemia in other organs and whether other non-pharmacological forms of stress, which do not produce ischemia but are capable of activating these potential mediators, are also cardioprotective. ***(Ann NY Acad Sci. 1998;874:178-191)***

Introduction

In the past, numerous attempts involving pharmacological agents have been undertaken to limit myocardial infarct size after a coronary artery occlusion. Although several compounds showed initially some promise, in the long run the results have been rather disappointing. In 1985, Murry amd colleagues[1] revived interest in cardioprotection when they showed that after four days of reperfusion, myocardial infarcts in dogs produced by a 45-min coronary artery occlusion were 75% smaller (29% versus 7%) when this 45-min coronary occlusion was preceded by four sequences of a 5-min coronary artery occlusion and a 5-min reperfusion. This phenomenon of protecting myocardium against irreversible damage during a period of sustained ischemia by preceding periods of brief ischemia has been termed ischemic preconditioning. The smaller infarct size observed after such an extended period of reperfusion was most encouraging as in most of the earlier studies the infarct size limitation proved only to be a delay in the development of infarct size and ultimate infarct size was not affected. Subsequent studies not only confirmed the occurrence of ischemic preconditioning in a large number of animal species, but also revealed that the time course of protection exhibited (at least in some species) a biphasic pattern.[2,3] Thus, while in all animal species studied, ischemic preconditioning triggered an early phase of cardioprotection lasting up to two hours, it could be shown that in some species there was a second window of protection 24 hours later that usually lasted a few days. This second window of protection against infarct size has remained controversial, however, as several groups of investigators failed to show its existence.[4,5] Reasons may be numerous and could include such factors as the intensity and the duration of the preconditioning stimulus, the duration of the infarct-producing coronary artery occlusion, the time interval between the preconditioning stimulus and the sustained coronary artery occlusion, the area at risk, the surgical preparation and the anesthetic regimen employed. When demonstrated, the protection during the second window usually proved to be considerably less than during the early phase and this smaller magnitude of protection could have contributed to the failure to observe a significant infarct size limitation in some of the negative studies. Furthermore, in a recent study Miki and colleagues[6] subjected instrumented conscious rabbits to a 30-min coronary artery occlusion and determined infarct size by either triphenyltetrazolium chloride (TTC) staining after 3 h of reperfusion or by histology after 72 h of reperfusion. Using TTC staining, they found that four cycles of 5-min coronary artery occlusion and 10-min reperfusion preceding the 30-min coronary artery occlusion by 24 h resulted in smaller infarct sizes (33% of the risk zone compared to 45% in control animals), whereas the infarct sizes of the preconditioned animals and the control animals were almost identical (57% versus 59%) when they were determined with histology. The authors hypothesized that increased levels of myocardial superoxide dismutase, which have been implicated in the mechanism of the second window of protection (not in the early protection), may cause necrotic tissue to be seen as viable tissue when TTC staining is performed.[6] They

therefore conclude that the existence of a second window of protection might be based on the failure of TTC staining to measure total infarct size.

Despite numerous efforts, the mechanisms underlying the protection by both early (classical) and delayed (second window) ischemic preconditioning are still incompletely understood. Nevertheless a large number of potential mediators, such as adenosine receptors, norepinephrine, activation of (isoenzymes of) protein kinase C, which all ultimately lead to activation of ATP-sensitive potassium channels, have been suggested to be involved in both phases of protection.[7-13]

Ischemic preconditioning has not only received wide attention in heart research, but has also been a topic of extensive studies involving other organs such as brain[14-16], kidney[17,18], skeletal muscle[19] and liver.[20] In several of these studies, it has been shown that despite differences in the endpoint used to assess protection the same mediators as in myocardial ischemic preconditioning may be involved. However, several of the putative mediators do not require ischemia to become activated. This has guided us and others to investigate whether the myocardium can also be protected by brief ischemia in other organs and whether other (not pharmacological) forms of stress that do not produce ischemia, but are capable of activating these potential mediators are also cardioprotective.

Cardioprotection by Non-Ischemic Stress

The first to describe that regional myocardium can become preconditioned without prior local ischemia were Przyklenk and colleagues[21] when they determined infarct size in anesthetized dogs subjected to a 60-min occlusion of the left anterior descending coronary artery (LAD). They observed that when the LAD-occlusion was preceded by four episodes of 5-min left circumflex coronary artery (LCX) occlusion and 5 min of reperfusion, infarct sizes were limited to the same extent as after local ischemic preconditioning. An explanation for this finding was not given but the authors suggested that this could involve factors that were produced or activated during ischemia and/or reperfusion and transported throughout the heart. If true, this could also imply that ischemia in other organs might produce or activate the same factors which could then be transported to the heart, and thereby protect the myocardium. Conversely, it is also feasible that myocardial ischemia could precondition other organs, an option that to our knowledge, has not yet been investigated. In a subsequent study of the same group,[22] it was shown that in the same canine model stretch produced by left atrial infusion of saline also limited infarct size in dogs subjected to a 60-min coronary artery occlusion. Volume loading was chosen such that it caused a 10-15% increase in end-diastolic segment length, which is similar to that observed in the study by Przyklenk and colleagues[21] in which the LAD distribution area was preconditioned by brief periods of left circumflex coronary artery occlusion. Because stretch activates ion channels located in the sarcolemma,[23] which appear to be selective for cations but are rather non-selective for K^+, Na^+ or Ca^{2+}, and because

these channels can be blocked by gadolinium,[24] Ovize and colleagues[22] investigated whether this compound affected the protection by stretch. They proved that pretreatment with gadolinium blocked the protection by stretch and attenuated the protection by ischemic preconditioning. Unfortunately, the investigators did not study the effect of gadolinium on the protection of the remote myocardium observed in the study of Przyklenk and colleagues,[21] as this might have led to an insight into the mechanism by which ischemia in the left circumflex coronary artery-perfused myocardium protected the adjacent virgin myocardium of the LAD distribution territory. In a follow up study, Gysembergh and colleagues[25] not only showed that volume loading was also as effective as ischemic preconditioning in protecting myocardium in anesthetized rabbits, but more importantly, showed that pretreatment with glibenclamide also abolished the protection by stretch, thereby suggesting that stretch ultimately protects the myocardium through activation of K^{+}_{ATP} channels. Although these studies suggest a common mechanism for the protection by stretch and by early ischemic preconditioning, there have been no studies investigating whether there is also a second window of protection by stretch. It is equally unknown if the time course of the protection of the remote myocardium in the study by Przyklenk and colleagues shows a biphasic pattern.[21]

Adenosine has been repeatedly found to play a major role in the mechanism underlying ischemic preconditioning. There are, however, several maneuvers that do not produce ischemia, but cause adenosine levels to increase. Thus Saito and colleagues[26] observed increased myocardial adenosine concentrations during exercise, while Watkinson and colleagues[27] found that adenosine concentrations in the pericardial fluid increased, with increasing workloads during treadmill exercise in dogs and finally Hall and colleagues[28] have reported that in pigs an increase in workload in the absence of ischemia was accompanied by an increase in interstitial adenosine concentrations. Nevertheless, Marber and colleagues[29] failed to show a protective effect of a single 5-min period of rapid atrial pacing against myocardial infarction in the rabbit heart. On the other hand, transient rapid ventricular pacing has been reported to protect against the occurrence of ventricular arrhythmias (a still controversial endpoint in ischemic preconditioning studies) during a subsequent coronary artery occlusion. However, the authors concluded that based on the ST-segment changes occurring during the pacing period, the pacing-induced protection was secondary to ischemia.[30] We have reported that in anesthetized pigs, infarct size after a 60-min coronary artery occlusion was less when the occlusion was preceded by 30-min ventricular pacing at a rate of 200 beats /min.[31] Assessment of ventricular performance during and after pacing by a number of metabolic, perfusion and functional parameters such as myocardial ATP and phosphocreatine levels, energy charge, and regional wall function data, revealed that pacing at this rate did *not* lead to ischemia. In that study, as with ischemic preconditioning, protection by ventricular pacing was abolished after pretreatment with the non-selective ATP-sensitive potassium channel blocker glibenclamide. Surprisingly, however, administration of

glibenclamide during the intermittent period of normal sinus rhythm between the period of ventricular pacing and the 60-min coronary artery occlusion did not abolish the protection by ventricular pacing. In a more recent study, Domenech and colleagues[32] reported on a series of similar experiments performed in anesthetized dogs. In that study arterial blood pressure was controlled at 80-90 mmHg to avoid effects of changes in hemodynamics during the five sequences of ventricular tachycardia at 213±12 cycles/min and the subsequent infarct-producing coronary artery occlusion. Based on the coronary flow reserve during the pacing period, it was also excluded that the period of tachycardia caused myocardial ischemia. Ventricular pacing again protected the myocardium, but this protection was abolished by treatment with the non-selective adenosine blocker 8-phenyltheophylline (8PT), independent of whether its administration occurred before or after the periods of tachycardia, suggesting a mechanism that is similar to ischemic preconditioning. Using microdialysis, the authors also showed that the tachycardia stimulus indeed increased interstitial adenosine concentrations twofold, and that in the preconditioned animals concentrations continued to rise during the first 40 min of ischemia, but started to decline during the last 20 min of ischemia and returned to baseline during the first hour of reperfusion. Because dialysis data were not obtained in the other series of experiments, it is unknown how treatment with 8PT, either before or after the intermittent pacing periods, affected the interstitial adenosine concentrations during and after the coronary artery occlusion. It is also of interest that Domenech and colleagues[32] did not find any changes in cytosolic or particulate protein kinase C (PKC) activity of translocation of the α, β, ε and ξ-protein kinase C isoenzymes, suggesting that (isoenzyme) protein kinase C activity or translocation does not play a role in the tachycardia-induced protection. These data on activity and translocation of isoenzymes of PKC are in agreement with unpublished studies from our laboratory, in which we also could not show any changes in the activity or translocation of the α- and ε-isoenzymes.

Although, both studies[31,32] suggest that similar mechanisms appear to be underlie the cardioprotection by ventricular tachycardia and ischemic preconditioning, the degree of protection by ventricular tachycardia was in both studies found to be less than with ischemic preconditioning. If only activation of adenosine receptors is involved in the mechanism underlying the protection by ischemic preconditioning these observations are difficult to explain as the increase in the interstitial adenosine concentrations during ventricular tachycardia observed in the study by Domenech and colleagues[32] should be sufficient to maximally activate adenosine A_1 receptors.[33]

Another difference in the protection by ventricular pacing and ischemic preconditioning relates to the time course of protection, as the protection by ventricular pacing appears to be more ephemeral than the first window of protection by ischemic preconditioning. Furthermore, while a second window of protection against the occurrence of ventricular arrhythmias has

been demonstrated after ventricular pacing,[34,35] no study has so far addressed whether ventricular pacing also produces a second window of protection against infarction.

The conclusions regarding the magnitude of protection and the mechanisms by which ventricular pacing and ischemic preconditioning protect the myocardium in both *in vivo* studies[31,32] have been challenged by Hearse and colleagues[36] employing isolated blood-perfused paced rat hearts. In this model ischemic preconditioning and rapid pacing produced, in contrast with the *in vivo* studies, a similar improvement of postischemic function at 40 min of reperfusion after 35-min ischemia as assessed by left ventricular developed pressure. The duration of the ischemic period and the time point of the postischemic measurements raise the question whether the results may have been the consequence of a mixture of stunning and irreversible damage. Hearse and colleagues[36] concluded that the mechanisms of protection are different because rapid pacing did not, but ischemic preconditioning did, decrease tissue levels of high energy phosphates. This conclusion is premature, however, in view of the involvement of K^{+}_{ATP} channels in protection by ischemic preconditioning and pacing in our studies.[31,37]

Myocardial infarct size limitation by ischemic stress in other organs

The first to report that brief ischemia in organs other than the heart itself could protect the heart during a coronary artery occlusion were McClanahan and colleagues,[38] who showed that a brief renal artery occlusion followed by reperfusion preceding a 45-min coronary artery occlusion limited myocardial infarct size in rabbits. These preliminary data led us to investigate in detail whether a 15-min occlusion of either renal artery or the mesenteric artery applied 10 minutes before a 60-min coronary artery occlusion also limited myocardial infarct size in anesthetized rats.[39,40] Since body temperature may influence infarct size and interventions may be protective only in combination with hypothermia, we studied animals under normothermic (36.5°C-37.5°C) and mild hypothermic (30°C-31°C) conditions. Table 1 shows that a 15-min mesenteric artery occlusion 10 min before the 60-min coronary artery occlusion limited myocardial infarct size in both temperature ranges, while brief renal ischemia was only effective in the hypothermic range. Pretreatment with the ganglion blocker hexamethonium abolished the protection by the transient mesenteric artery occlusion, but the protection by classical ischemic preconditioning was not affected. When the 60-min coronary artery occlusion was repeated in the presence of a permanent mesenteric artery occlusion, infarct size was not different from the control group, strongly suggesting that a mediator released upon reperfusion of the mesenteric artery may be involved. The reason we failed to observe protection by the renal artery occlusion at normothermia is unclear. Other groups of investigators have also found positive results with brief renal ischemia in rabbits, and suggest the involvement of adenosine in the protection.[41,42] Preliminary data by Pell and colleagues[42] obtained in rabbits preconditioned with a 10-min renal artery occlusion and 10-min reperfusion before a 30-min coronary artery occlusion suggest that brief renal ischemia and

preconditioning by local myocardial ischemia protect the myocardium by similar mechanisms as pretreatment with both the non-selective adenosine blocker 8-(*p*-sulphophenyl) theophylline and the ATP-sensitive K^+ channel blocker 5-hydroxydecanoate abolished the protection by either mode of protection.

Table 1 Effect of remote organ ischemia on infarct size produced by 60-min 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)				
Shem+60-min CAO	31±4 (*N*=11)	36±4 (*N*=11)	68±2	67±3
15-min CAO + 10 min Rep + 60 min CAO	47±4 (*N*=9)	40±5 (*N*=8)	50±3^a	22±3^{a,b}
15-min MAO + 10 min Rep + 60 min CAO	42±4 (*N*=10)	41±3 (*N*=11)	50±3^a	44±5^a
15-min RAO + 10 min Rep + 60 min CAO	35±8 (*N*=8)	37±2 (*N*=9)	72±5	46±6^{a,b}
Protocol II				
(After ganglion blockade)				
Shem+60-min CAO	37±5 (*N*=7)	35±3 (*N*=7)	68±3	67±3
15-min CAO + 10 min Rep + 60 min CAO	45±3 (*N*=7)	35±2 (*N*=7)	54±3^a	18±4^{a,b}
15-min MAO + 10 min Rep + 60 min CAO	40±3 (*N*=7)	37±3 (*N*=7)	74±2	69±3
Protocol III				
Permanent MAO + 60-min CAO	36±4 (*N*=6)	34±2 (*N*=8)	70±3	63±3

Sham, control group undergoing 60-min CAO without stimulus; CAO, coronary artery occlusion; MAO, mesenteric artery occlusion; RAO, renal artery occlusion; Rep, reperfusion. Permanent MAO started 25 min before the onset of 60-min CAO and was maintained until the end of the 3-h reperfusion period. Data are mean±SEM. a $p<0.05$ versus control. b $p<0.05$ versus corresponding 36.5-37.5 °C group. (After Gho et al[40])

Since ischemia produced by a partial coronary artery occlusion can also precondition the myocardium,[43-46] Birnbaum and colleagues[47] investigated whether a 55-65% reduction in femoral artery blood flow for 30 min and a 30-min stimulation of the gastrocnemius muscle (at a rate of one pulse per second for 20 msec with a stimulus of 9V) could also limit infarct size produced by a 30-min coronary artery occlusion. The results revealed that if performed alone, neither of these two interventions limited myocardial infarct size when compared to the infarct size of a group of control animals (26 ± 3%). However, when both maneuvers were combined, infarct size was limited to 9 ± 2%. At variance with abrupt brief renal and mesenteric artery occlusion and reperfusion, the combination of flow restriction in the femoral artery and stimulation of the gastrocnemius muscle may have a clinical analog in patients with peripheral vascular disease. This investigation therefore emphasizes the need for further studies exploring the possibilities of how the myocardium can be preconditioned by transient ischemia outside the heart.

In this respect the influence of cerebral ischemia on the heart may be of special interest. This topic has already been the subject of many studies because donor hearts for transplantation are often obtained from patients with brain death. In several of these studies it has been noted that brain death leads to severe impairment in myocardial contractility and even to irreversible myocardial damage.[48,49] In the experimental animal, cell damage is usually produced by increasing intracranial pressure (ICP). Shivalkar and colleagues[50] have shown that rapid, rather than gradual, increases in ICP (producing a 1,000–fold and a 200–fold increase in epinephrine levels, respectively) cause irreversible myocardial damage. In recent studies, the same group of investigators has shown that sympathetic withdrawal rather than myocardial damage, if present at all, is responsible for the changes in hemodynamic profile after brain death in rats.[51,52] In contrast to the influence of prolonged cerebral ischemia on myocardial function and structure, the effect of brief cerebral ischemia on the former has not been studied. It is of interest, however, that the brain can be preconditioned by brief cerebral ischemia, but in contrast to the heart, the protection is observed only 2-5 days after the preconditioning stimulus, a time interval that resembles that of the second window of protection in myocardial ischemic preconditioning.[16]

Hypothermia

The effect of body temperature on the development of infarction after a coronary artery occlusion has been the topic of several investigations in recent years. For instance, Chien and colleagues[53] reported a steep relation between body core temperature in the range of 35°C - 42°C and infarct size after a 30 min-coronary artery occlusion for rabbits. Thus a decrease in temperature of 1°C resulted in 12% less infarction of the area at risk, with no infarction occurring at a body core temperature of 34.5°C. An even more temperature sensitive relation was found by Duncker and colleagues[54] when they subjected anesthetized pigs to a 45-min coronary artery occlusion. Similarly, Schwartz and colleagues[55] also observed that in dog hearts the infarct size after a 60-min coronary artery occlusion was modulated by epicardial temperature. Because we did not observe a difference in infarct size when we subjected anesthetized rats to a 60-min coronary artery occlusion at body temperature of 31°C and 37°C, we determined in anesthetized rats infarct size after coronary artery occlusions of different duration.[56] Figure 1 shows that in rats infarct sizes are smaller at lower body temperatures, provided that the coronary artery is occluded for less than 60 minutes. For coronary artery occlusions exceeding 60 minutes, infarct size development was complete at both body temperatures and no beneficial effect of hypothermia was noticeable. In all the aforementioned studies the total body was cooled and cooling was started before the coronary artery occlusion. In order to assess whether regional myocardial hypothermia also affected infarct size and the importance of the timing of the onset of regional cooling, Hale and colleagues[57,58] studied infarct sizes in rabbits in which the entire anterior portion of the heart was cooled using a bag

containing ice and water. This procedure permitted a local temperature reduction of approximately 6°C within 5 minutes. These studies showed that regional cooling can be protective even when started 10 min after the onset of ischemia and that changes in hemodynamic parameters are not responsible for the protection. As in man infarct size development is slower than in rabbits, it could very well be that the delay in the onset of cooling with respect to the onset of the development of infarction may be longer than 10 minutes. This approach of regional cooling may therefore have therapeutic value if the procedure can be done safely and without surgical intervention in patients developing an infarction.

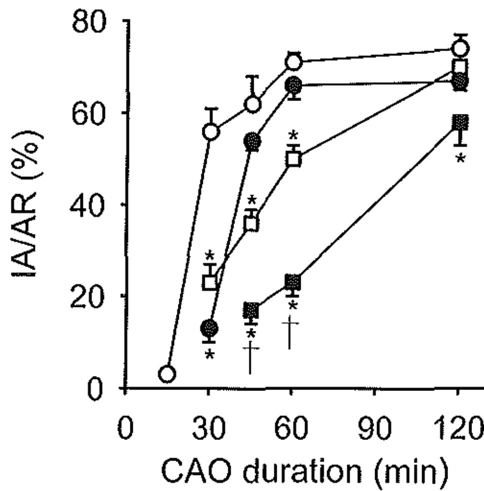


*Figure 1 Effect of mild hypothermia and ischemic preconditioning alone and in combination on infarct size (expressed as percentage of area at risk, IA/AR) produced by coronary artery occlusions of different duration. Notice that the slopes of the relation between infarct size (IA/AR) and the duration of the coronary artery occlusion (CAO) is less steep and linear (over the CAO range tested) in the preconditioned animals and that combined hypothermia and ischemic preconditioning were also protective for CAO's of 120 min. ○=normothermia (36.5-37.5°C); ●=hypothermia (30-31°C); □=preconditioning at normothermia; ■=preconditioning at hypothermia; IA=infarcted area; AR=area at risk. Data are mean ± SEM. *p<0.05 vs ○ at corresponding CAO duration. =p<0.05 vs □ at corresponding CAO duration.* (Reproduced with permission of *Cardiovascular Research.*)

To investigate whether the protection by ischemic preconditioning also depends on the body temperature, we determined infarct sizes after 3 hours of reperfusion following coronary artery occlusions varying from 15 min to 120 min which were preceded by an ischemic preconditioning stimulus consisting of a 15-min coronary artery occlusion and 10-min reperfusion.[56] Figure 1 shows that ischemic preconditioning had a protective effect at both temperatures. Furthermore, the steep parts of the sigmoid curves describing the relation

between infarct size and coronary artery occlusion are less steep for the preconditioned animals (at 37°C) than for the hypothermic animals, suggesting a different mechanism of action for ischemic preconditioning and lowering of temperature. Figure 1 also shows that the hypothermic animals still benefit from ischemic preconditioning and that the duration of the coronary artery occlusion at which there is still infarct limitation can be expanded to 120 minutes when hypothermia and ischemic preconditioning are combined.

Hypothermia and ischemic preconditioning have also been combined in an isolated perfused rat heart model by Lu and colleagues.[59] After preconditioning with a single 5-min period of ischemia and 10-min reperfusion, hearts were arrested by a 4°C St Thomas Hospital cardioplegic solution for 3 hours and then reperfused by a 37°C Krebs-Henseleit buffer solution for 45 min. The authors observed that creatine kinase activity in the effluent was higher when preconditioning was performed at 31°C than at 37°C and therefore concluded that hypothermia during preconditioning attenuated the myocardial protection of preconditioning. However control groups for either the hypothermic and normothermic preconditioning experiments were lacking in this study. Furthermore, the reperfusion period lasted only 45 min and it can therefore not be excluded that the lesser recovery in the hypothermic group reflected only a delay of recovery. Nevertheless, the data by Lu and colleagues[59] are of interest as they point toward a potential temperature-dependent effect of the ischemic preconditioning stimulus. Such an effect is also supported by the observations of Dote and colleagues[60] who reported that an ischemic preconditioning stimulus at 25°C was less effective than an ischemic preconditioning stimulus at 38°C in protecting against infarction produced by a 45-min coronary artery occlusion at 38°C. These findings suggest that in our study[56] an ischemic preconditioning stimulus at 37°C could have protected the myocardium more during the 60-min coronary artery occlusion at 31°C than the preconditioning stimulus at 31°C.

Can pathological hearts be preconditioned?

In all ischemic preconditioning studies, but also in all studies employing stimuli which do not lead to ischemia, infarct-size limitation had been investigated in animals with normal healthy hearts, until Speechly-Dick and colleagues[61] reported that ischemic preconditioning also occurred in hypertrophied rabbit hearts.

It is also of interest that Ferdinandy and colleagues[62] have shown that the protection by ventricular pacing could not be demonstrated in rats that had been fed a high cholesterol diet for 24 weeks. One should also keep in mind that the authors evaluated their preconditioning protocol with rapid ventricular pacing in a setting of a 10-min coronary artery occlusion in an isolated rat heart preparation and that evaluation was therefore based on recovery of performance rather than infarct size. A major reason for the failure to observe a protective effect in the hypercholesterolemic animals may be that an improved recovery of the evaluated parameters involves myocardial production of nitric oxide.[62] Because that same group of

investigators also showed that ischemic preconditioning was lost in rabbits fed a high cholesterol diet,[63] such data strongly suggest that the capability of the myocardium to adapt to several forms of stress may be impaired under pathological conditions.

In contrast to the negative studies,[62-64] Iliodromitis and colleagues[65] have recently shown that ischemic preconditioning with one cycle of 5-min ischemia and 10-min reperfusion was equally effective in normocholesterolemic as in hypercholesterolemic (0.2% cholesterol rich diet for 8 weeks caused cholesterol increase from 58 ± 9 mg% to 1,402 ± 125 mg%) rabbits in limiting infarct size produced by 30-min ischemia. Similarly, Cokkinos and colleagues[66] reported that in isolated hearts obtained from hyperthyroid rats (L-thyroxine administration for 2 weeks causing an increase in left ventricular mass of 15%) recovery of left ventricular developed pressure was similar (38%) in control and hyperthyroid hearts after 20-min ischemia and 45-min reperfusion. After preconditioning with two periods of no flow ischemia, recovery of function improved in both animals to the same extent (59% and 69%, respectively). The currently available data must, however, be interpreted with caution as it can not yet be excluded that in the negative studies only the time course of protection may be shifted or that more intense stress or stress of a longer duration is still capable of eliciting a cardioprotective response. It is, however, equally possible that under certain pathological conditions more severe stress or stress of the longer duration preceding a sustained coronary artery occlusion may prove to be deleterious rather than protective.

Conclusions

The evidence that myocardium can be protected against the development of irreversible damage is increasing. Local ischemic myocardial preconditioning is the most potent endogenous stimulus, but transient ischemia in remote organs is and non-pharmacological stimuli that do not lead to ischemia have also been proven to be cardioprotective. A major question remains whether cardioprotection can still be shown in models that mimic more closely the pathological conditions in man. Major efforts should therefore be directed to the study of ischemic and non-ischemic preconditioning in more pathological models. In earlier studies we have shown that partial coronary artery occlusions are capable of preconditioning normal myocardium without an intermittent reperfusion period between the partial and complete occlusion,[45,46] analogous to the two-stage Harris model.[67] In these studies we used complete reperfusion after release of the infarct producing 60-min coronary artery occlusion. Kapadia and colleagues[68] have extended these studies by showing that myocardium can also be preconditioned in the presence of a permanent stenosis, which is thus also present during the reperfusion period. Studies in hypercholesterolemic animals, in particular when they have moderate to severe coronary artery lesions, and in animals with hypertrophied and failing hearts are another step forward in assessing whether ischemic preconditioning or other stimuli may be protective in man. A major reason for undertaking such studies is that we believe that

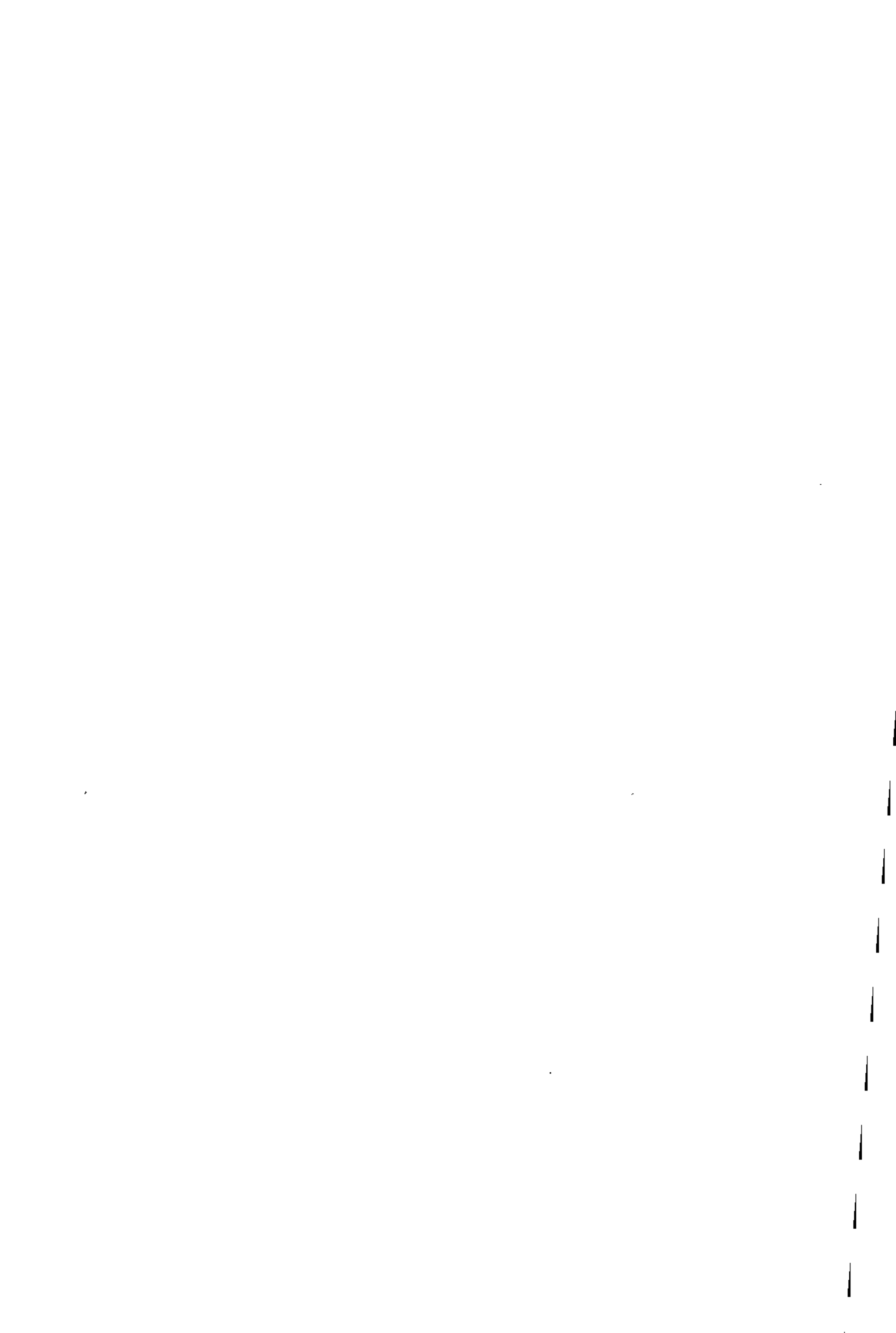
the definitive proof of ischemic preconditioning in man cannot be achieved,[69,70] because of the numerous uncontrolled and confounding factors. If the human myocardium can protect itself against the development of irreversible damage, one must also realize that this could be a confounding factor when one wants to evaluate the salvaging effects of therapeutic interventions such as thrombolysis.

References

1. Murry CE, Jennings RB, Reimer KA. Preconditioning with ischemia: a delay of lethal cell injury in ischemic myocardium. *Circulation*. 1986;74:1124-1136.
2. Kuzuya T, Hoshida S, Yamashita N, Fuji H, Oe H, Hori M, Kamada T, Tada M. Delayed effects of sublethal ischemia on the acquisition of tolerance to ischemia. *Circ Res*. 1993;72:1293-1299.
3. Marber MS, Latchman DS, Walker JM, 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.
4. Qiu Y, Tang XL, Park SW, Sun JZ, Kalya A, Bolli R. The early and late phases of ischemic preconditioning: a comparative analysis of their effects on infarct size, myocardial stunning, and arrhythmias in conscious pigs undergoing a 40-minute coronary occlusion. *Circ Res*. 1997;80:730-742.
5. 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.
6. Miki T, Swafford AN, Cohen MV, Downey JM. Second window of protection in conscious rabbits: real or artifactual. *J Mol Cell Cardiol*. 1998;30:285.
7. Yellon DM, Baxter GF. A "second window of protection" or delayed preconditioning phenomenon: future horizons for myocardial protection? *J Mol Cell Cardiol*. 1995;27:1023-1034.
8. Meng X, Brown JM, Ao L, Banerjee A, Harken AH. Norepinephrine induces cardiac heat shock protein 70 and delayed cardioprotection in the rat through $alpha_1$ adrenoceptors. *Cardiovasc Res*. 1996;32:374-383.
9. Light PE, Sabir AA, Allen BG, Walsh MP, French RJ. Protein kinase C-induced changes in the stoichiometry of ATP binding activate cardiac ATP-sensitive K^+ channels. A possible mechanistic link to ischemic preconditioning. *Circ Res*. 1996;79:399-406.
10. Baxter GF, Goma FM, Yellon DM. Involvement of protein kinase C in the delayed cytoprotection following sublethal ischaemia in rabbit myocardium. *Br J Pharmacol*. 1995;115:222-224.
11. Lawson CS, Downey JM. Preconditioning: state of the art myocardial protection. *Cardiovasc Res*. 1993;27:542-550.
12. Brooks G, Hearse DJ. Role of protein kinase C in ischemic preconditioning: player or spectator? *Circ Res*. 1996;79:627-630.
13. Gho BCG, Eskildsen-Helmond YEG, De Zeeuw S, Lamers JMJ, Verdouw PD. Does protein kinase C play a pivotal role in the mechanisms of ischemic preconditioning? *Cardiovasc Drugs Ther*. 1996;10:775-786.
14. Kitagawa K, Matsumoto M, Tagaya M, Hata R, Ueda H, Niinobe M, Handa N, Fukunaga R, Kimura K, Mikoshiba K, et al. 'Ischemic tolerance' phenomenon found in the brain. *Brain Res*. 1990;528:21-24.
15. Kawai K, Nakagomi T, Kirino T, Tamura A, Kawai N. Preconditioning *in vivo* ischemia inhibits anoxic long-term potentiation and functionally protects CA_1 neurons in the gerbil. *J Cereb Blood Flow Metab*. 1998;18:288-296.
16. Chen J, Simon R. Ischemic tolerance in the brain. *Neurology*. 1997;48:306-311.
17. 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.
18. Islam CF, Mathie RT, Dinneen MD, Kiely EA, Peters AM, Grace PA. Ischaemia-reperfusion injury in the rat kidney: the effect of preconditioning. *Br J Urol*. 1997;79:842-847.
19. Pang CY, Neligan P, Zhong A, He W, Xu H, Forrest CR. Effector mechanism of adenosine in acute ischemic preconditioning of skeletal muscle against infarction. *Am J Physiol*. 1997;273:R887-R895.
20. Peralta C, Closa D, Hotter G, Gelpi E, Prats N, Rosello-Catafau J. Liver ischemic preconditioning is mediated by the inhibitory action of nitric oxide on endothelin. *Biochem Biophys Res Commun*. 1996;229:264-270.
21. 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.
22. Ovize M, Kloner RA, Przyklenk K. Stretch preconditions canine myocardium. *Am J Physiol*. 1994;266:H137-H146.

23. Guharay F, Sachs F. Stretch-activated single ion channel currents in tissue-cultured embryonic chick skeletal muscle. *J Physiol.* 1984;352:685-701.
24. Yang XC, Sachs F. Block of stretch-activated ion channels in Xenopus oocytes by gadolinium and calcium ions. *Science.* 1989;243:1068-1071.
25. Gysembergh A, Margonari H, Loufoua J, Ovize A, Andre-Fouet X, Minaire Y, Ovize M. Stretch-induced protection shares a common mechanism with ischemic preconditioning in rabbit heart. *Am J Physiol.* 1998;274:H955-H964.
26. Saito D, Nixon DG, Vomacka RB, Olsson RA. Relationship of cardiac oxygen usage, adenosine content, and coronary resistance in dogs. *Circ Res.* 1980;47:875-882.
27. Watkinson WP, Foley DH, Rubio R, Berne RM. Myocardial adenosine formation with increased cardiac performance in the dog. *Am J Physiol.* 1979;236:H13-H21.
28. Hall JL, Van Wylen DG, Pizzurro RD, Hamilton CD, Reiling CM, Stanley WC. Myocardial interstitial purine metabolites and lactate with increased work in swine. *Cardiovasc Res.* 1995;30:351-356.
29. 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.
30. Vegh A, Szekeres L, Parratt JR. Transient ischaemia induced by rapid cardiac pacing results in myocardial preconditioning. *Cardiovasc Res.* 1991;25:1051-1053.
31. Koning MM, Gho BC, van Klaarwater E, Opstal RL, Duncker DJ, Verdouw PD. Rapid ventricular pacing produces myocardial protection by nonischemic activation of K^{+}_{ATP} channels. *Circulation.* 1996;93:178-86.
32. Domenech RJ, Macho P, Velez D, Sanchez G, Liu X, Dhalla N. Tachycardia preconditions infarct size in dogs: role of adenosine and protein kinase C. *Circulation.* 1998;97:786-794.
33. Londos C, Cooper DM, Schlegel W, Rodbell M. Adenosine analogs inhibit adipocyte adenylate cyclase by a GTP- dependent process: basis for actions of adenosine and methylxanthines on cyclic AMP production and lipolysis. *Proc Natl Acad Sci U S A.* 1978;75:5362-5366.
34. Vegh A, Papp JG, Parratt JR. Prevention by dexamethasone of the marked antiarrhythmic effects of preconditioning induced 20 h after rapid cardiac pacing. *Br J Pharmacol.* 1994;113:1081-1082.
35. Kaszala K, Vegh A, Papp JG, Parratt JR. Time course of the protection against ischaemia and reperfusion-induced ventricular arrhythmias resulting from brief periods of cardiac pacing. *J Mol Cell Cardiol.* 1996;28:2085-2095.
36. Hearse DJ, Ferrari R, Sutherland FJ. Preconditioning by ischemia, rapid pacing and ventricular fibrillation: studies of catecholamines and high energy phosphates. *J Mol Cell Cardiol.* 1998;30:A16.
37. Rohmann S, Weygandt H, Schelling P, Kie Soei L, Verdouw PD, Lues I. Involvement of ATP-sensitive potassium channels in preconditioning protection. *Basic Res Cardiol.* 1994;89:563-576.
38. 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.
39. Verdouw PD, Gho BC, Koning MM, Schoemaker RG, Duncker DJ. Cardioprotection by ischemic and nonischemic myocardial stress and ischemia in remote organs. Implications for the concept of ischemic preconditioning. *Ann N Y Acad Sci.* 1996;793:27-42.
40. Gho BC, Schoemaker RG, van den Doel MA, Duncker DJ, Verdouw PD. Myocardial protection by brief ischemia in noncardiac tissue. *Circulation.* 1996;94:2193-2200.
41. Takaoka A, Nakae I, Mitsunami K, Yabe T, Morikawa S, Inubushi T, Kinoshita M. Renal ischemia/reperfusion remotely improves myocardial energy metabolism during myocardial ischemia via adenosine receptors in rabbits: effects of "remote preconditioning". *J Am Coll Cardiol.* 1999;33:556-564.
42. Pell TJ, Baxter GF, Yellon DM, Drew GM. Renal ischemia preconditions myocardium: role of adenosine receptors and ATP-sensitive potassium channels. *Am J Physiol.* 1998;275:H1542-H1547.
43. Ovize M, Kloner RA, Hale SL, Przyklenk K. Coronary cyclic flow variations "precondition" ischemic myocardium. *Circulation.* 1992;85:779-789.
44. Iwamoto T, Bai XJ, Downey HF. Preconditioning with supply-demand imbalance limits infarct size in dog heart. *Cardiovasc Res.* 1993;27:2071-2076.
45. Koning MM, Simonis LA, de Zeeuw S, Nieukoop S, Post S, Verdouw PD. Ischaemic preconditioning by partial occlusion without intermittent reperfusion. *Cardiovasc Res.* 1994;28:1146-1151.
46. Koning MM, Gho BC, 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.
47. Birnbaum Y, Hale SL, Kloner RA. Ischemic preconditioning at a distance: reduction of myocardial infarct size by partial reduction of blood supply combined with rapid stimulation of the gastrocnemius muscle in the rabbit. *Circulation.* 1997;96:1641-1646.

48. Galinanes M, Hearse DJ. Brain death-induced impairment of cardiac contractile performance can be reversed by explantation and may not preclude the use of hearts for transplantation. *Circ Res.* 1992;71:1213-1219.
49. Shanlin RJ, Sole MJ, Rahimifar M, Tator CH, Factor SM. Increased intracranial pressure elicits hypertension, increased sympathetic activity, electrocardiographic abnormalities and myocardial damage in rats. *J Am Coll Cardiol.* 1988;12:727-736.
50. Shivalkar B, Van Loon J, Wieland W, Tjandra-Maga TB, Borgers M, Plets C, Flameng W. Variable effects of explosive or gradual increase of intracranial pressure on myocardial structure and function. *Circulation.* 1993;87:230-239.
51. Herijgers P, Borgers M, Flameng W. The effect of brain death on cardiovascular function in rats. Part I. Is the heart damaged? *Cardiovasc Res.* 1998;38:98-106.
52. Herijgers P, Flameng W. The effect of brain death on cardiovascular function in rats. Part II. The cause of the *in vivo* haemodynamic changes. *Cardiovasc Res.* 1998;38:107-115.
53. Chien GL, Wolff RA, Davis RF, van Winkle DM. "Normothermic range" temperature affects myocardial infarct size. *Cardiovasc Res.* 1994;28:1014-1017.
54. Duncker DJ, Klassen CL, Ishibashi Y, Herrlinger SH, Pavek TJ, Bache RJ. Effect of temperature on myocardial infarction in swine. *Am J Physiol.* 1996;270:H1189-H1199.
55. Schwartz LM, Verbinski SG, Vander Heide RS, Reimer KA. Epicardial temperature is a major predictor of myocardial infarct size in dogs. *J Mol Cell Cardiol.* 1997;29:1577-1583.
56. Van den Doel MA, Gho BC, Duval SY, Schoemaker RG, Duncker DJ, Verdouw PD. Hypothermia extends the cardioprotection by ischaemic preconditioning to coronary artery occlusions of longer duration. *Cardiovasc Res.* 1998;37:76-81.
57. Hale SL, Kloner RA. Myocardial temperature in acute myocardial infarction: protection with mild regional hypothermia. *Am J Physiol.* 1997;273:H220-H227.
58. Hale SL, Dave RH, Kloner RA. Regional hypothermia reduces myocardial necrosis even when instituted after the onset of ischemia. *Basic Res Cardiol.* 1997;92:351-357.
59. Lu EX, Ying GL, Guo X. Hypothermia during preconditioned ischemia-reperfusion attenuates the myocardial protection of preconditioning. *J Thorac Cardiovasc Surg.* 1997;114:514-516.
60. Dote K, Wolff RA, Van Winkle DM. Hypothermia during transient antecedent ischemia attenuates preconditioning. *FASEB J.* 1996;10:A36.
61. Speechly-Dick ME, Baxter GF, Yellon DM. Ischaemic preconditioning protects hypertrophied myocardium. *Cardiovasc Res.* 1994;28:1025-1029.
62. Ferdinandy P, Szilvassy Z, Horvath LI, Csont T, Csonka C, Nagy E, Szentgyorgyi R, Nagy I, Koltai M, Dux L. Loss of pacing-induced preconditioning in rat hearts: role of nitric oxide and cholesterol-enriched diet. *J Mol Cell Cardiol.* 1997;29:3321-3333.
63. Szilvassy Z, Ferdinandy P, Szilvassy J, Nagy I, Karcsu S, Lonovics J, Dux L, Koltai M. The loss of pacing-induced preconditioning in atherosclerotic rabbits: role of hypercholesterolaemia. *J Mol Cell Cardiol.* 1995;27:2559-2569.
64. Ferdinandy P, Csont T, Szilvassy Z, Tosaki. A, Dux L. Inhibition of HMG-CoA reductase, similarly to hypercholesterolemia, inhibits preconditioning in rat hearts. *J Mol Cell Cardiol.* 1998;30:A77.
65. Iliodromitis EK, Bofilis E, Kaklamanis L, Papalois A, Kremastinos DT. Precondtioning in hypercholesterolemic rabbits *in vivo*. *J Mol Cell Cardiol.* 1998;30:A76.
66. Cokkinos DD, Pantos C, Tzeis S, Malliopoulou V, Karageorgiou C, Davos C, Varanos D, Cokkinos DV. Ischemic precondtioning protect against myocardial dysfunction caused by ischeamia in isolated thyroxine induced hypertrophied rat hearts. *J Mol Cell Cardiol.* 1988;30:A76.
67. Harris AS. Delayed development of ventricular ectopic rhythms following experimental coronary occlusion. *Circulation.* 1950;1:1318-1328.
68. Kapadia SJ, Terlato JS, Most AS. Presence of a critical coronary artery stenosis does not abolish the protective effect of ischemic preconditioning. *Circulation.* 1997;95:1286-1292.
69. Verdouw PD, van den Doel MA, de Zeeuw S, Duncker DJ. On the relevance of ischemic preconditioning in humans. *Basic Res Cardiol.* 1997;92:51-53.
70. Verdouw PD, Gho BC, Duncker DJ. Ischaemic preconditioning: is it clinically relevant? *Eur Heart J.* 1995;16:1169-1176.



Involvement of both adenosine A_1 and A_3 receptors in ischemic preconditioning in rats

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Involvement of both Adenosine A_1 and A_3 receptors in ischemic preconditioning in rats

Background The role of adenosine in ischemic preconditioning has been confirmed in several species such as rabbits, dogs, swine and human. However, in the rat heart its role is not clear as several studies show controversial evidence using adensine antagonists. This could be explained by the finding that during myocardial ischemia interstitial adenosine levels are higher in rats compared to other species. We therefore studied the role of adenosine receptors in an *in vivo* rat model using both low and high dose of 8-SPT.

Methods and Results Area at risk (AR) and infarcted area (IA) were determined in anesthetized rats after 180 min of reperfusion following a 60-min coronary artery occlusion (CAO). In control rats IA/AR was 70±2%. Ischemic preconditioning by a single 15-min CAO (PC) decreased IA/AR to 47±4% (P<0.05 vs control). The adenosine receptor antagonist 8-SPT had no effect on infarct size by itself (IA/AR = 74±1%) and blunted the protection by PC at a dose of 10 mg/kg i.v. (IA/AR = 59±2%, P<0.05 vs PC), whereas 50 mg/kg i.v. of 8-SPT abolished the protection (IA/AR = 70±1%, P<0.05 vs PC). The selective adenosine A_3 agonist IB-MECA administered prior to the 60-min CAO limited IS (49±4%, *P*<0.05 vs control) to the same extent as ischemic preconditioning. The protection by adenosine A_3 receptor stimulation, however, was partly blocked by high dosage of 8-SPT (62±2%, *P*<0.05 vs IB-MECA), but not by low dosage of 8-SPT (56±2%, *P*=0.157), suggesting that 8-SPT also blocks adenosine A_3 receptors at high dose.

Conclusions 8-SPT dose-dependently inhibited the cardioprotection by ischemic preconditioning indicating that adenosine is involved in ischemic preconditioning in the rat heart *in vivo* via A_1 receptor stimulation. The higher dose of 8-SPT abolished the protection by IP and blunted the cardioprotection by IB-MECA, suggesting that the A_3 receptor also contributes to ischemic preconditioning.

Introduction

Since the original observation by Downey and coworkers[1] in an *in vivo* rabbit model, that the nonselective adenosine receptor blockers 8-SPT and PD115,199 were able to abolish the cardioprotection afforded by ischemic preconditioning, the role of adenosine in ischemic preconditioning has also been confirmed in other species such as dogs[2] and swine.[3,4]

The rabbit has been extensively used for studies on the role of adenosine and has shown that different adenosine receptor subtypes like A_1 and A_3 are involved. Agonists selective for adenosine A_1 (rPIA[5,6] and CCPA[6]) or A_3 (APNEA[7,8] and IB-MECA[9]) receptors showed infarct size reduction to the same extent as with ischemic preconditioning in both *in vitro* and *in vivo* studies. Moreover, in isolated rabbit myocytes ischemic preconditioning could only be partially blocked by the adenosine A_1 selective antagonist DPCDX, but was completely blocked by the combination of DPCPX with either adenosine A_1/A_3 antagonist BWA1433 or 8-SPT, indicating that both adenosine A_1 and A_3 receptors are involved.[10]

In the rat heart, however, evidence for the role on adenosine receptors in ischemic preconditioning is still incompletely understood. In this species cardioprotection can be induced by adenosine or selective A_1 receptor agonists CCPA[11,12] or rPIA.[13] However, Li et al[14] failed to observe a blunting of the protective effect of ischemic preconditioning *in vivo* by the adenosine receptor antagonist 8-SPT at a concentration of 10 mg/kg. Other investigators also failed to abolish ischemic preconditioning using the non-selective adenosine receptor blockers 8-SPT[15] or PD115,199.[11] It is possible that blockade of adenosine receptors was inadequate because not all receptor types, for example adenosine A_3 receptors, were blocked. Another possible explanation is that the interstitial level of the antagonist is not high enough to block all receptors, which is supported by the *in vitro* study of Headrick.[16] In that study it was shown that during myocardial ischemia interstitial adenosine levels rose 6-fold to ~2 μM in isolated rabbit hearts, whereas they rose 27-fold to ~7 μM in isolated rat hearts. Using a higher concentration of 8-SPT, Headrick was able to block the protective effect of ischemic preconditioning. Taken into account the K_i value of 8-SPT for A_1 and A_3 receptors (K_i (A_1) = 2600 nM, K_i (A_3) = 10000 nM) it is likely that Headrick, using a higher dose, also blocked the A_3 receptors in addition to the A_1 receptors. Thus, suggesting that adenosine A_3 receptors may also be involved in ischemic preconditioning.

In view of these considerations, we investigated the role of the adenosine A_1 and A_3 receptors in ischemic preconditioning in an *in vivo* rat model. Taking into account the higher interstitial levels of ADO in the rat heart, we used both a low (10 mg/kg) and a high concentration (50 mg/kg) of 8-SPT during ischemic preconditioning. Since high doses of 8-SPT may also block adenosine A_3 receptors, additional experiments were performed using the selective adenosine A_3 agonist IB-MECA with and without 8-SPT.

Methods

All experiments were performed in accordance with the Guiding Principles in the Care and Use of Laboratory Animals, as approved by the American Physiological Society and with prior approval of the Animal Care Committee of the Erasmus University Rotterdam.

Surgical and experimental procedures

Male Wistar rats weighing approximately 300 g, fed ad libitum, were anesthetized with pentobarbital (60 mg/kg) and intubated for positive pressure ventilation (Harvard rodent ventilator, Hilliston, Mass, USA) with room air. Through the carotid artery a PE-50 catheter was positioned in the thoracic aorta for measurement of arterial blood pressure and heart rate. In the inferior caval vein a PE-50 catheter was placed for infusion of Haemaccel (Behringwerke AG, Marburg, Germany) to compensate for blood loss during surgery. After thoracotomy via the left third intercostal space and opening of the pericardium, a silk 6-0 suture was looped under the coronary artery for later production of coronary artery occlusion. Following laparotomy, a catheter was positioned in the abdominal cavity to allow intraperitoneal infusion of pentobarbital for maintenance of anesthesia.

Rectal temperature was continuously measured with an electronic thermometer (Electromedics Inc.) and was maintained at 36.5-37.5 °C by either heating pads or ice-filled packages. Except during production of coronary artery occlusion and reperfusion, the thoracotomy site was covered with aluminum foil to prevent heat loss from the thoracic cavity. In an earlier study[17] we verified the adequacy of this procedure in five rats in which simultaneous measurements of rectal and intrathoracic temperature showed no differences at baseline and at the end of a 60-min coronary artery occlusion.

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

Experimental protocols

All protocols were performed at a body temperature of 36.5-37.5 °C. In all animals the hearts were subjected to a 60-min coronary artery occlusion (CAO) followed by 180 min of reperfusion (Rep) (Fig 1). In the control groups, animals received either no pretreatment or pretreatment with the H_1 antagonist mepyramine (MEP) or the non-selective adenosine antagonist 8-(*p*-Sulfophenyl)theophylline (8-SPT) to exclude an effect of these agents on infarct size. In groups 4,5 and 6, ischemic preconditioning was produced by a 15-min CAO followed by 10 min of Rep before the 60-min CAO. To investigate the contribution of adenosine receptors in ischemic preconditioning groups 5 and 6 were pretreated with a low (10

mg/kg) and high (50 mg/kg) dose of the non-selective adenosine antagonist 8-SPT, respectively. In groups 7 and 8, the effect of A_3 receptor stimulation on infarct size was studied using the selective A_3 agonist N^6-(3-iodobenzyl)adenosine-5'-N-methyluronamide (IB-MECA, 100 μg/kg). Since infusion of IB-MECA induced a marked decrease in arterial blood pressure, which has been ascribed to release of histamine from mast cells,[18] the animals in group 8 were pretreated with the H_1-antagonist mepyramine (5 mg/kg). In groups 9 and 10 the selectivity of 8-SPT was tested by infusion of 10 or 50 mg/kg of 8-SPT before administration of IB-MECA.

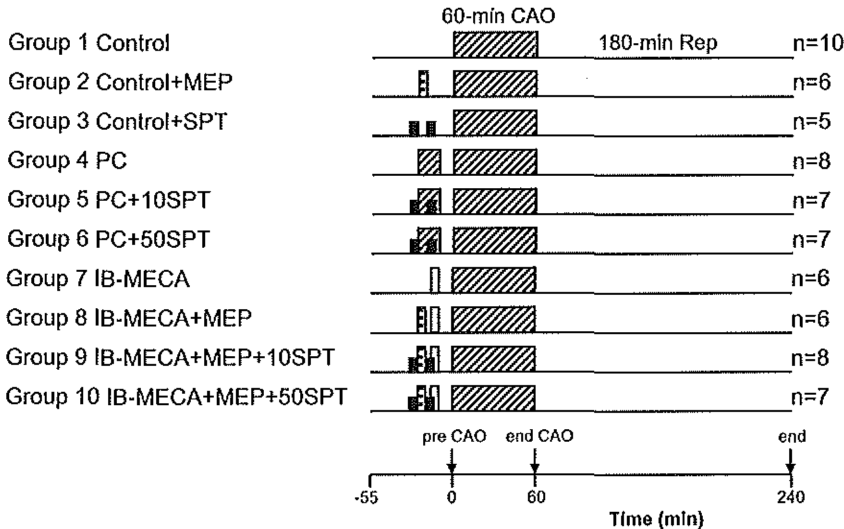


Figure 1 Diagram showing the 10 experimental groups. Ischemic preconditioning (PC, hatched bars) was produced by 15 min of total coronary artery occlusion (CAO) followed by 10 min of reperfusion (Rep). IB-MECA (100 μg/kg, open bars), low (10 mg/kg) and high (50 mg/kg) dose of 8-SPT (black bars) and mepyramine (5 mg/kg, horizontal striped bars) were given intravenously. Infarct size was determined at the end of the 180 min of reperfusion in all groups.

Measurement of infarct size

At the end of each experiment the coronary artery was reoccluded and the area at risk was demarcated by infusion of 10 ml Trypan Blue (0.4%, Sigma Chemical Co.) to stain the normally perfused myocardium dark blue and delineate the nonstained area at risk (AR). The heart was then quickly excised, cooled in ice-cold saline and cut into slices of 1 mm from apex to base. From each slice, the right ventricle was removed and the left ventricle was divided into the AR and the remaining left ventricle, using microsurgical scissors. The AR was then incubated for 10 min 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 noninfarcted area, the different areas of the

LV were dried and weighed separately. Infarct size was expressed as a percentage of the AR. Animals in which AR was below 15% of left ventricular weight were excluded.

Data analysis and presentation

Infarct size (IA/AR, in percent) was analyzed by one-way ANOVA followed by Dunnett's test. Hemodynamic variables were compared by two-way ANOVA for repeated measures followed by the paired or unpaired *t* test. Statistical significance was accepted when $p<0.05$. Data are presented as mean±SEM.

Results

Mortality

Of the 100 rats that had entered the study 19 animals were excluded because of technical failure and three because of sustained ventricular fibrillation during the 60-min CAO (Group 1 Control, Group 2 Control + MEP and Group 4 PC). In 8 animals the area at risk was less than 15% of total left ventricular mass and therefore also excluded.

Hemodynamics

Table 1 shows that at baseline there were no significant differences in mean arterial pressure (MAP) among the 10 groups. Heart rate (HR) was significant only between group 1 (control) and group 8 (IB-MECA+MEP), but similar just before the sustained occlusion. Fifteen minutes of ischemia (group 4) resulted in a slight decrease in both HR and MAP, which was not significant. Infusion of 8-SPT also had no effect on heart rate, but increased MAP by 15-35%. Stimulation of the adenosine A_3 receptors by infusion of IB-MECA (group 7), however, resulted in severe hypotension as MAP decreased from 116±8 mmHg to 59±7 mmHg, most likely via A_3 receptor-mediated histamine release. A bolus of MEP, which partly attenuated the hypotensive response to IB-MECA (group 8), also had a negative effect on HR, as in the control group (group 2) HR decreased from 410±13 bpm to 371±11 bpm and in the IB-MECA treated group from 419±6 bpm to 369±12 bpm. Despite infusion of both MEP and 8-SPT in groups 9 and 10, IB-MECA stil decreased MAP, but to a lesser extent.

The 60-min coronary artery occlusion followed by 180 min of reperfusion resulted in a decrease in both HR and MAP in the control groups (groups 1, 2 and 3). In preconditioned animals HR and MAP were maintained, while the increase in MAP produced by both doses of 8-SPT was lost during the 60-min CAO and 180-min Rep. After infusion of IB-MECA the 60-min coronary artery occlusion did not further decrease MAP with or without MEP or 8-SPT.

Table 1 Systemic hemodynamics

		BL	Pre Stimulus	Pre CAO	End CAO	End Rep
	Control					
Group 1	60-min CAO					
	HR (bpm)	363±13	-	360±12	362±12	363±14
	MAP (mmHg)	105±6	-	102±5	93±6*	85±6*†
Group 2	MEP+60-min CAO					
	HR (bpm)	410±13	365±11*	371±11*	366±8*	360±14*
	MAP (mmHg)	115±6	112±5	108±6	102±5	85±7*†
Group 3	SPT+60-min CAO					
	HR (bpm)	381±16	382±22	382±18	345±13*†	326±8*†
	MAP (mmHg)	98±4	137±7*	133±5*	86±8†	71±9*†
	PC					
Group 4	15-min CAO+10 min Rep+60-min CAO					
	HR (bpm)	391±10	391±9	377±11	380±8	377±8
	MAP (mmHg)	109±6	108±6	92±3*	93±5	89±6*
Group 5	10SPT+15-min CAO+10 min Rep+60-min CAO					
	HR (bpm)	367±6	366±8	356±8	340±11*	323±9*†
	MAP (mmHg)	99±5	129±4*	102±10	83±8†	74±4*†
Group 6	50SPT+15-min CAO+10 min Rep+60-min CAO					
	HR (bpm)	369±10	362±8	360±10	345±10*	348±10
	MAP (mmHg)	119±6	137±9	111±6	96±4†	86±5*†
	IB-MECA					
Group 7	IB-MECA+60-min CAO					
	HR (bpm)	386±17	379±17	363±16	337±21*	311±16*
	MAP (mmHg)	116±8	108±10	59±7*	63±10*	66±8*
Group 8	MEP+IB-MECA+60-min CAO					
	HR (bpm)	419±6	352±14*	369±12*	368±10*	351±8*
	MAP (mmHg)	120±5	98±11*	76±10*	76±7*	73±7*
Group 9	MEP+10SPT+IB-MECA+60-min CAO					
	HR (bpm)	403±10	363±11*	366±14*	340±11*	336±19*
	MAP (mmHg)	110±5	133±7*	67±5*	72±7*	72±7*
Group 10	MEP+50SPT+IB-MECA+60-min CAO					
	HR (bpm)	390±9	369±13	367±14	341±16*	316±10*†
	MAP (mmHg)	105±8	135±5*	86±4	72±7*	65±6*†

AR, area at risk as percentage of left ventricular total mass; IA/AR, infarct size as percentage of area at risk; CAO, coronary artery occlusion; Rep, coronary reperfusion; MEP, Mepyramine (5 mg/kg); 10SPT, 8-(*p*-Sulfophenyl)theophylline (10mg/kg); 50SPT, 8-(*p*-Sulfophenyl)theophylline (50mg/kg); IB-MECA (100μg/kg); PC, preconditioning. Data are mean±SEM. *$P<0.05$ vs BL; †$P<0.05$ vs pre CAO.

Infarct size

There were no significant differences in AR among the experimental groups (Table 2). In the control rats, 60-min CAO resulted in an IA/AR of 70±2%. Pretreatment with MEP or 8-SPT had no effect on infarct size (71±1% and 74±1%, respectively). Ischemic preconditioning by a single 15-min CAO (PC) decreased IA/AR to 47±4% ($P<0.05$ vs control). This protection was blunted by 10 mg/kg 8-SPT (59±2%, $P<0.05$) and abolished by 50 mg/kg 8-SPT (70±1%, $P<0.05$). Pharmacological stimulation of the adenosine A_3 receptors by IB-MECA also decreased IA/AR during the 60-min CAO (49±4%, $P<0.05$ vs control) to a similar extent as ischemic preconditioning. MEP had no effect on this protection (51±2%, $P<0.05$ vs control). The protection by adenosine A_3 receptor stimulation, however, was partly blocked by high dosage of 8-SPT (62±2%, $P<0.05$ vs IB-MECA), but not by low dosage of 8-SPT (56±2%, $P=0.16$), suggesting that 8-SPT at high doses blocks adenosine A_3 receptors in addition to A_1 receptors.

Table 2 Myocardial infarct size

		AR, % LV mass	IA/AR, %
	Control		
Group 1	60-min CAO	39±3	70±2
Group 2	MEP+60-min CAO	36±2	71±1
Group 3	SPT+60-min CAO	35±5	74±1
	PC		
Group 4	15-min CAO+10-min Rep+60-minCAO	38±4	47±4*
Group 5	10SPT+15-min CAO+10-min Rep+60-min CAO	36±4	59±2*†
Group 6	50SPT+15-min CAO+10-min Rep+60-minCAO	34±2	70±1†
	IB-MECA		
Group 7	IB-MECA+60-min CAO	33±5	49±4*
Group 8	MEP+IB-MECA+60-min CAO	33±1	51±2*
Group 9	MEP+10SPT+IB-MECA+60-min CAO	32±3	56±2*
Group 10	MEP+50SPT+IB-MECA+60-min CAO	34±3	62±2*‡

AR, area at risk as percentage of left ventricular total mass; IA/AR, infarct size as percentage of area at risk; Sham indicates control group undergoing 60-min CAO without pretreatment; CAO, coronary artery occlusion; Rep, coronary reperfusion; MEP, Mepyramine (5 mg/kg); 10SPT, 8-(*p*-Sulfophenyl)theophylline (10mg/kg); 50SPT, 8-(*p*-Sulfophenyl)theophylline (50mg/kg); IB-MECA (100μg/kg); PC, preconditioning. Data are mean±SEM. *$P<0.05$ vs control; †$P<0.05$ vs PC; ‡$P<0.05$ vs IB-MECA.

Discussion

The major findings of the present study are that (i) 8-SPT, at a dose which had no effect on the cardioprotection by the selective A_3 agonist IB-MECA, attenuated the protection by ischemic preconditioning in the rat heart *in vivo*, indicating the involvement of A_1 receptors in ischemic preconditioning. (ii) A higher dose of 8-SPT abolished the cardioprotection produced by ischemic preconditioning, but also blunted the protection by IB-MECA, suggesting that

both A_1 and A_3 receptors are involved in the cardioprotection afforded by ischemic preconditioning in the rat heart *in vivo*.

Both *in vitro* and *in vivo* studies on the role of adenosine in ischemic preconditioning in rats have shown controversial results. In Langendorff perfused rat hearts cardioprotection, defined as increased recovery of function, could be induced by activation of the adenosine receptors by either adenosine[13,19] or the adenosine A_1 agonist rPIA.[13] On the other hand, using comparable concentrations of agonists in the same model Cave et al[20] and Asimakis et al[21] were unable to induce cardioprotection. Similar controversies were found using non-selective adenosine antagonists 8-SPT and BWA1433 before ischemic preconditioning, which did not block ischemic preconditioning at low dose of 10 μM[13,20,21], but at high dose (50 μM) cardioprotection was completely abolished.[16,22] Moreover, in *in vivo* studies adenosine infusion decreased infarct size,[11] but ischemic preconditioning could not be abolished by the adenosine antagonists 8-SPT[14] or PD-155199.[11] These results can be explained by the very high interstitsial adenosine levels found in rat heart and as a result, to competitively overcome the elevated levels of adenosine, high concentrations of potent antagonists must be delivered to the rat heart receptor site.[16] Thus, conclusive evidence on the role of adenosine in ischemic preconditioning in the rat heart is still lacking.

In the present study, cardioprotection by ischemic preconditioning was dose dependently inhibited by the non-selective adenosine receptor antagonist 8-SPT indicating that adenosine is involved in ischemic preconditioning in the rat heart *in vivo*. Although Li et al[14] also used a dose of 10 mg/kg 8-SPT *in vivo*, blockade of ischemic preconditioning could not be exhibited. An explanation for this discrepancy could be attributed to the use of different anesthesia in that study, a combination of ketamine/xylazine, which can interfere with the process of ischemic preconditioning[23], or to the use of female rats in that study as estrogen has been shown to be cardioprotective,[24] even at physiological levels.[25] Still, the dose dependent inhibition by 8-SPT in this study is consistent with the hypothesis of Headrick[16] that in preceding studies in the rat the 8-SPT dose was insufficient to block adenosine receptors as interstitial adenosine levels increased more in rat hearts compared to rabbit hearts. That study was the first to measure interstitial adenosine in the rat heart during ischemia and have put forward an explanation for the negative findings on the role of adenosine in ischemic preconditioning, as in these studies[13,20,21] adenosine receptors were not completely blocked.

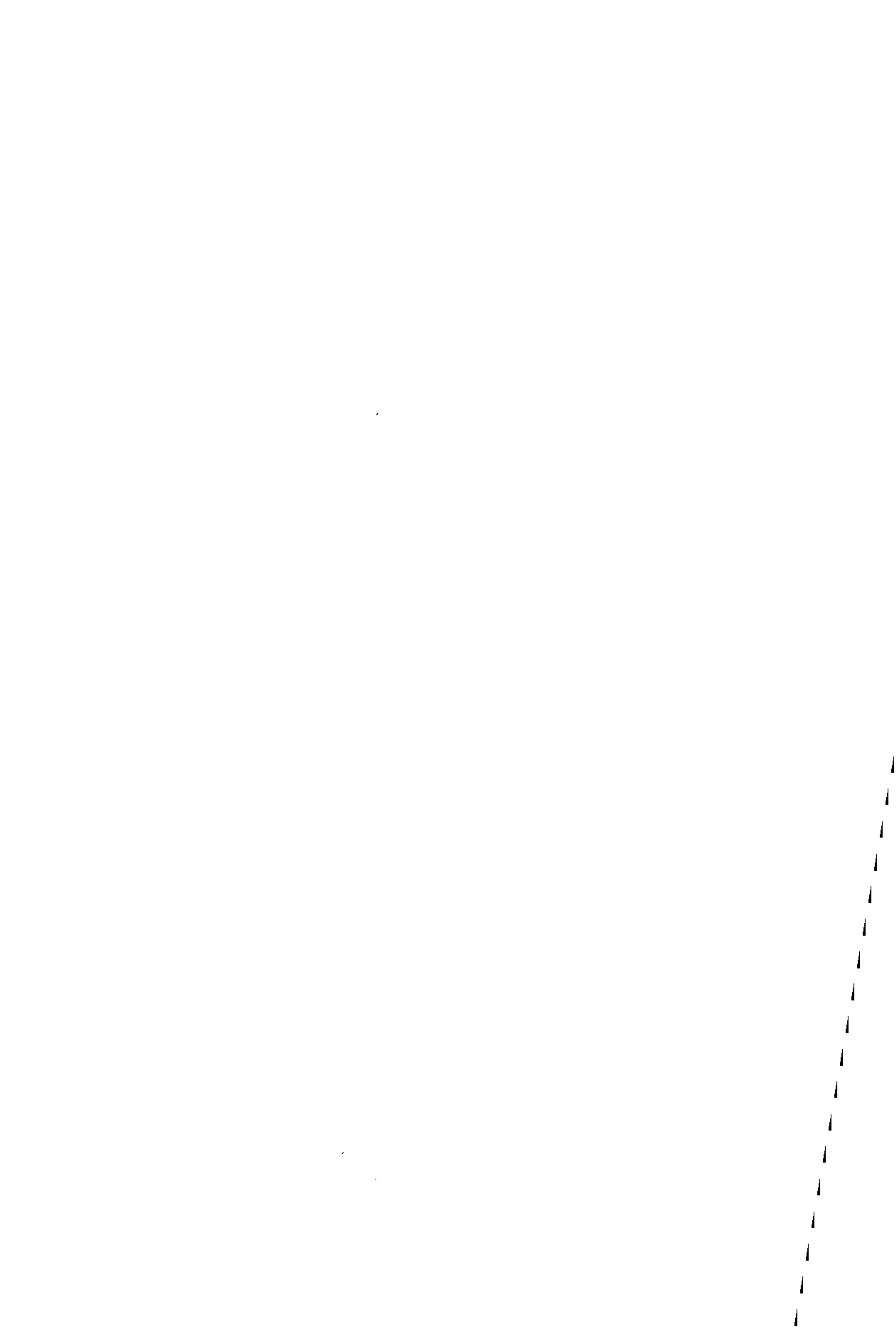
Based on the higher interstitial adenosine levels during myocardial ischemia, Headrick[16] used a dose of 50 μM 8-SPT instead of a dose of 10 μM 8-SPT, but did not make a distinguish between A_1 or A_3 adenosine receptors. Taken into account the K_i value of 8-SPT for A_1 and A_3 receptors (K_i (A_1) = 2600 nM, K_i (A_3) = 10000 nM) it is likely that a higher dose also blocked the A_3 receptors in addition to the A_1 receptors. This is supported by our finding that low dose of 8-SPT (10 mg/kg) is unable to block cardioprotection afforded by the adenosine A_3 receptor agonist IB-MECA, and thus only blocks adenosine A_1 receptors. The cardioprotection by IB-

MECA was, however, partly blocked by a dose which is five times higher (50 mg/kg), whereas the K_i value for A_3 receptors is only 4 times higher, suggesting that 50 mg/kg 8-SPT also blocks the adenosine A_3 receptors. In conclusion, the complete abolishment of ischemic preconditioning in this study by the high dose of 8-SPT, suggests a role for both adenosine A_1 and A_3 receptors in ischemic preconditioning in rats *in vivo*.

References

1. Liu GS, Thornton J, Van Winkle DM, Stanley AW, Olsson RA, Downey JM. Protection against infarction afforded by preconditioning is mediated by A_1 adenosine receptors in rabbit heart. *Circulation.* 1991;84:350-356.
2. Auchampach JA, Gross GJ. Adenosine A_1 receptors, K^+_{ATP} channels, and ischemic preconditioning in dogs. *Am J Physiol.* 1993;264:H1327-H1336.
3. 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.* 1994;266:H829-H839.
4. Schulz R, Rose J, Post H, Heusch G. Involvement of endogenous adenosine in ischaemic preconditioning in swine. *Pflugers Arch.* 1995;430:273-282.
5. Sakamoto J, Miura T, Goto M, Iimura O. Limitation of myocardial infarct size by adenosine A_1 receptor activation is abolished by protein kinase C inhibitors in the rabbit. *Cardiovasc Res.* 1995;29:682-688.
6. Thornton JD, Liu GS, Olsson RA, Downey JM. Intravenous pretreatment with A1-selective adenosine analogues protects the heart against infarction. *Circulation.* 1992;85:659-665.
7. Liu GS, Richards SC, Olsson RA, Mullane K, Walsh RS, Downey JM. Evidence that the adenosine A_3 receptor may mediate the protection afforded by preconditioning in the isolated rabbit heart. *Cardiovasc Res.* 1994;28:1057-1061.
8. Rice PJ, Armstrong SC, Ganote CE. Concentration-response relationships for adenosine agonists during preconditioning of rabbit cardiomyocytes. *J Mol Cell Cardiol.* 1996;28:1355-1365.
9. Auchampach JA, Rizvi A, Qiu Y, Tang XL, Maldonado C, Teschner S, Bolli R. Selective activation of A3 adenosine receptors with N6-(3-iodobenzyl)adenosine-5'-N-methyluronamide protects against myocardial stunning and infarction without hemodynamic changes in conscious rabbits. *Circ Res.* 1997;80:800-809.
10. Wang J, Drake L, Sajjadi F, Firestein GS, Mullane KM, Bullough DA. Dual activation of adenosine A_1 and A_3 receptors mediates preconditioning of isolated cardiac myocytes. *Eur J Pharmacol.* 1997;320:241-248.
11. Liu Y, Downey JM. Ischemic preconditioning protects against infarction in rat heart. *Am J Physiol.* 1992;263:H1107-H1112.
12. Ganote CE, Armstrong S, Downey JM. Adenosine and A_1 selective agonists offer minimal protection against ischaemic injury to isolated rat cardiomyocytes. *Cardiovasc Res.* 1993;27:1670-1676.
13. Lasley RD, Rhee JW, Van Wylen DG, Mentzer RM, Jr. Adenosine A_1 receptor mediated protection of the globally ischemic isolated rat heart. *J Mol Cell Cardiol.* 1990;22:39-47.
14. Li Y, Kloner RA. The cardioprotective effects of ischemic 'preconditioning' are not mediated by adenosine receptors in rat hearts. *Circulation.* 1993;87:1642-1648.
15. Bugge E, Ytrehus K. Ischaemic preconditioning is protein kinase C dependent but not through stimulation of alpha adrenergic or adenosine receptors in the isolated rat heart. *Cardiovasc Res.* 1995;29:401-406.
16. Headrick JP. Ischemic preconditioning: bioenergetic and metabolic changes and the role of endogenous adenosine. *J Mol Cell Cardiol.* 1996;28:1227-1240.
17. Gho BC, Schoemaker RG, van den Doel MA, Duncker DJ, Verdouw PD. Myocardial protection by brief ischemia in noncardiac tissue. *Circulation.* 1996;94:2193-2200.
18. Fozard JR, Pfannkuche HJ, Schuurman HJ. Mast cell degranulation following adenosine A_3 receptor activation in rats. *Eur J Pharmacol.* 1996;298:293-297.
19. Fralix TA, Murphy E, London RE, Steenbergen C. Protective effects of adenosine in the perfused rat heart: changes in metabolism and intracellular ion homeostasis. *American Journal of Physiology.* 1993;264:C986-C994.
20. Cave AC, Collis CS, Downey JM, Hearse DJ. Improved functional recovery by ischaemic preconditioning is not mediated by adenosine in the globally ischaemic isolated rat heart. *Cardiovasc Res.* 1993;27:663-668.

21. Asimakis GK, Inners-McBride K, Conti VR. Attenuation of postischaemic dysfunction by ischaemic preconditioning is not mediated by adenosine in the isolated rat heart. *Cardiovasc Res.* 1993;27:1522-1530.
22. de Jonge R, de Jong JW. Ischemic preconditioning and glucose metabolism during low-flow ischemia: role of the adenosine A_1 receptor. *Cardiovasc Res.* 1999;43:909-918.
23. Haessler R, Kuzume K, Chien GL, Wolff RA, Davis RF, Van Winkle DM. Anaesthetics alter the magnitude of infarct limitation by ischaemic preconditioning. *Cardiovasc Res.* 1994;28:1574-1580.
24. Lee TM, Su SF, Tsai CC, Lee YT, Tsai CH. Cardioprotective effects of 17 beta-estradiol produced by activation ofmitochondrial ATP-sensitive K^+ Channels in canine hearts. *J Mol Cell Cardiol.* 2000;32:1147-1158.
25. Kolodgie FD, Farb A, Litovsky SH, Narula J, Jeffers LA, Lee SJ, Virmani R. Myocardial protection of contractile function after global ischemia by physiologic estrogen replacement in the ovariectomized rat. *J Mol Cell Cardiol.* 1997;29:2403-2414.



Time course and mechanism of myocardial catecholamine release during transient ischemia *in vivo*

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Time course and mechanism of myocardial catecholamine release during transient ischemia *in vivo*

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Background Elevated concentrations of norepinephrine (NE) have been observed in ischemic myocardium. We investigated the magnitude and mechanism of catecholamine release in the myocardial interstitial fluid (MIF) during ischemia and reperfusion *in vivo* through the use of microdialysis.

Methods and Results In 9 anesthetized pigs, interstitial catecholamine concentrations were measured in the perfusion areas of the left anterior descending coronary artery (LAD) and the left circumflex coronary artery. After stabilization, the LAD was occluded for 60 minutes and reperfused for 150 minutes. During the final 30 minutes, tyramine (154 nmol·kg^{-1}·min^{-1}) was infused into the LAD. During LAD occlusion, MIF NE concentrations in the ischemic region increased progressively from 1.0±0.1 to 524±125 nmol/L. MIF concentrations of dopamine and epinephrine rose from 0.4±0.1 to 43.9±9.5 nmol/L and from <0.2 (detection limit) to 4.7±0.7 nmol/L, respectively. Local uptake-1 blockade attenuated release of all 3 catecholamines by >50%. During reperfusion, MIF catecholamine concentrations returned to baseline within 120 minutes. At that time, the tyramine-induced NE release was similar to that seen in nonischemic control animals despite massive infarction. Arterial and MIF catecholamine concentrations in the left circumflex coronary artery region remained unchanged.

Conclusions Myocardial ischemia is associated with a pronounced increase of MIF catecholamines, which is at least in part mediated by a reversed neuronal reuptake mechanism. The increase of MIF epinephrine implies a (probably neuronal) cardiac source, whereas the preserved catecholamine response to tyramine in postischemic necrotic myocardium indicates functional integrity of sympathetic nerve terminals. *(Circulation. 2000;101:2645-2650.)*

Introduction

Myocardial ischemia is associated with a marked accumulationof norepinephrine (NE) in ischemic tissue.[1–4] *In vitro* studies suggest that this is caused by nonexocytotic release of NE from cardiac sympathetic nerves.[5–7] In contrast to the normally occurring exocytotic NE release, this non-exocytotic NE release is (1) calcium independent, (2) not under influence of local or central sympathetic stimulation, and (3) not affected by presynaptic inhibition.[7] Interestingly, these *in vitro* studies in the sympathetically dominant rat heart also suggest that the ischemia-induced nonexocytotic NE release can be attenuated by neuronal uptake-1 (U1) blockade, indicating that under ischemic conditions, the U1 mechanism is reversed and can operate as a carrier for outward instead of inward NE transport.[5,6] However, this has not been investigated in parasympathetically dominant human and porcine hearts *in vivo*. Furthermore, little is known about the myocardial release of epinephrine (E) and dopamine (DA) in the ischemic heart *in vivo*; this is of particular interest because cardiac E release has been reported in *in vitro* studies and in healthy elderly men and patients with severe congestive heart failure at rest and during exercise.[8–11]

Microdialysis allows the measurement of catecholamine concentrations in the myocardial interstitial fluid (MIF) *in vivo* and the investigation of the mechanisms that underlie their local release and clearance.[12] Using an *in vivo* porcine model, we investigated the time course and magnitude of changes in MIF concentrations of catecholamines during severe myocardial ischemia and reperfusion. To determine the contribution of reversal of the U1 mechanism to ischemia-induced NE release, 1 of the microdialysis probes in the ischemic myocardium was coperfused with the U1 inhibitor desipramine (DMI).[12,13]

We also determined whether sympathetic nerve endings are functionally impaired during reperfusion after severe myocardial ischemia, as has been suggested for the isolated rat hearts.[2] For this purpose, local NE response to an intracoronary infusion of tyramine in the postischemic myocardium was compared with the response observed in the nonischemic porcine myocardium of control animals previously studied under similar experimental conditions.[12] Tyramine is taken up via U1 into the sympathetic nerve endings, where it releases NE. Tyramine thus provides information on the NE content as well as on the U1 function of sympathetic nerve endings.[12]

Methods

Animal Care

All experiments were performed in accordance with the *Guiding Principles for Research Involving Animals and Human Beings* as approved by the Council of the American Physiological Society and under the regulations of the Animal Care Committee of Erasmus University Rotterdam.

Surgical Procedure

After an overnight fast, crossbred Landrace ×Yorkshire pigs of either sex (weight 30 to 35 kg, n=59) were sedated with ketamine (20 to 25 mg/kg IM), anesthetized with sodium pentobarbital (20 mg/kg IV), intubated, and connected to a respirator for intermittent positive pressure ventilation with a mixture of oxygen and nitrogen. Respiratory rate and tidal volume were set to keep arterial blood gases within the normal range.[12,14] Catheters were positioned in the superior caval vein for the continuous administration of sodium pentobarbital (10 to 15 mg $\cdot kg^{-1} \cdot h^{-1}$) and saline to replace blood withdrawn during sampling. In the descending aorta, a fluid-filled catheter was placed to monitor aortic blood pressure and blood sampling. Through a carotid artery, a micromanometer-tipped catheter (B. Braun Medical BV) was inserted into the left ventricle (LV) for the measurement of LV pressure and its first derivative, LVdP/dt. After the administration of pancuronium bromide (4 mg), a midsternal thoracotomy was performed, and the heart was suspended in a pericardial cradle. An electromagnetic flow probe (Skalar) was then placed around the ascending aorta for the measurement of cardiac output. After a Doppler flow probe was placed on a proximal segment of the left anterior descending coronary artery (LAD), a cannula (outer diameter=1.3 mm) was inserted distal to this site into the LAD for the administration of tyramine.

Microdialysis probes were implanted in the LV myocardium through the use of a steel guiding needle and split plastic tubing: 1 probe in the region perfused by the left circumflex coronary artery (LCx) and 2 probes in the area perfused by the LAD. To achieve local U1 inhibition, 1 of the LAD probes was coperfused with DMI (100 mmol/L).13 In addition, a microdialysis probe was placed in the interventricular coronary vein that drains the LAD region.[15]

Dialysis Methodology

The polycarbonate dialysis membrane of the microdialysis probes (CMA/20; Carnegie Medicine AB) has a cutoff value of 20 kDa, a length of 10 mm, and a diameter of 0.5 mm. Probes were perfused with an isotonic Ringer's solution at a rate of 2 μL/min with a CMA/100 microinjection pump. Dialysate volumes of 20 mL (sampling time 10 minutes) were collected in microvials containing 20 μL of a solution of 2% (wt/vol) EDTA and 30 nmol/L *l*-erythro-α-methyl-NE (AMN) as internal standard in 0.08 N acetic acid. Sampling started immediately after insertion of the probes. Plasma samples were drawn into chilled heparin-containing tubes containing 12 mg glutathione. Microdialysis and plasma samples were stored at -80°C until analysis within the next 5 days.[12,16]

In vivo probe recovery of NE (5261%) has been determined through retrodialysis with AMN as a calibrator and direct comparison of hemomicrodialysis and plasma samples.[12,17] *In vivo* probe recovery for E (68±3%) was determined through a comparison of E concentrations in arterial plasma with E concentrations in the dialysate obtained from the carotid artery probe.

The *in vivo* probe recovery for DA was not determined directly but was assumed to be similar to the probe recovery of NE because of the similarities of NE and DA in size and charge distribution.

Experimental Protocol

After a 120-minute stabilization period,[12] baseline measurements were obtained during a 30-minute period before the LAD was occluded distal to the first diagonal branch for 60 minutes, with an atraumatic clip, and then reperfused for 150 minutes. During the final 30 minutes of reperfusion, tyramine (154 nmol $\cdot$ kg^{-1} $\cdot min^{-1}$) was infused directly into the LAD. At the end of the experiment, the perfusion area of the LAD was determined with an intraatrial infusion of 30 mL of a 5% (wt/wt) solution of fluorescein sodium during reocclusion of the LAD. During occlusion, ventricular arrhythmias were counted and distinguished as premature ventricular contractions, ventricular tachycardia, or ventricular fibrillation.[18] After the induction of ventricular fibrillation with a 9-V battery, the heart was excised and infarct size was determined with the use of *para*–nitro blue tetrazolium.[14]

Analytical Procedures

Plasma catecholamines were determined through HPLC with fluorometricdetection after liquid-liquid extraction and derivatization with the fluorogenic agent 1,2-diphenyl-ethylenediamine.[19] For microdialysis samples, the catecholamines are not extracted before fluorometric detection with HPLC but instead were directly derivatized according to the procedure described by Alberts et al.[17] This method suppresses the interference of sulfhydryl compounds on derivatization, thus improving sensitivity.

Reagents and Pharmaceuticals

Ketamine and sodium pentobarbital were obtained from Apharmo BV. Pancuronium bromide was obtained from Organon Teknica BV. Ringer's solution was purchased from Baxter. Tyramine was obtained from the Department of Pharmacy, University Hospital Rotterdam. Fluorescein sodium, *para*–nitro blue tetrazolium, DMI, NE, E, DA, and AMN were purchased from Sigma Chemical Co. EDTA was purchased from Merck. L-Glutathione was obtained from Fluka. Acetic acid was obtained from Baker. 1,2-Diphenyl-ethylenediamine was prepared as reported previously.[19]

Statistical Analysis

Five of the 9 animals experienced ventricular fibrillation during LAD occlusion (between 10 and 30 minutes of ischemia) but were successfully defibrillated within 1 minute with the use of 20- to 30-W countershocks and therefore were included in the analysis. Because there were no differences in the hemodynamic and catecholamine responses between animals that

fibrillated and the animals that maintained sinus rhythm, the data for all 9 animals were pooled. Catecholamine concentrations obtained with microdialysis were corrected for probe recovery. Lower limits of detection for catecholamines measured with microdialysis and those measured in arterial plasma were 0.2 and 0.02 nmol/L, respectively. Baseline values were determined by averaging the 3 measurements during the 30-minute period before occlusion.[12] Results are expressed as mean±SEM. For statistical analysis, 2-way ANOVA, 1-way ANOVA for repeated measures with Dunnett's multiple comparison test as post hoc test, and Student's *t* tests were used as appropriate.

Results

Systemic Hemodynamics During Ischemia and Reperfusion

Baseline hemodynamic data and the changes produced with 60-minute LAD occlusion and 120 minutes of reperfusion (Table 1) are in accordance with previously published data from our laboratory.[14]

Table 1 Cardiac and Systemic Hemodynamics at Baseline, at 60 Min of Ischemia, and at 120 Min of Reperfusion

	Baseline	Ischemia 60 min	Reperfusion 120 min
Mean Aortic Pressure, mmHg	97±2	82±2*	79±5
Cardiac Output, L/min	2.4±0.2	2.1±0.1	1.8±0.1*
Heart Rate, bpm	114±4	118±7	137±11*
Systemic Vascular Resistance, mmHg.min.L^{-1}	42±4	41±4	43±5
Stroke Volume, mL	22±2	18±1*	15±2*
$LVdP/dt_{max}$, mmHg/s	1680±100	1460±110	1380±150
Left Ventricular End Diastolic Pressure, mmHg	6.6±0.8	9.3±1.4*	10.0±1.9*
LADCA Flow, mL/min	34±3	0	46±8

Data are presented as mean±SEM, n=9; **P*<0.05 vs Baseline.

Infarct Size

The LAD occlusion resulted in an ischemic area (area at risk) that composed 29±2% of the LV mass. Infarct size determined at the end of reperfusion was 84±4% of the area at risk.

Catecholamine Concentrations During Ischemia and Reperfusion

At baseline, NE concentrations in MIF (NE_{MIF}) in the LAD and LCx regions were similar to concentrations in the coronary vein (NE_{CV}) but were 3 times the concentrations in arterial plasma (NE_{art})($P<0.05$; Table 2). DA concentrations followed a similar pattern, whereas E was detectable only in arterial plasma. Under U1 blockade, NE_{MIF} increased 5-fold, whereas DA_{MIF} did not change and E_{MIF} remained undetectable.

During the first 10 minutes of ischemia, NE_{MIF} in theLAD region tripled and continued to rise progressively, so NE_{MIF} increased 500-fold by the end of ischemia (Table 2, Figure 1). In the presence of U1 blockade, the rate of rise of NE_{MIF} was attenuated so that after 20 minutes of ischemia NE_{MIF} under U1 blockade was similar to, and at 60 minutes was less than half of

NE_{MIF} in the absence of U1 blockade. NE_{CV} increased progressively to 100-fold its baseline value. On reperfusion, NE_{MIF} under U1 blockade and NE_{CV} declined rapidly, with the early rate of decline being most pronounced for NE_{MIF} in the absence of U1 blockade (Figure 1). Within 120 minutes of reperfusion, catecholamine concentrations in MIF and coronary vein had returned to baseline values. NE_{MIF} in the LCx perfused area and NE_{art} remained unchanged during the course of the experiment.

In the LAD region, E_{MIF} and DA_{MIF} in the absence and presence of U1 blockade and E_{CV} and DA_{CV} followed qualitatively similar patterns as NE, but absolute increments during ischemia were substantially less pronounced (Table 2, Figure 1). Again, E_{art},DA_{art}, and DA_{MIF} in the LCx region did not change over the course of the experiment, whereas E_{MIF} in the LCx region remained undetectable.

Table 2 Plasma and MIF catecholamine concentrations at Baseline during Ischemia and Reperfusion

	Arterial Plasma nmol/L	Coronary Vein nmol/L	MIF LADCA nmol/L	MIF LADCA + DMI nmol/L	MIF LCXCA nmol/L
Norepinephrine					
Baseline	0.30±0.08	0.86±0.22†	0.97±0.11†	5.76±0.77†‡§	0.96±0.13†
60 min Ischemia	0.32±0.07	90±36*†	524±125*†‡	198±46*†‡§	0.79±0.27†‡§
120 min Reperfusion	0.54±0.23	0.62±0.30	0.54±0.11	1.99±0.28*†‡§	0.62±0.16
Epinephrine					
Baseline	0.04±0.01	< 0.20	< 0.20	< 0.20	< 0.20
60 min Ischemia	0.03±0.01	2.55±1.07*†	4.74±0.71*†‡	2.09±0.43*†§	< 0.20‡§
120 min Reperfusion	0.07±0.02	< 0.20	< 0.20	< 0.20	< 0.20
Dopamine					
Baseline	0.06±0.01	0.71±0.45†	0.40±0.12†	0.57±0.16†	0.59±0.27†
60 min Ischemia	0.06±0.01	11.6±4.3*†	43.9±9.5*†‡	16.5±5.6*†§	0.47±0.04†‡§
120 min Reperfusion	0.15±0.07	0.27±0.11	0.30±0.10†	0.34±0.13†	0.21±0.03

MIF; myocardial interstitial fluid; LADCA; left anterior descending coronary artery; LCXCA; left circumflex coronary artery; DMI; desipramine, U1 inhibitor. Data are presented as mean±SEM, n=9, *P<0.05 vs Baseline; †P<0.05 vs Arterial Plasma; ‡P<0.05 MIF LADCA vs Coronary Vein; § P<0.05 vs MIF LADCA.

Ventricular Arrhythmias During Ischemia

Most of the ventricular arrhythmias occurred within the first 30 minutes of ischemia. The incidence of premature ventricular contractions was particularly high between 20 to 30 minutes of ischemia (a total of 661 and a mean of 73±19 per animal). Five animals experienced ventricular fibrillation but were defibrillated successfully within 1 minute. There was no correlation between NE_{MIF} concentrations and the occurrence of ventricular arrhythmias in general or ventricular fibrillation in particular.

Postischemic Catecholamine Release With Tyramine

Figure 2 shows that the infusion of tyramine directly into the LAD after 120 minutes of reperfusion caused an increase in NE_{MIF} in the postischemic myocardium from 0.6±0.1 to 11.5±1.9 nmol/L (*P*<0.05). This increment was not different from the increase of from 0.9±0.2 to 13.4±3.2 nmol/L in the LAD region of the normal (nonischemic) swine heart.[12]

Furthermore, compared with the increment seen in the absence of U1 blockade, the increase in the presence of U1 blockade was minimal.

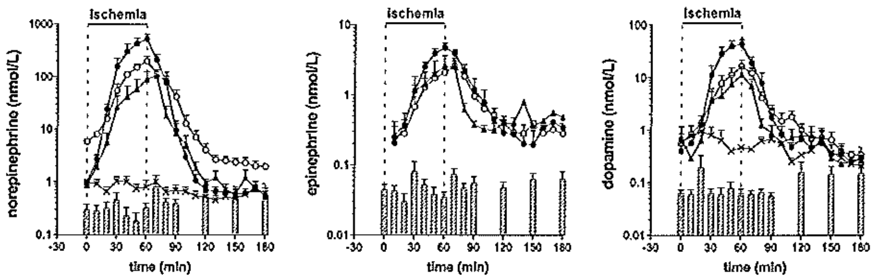

Figure 1 Time course of changes in catecholamine concentrations during ischemia and reperfusion. Data are shown for MIF in LAD region in absence (•) and presence (○) of U1 blockade, MIF in LCx region in absence of U1 blockade (3), and concentrations in arterial plasma (hatched bars) and in coronary vein (▲). Data are given as mean±SEM (n=9).

Discussion

The results of the present study demonstrate that myocardial ischemia is associated with a rapid and massive increase in the concentration of all 3 endogenous catecholamines (NE, E, and DA) in the myocardial interstitial fluid as measured with the microdialysis technique *in vivo*. As suggested for NE in *in vitro* studies,[5,6] the reversed U1 mechanism plays an important role in the release of all 3 catecholamines during ischemia *in vivo*. Furthermore, our study shows that after 60 minutes of ischemia, which results in massive infarction of the jeopardized myocardium, the functional integrity of sympathetic nerve terminals remains intact. Finally, our results suggest that cardiac interstitial E has a neuronal origin.

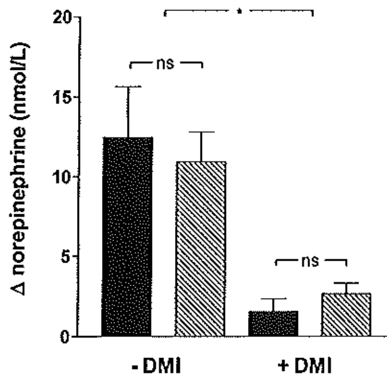

*Figure 2 Tyramine-induced NE release in perfusion area of LAD with and without U1 inhibition. Data are shown for postischemic myocardium (n=4, hatched bars) and for historic nonischemic control animals (n=4, solid bars).[12] Data are given as mean±SEM (n=4). *P<0.05.*

Interstitial Catecholamine Concentrations During Basal Conditions

The present study confirms that at baseline, NE_{MIF} is ≈3 times the NE_{art} and increases ≈6-fold in response to U1 blockade,[1,12,20] whereas E_{MIF} was below the detection limit regardless of the presence of U1 inhibition. DA_{MIF} did not rise under U1 blockade, suggesting that U1 does not play a predominant role in the clearance of DA from the interstitial compartment under baseline conditions in the heart. Little is known about the affinity of DA for U1 and the relevance of U1 to DA clearance in the heart. Because the main purpose of the U1 mechanism is to modulate synaptic transmission, it is hard to envisage a substantial role for U1 in the clearance of DA in the absence of any cardiac dopaminergic synaptic transmission. Furthermore, in tissues with known dopaminergic transmission, like brain and kidney, DA is taken up by a specific DA neuronal uptake mechanism that does not take up NE and is poorly inhibited by DMI.[21,22]

Interstitial Catecholamine Concentrations During Ischemia and Reperfusion

In *in vitro* experiments in isolated rat hearts, 3 phases of ischemia-induced release of NE, each with a different mechanism, have been recognized.[2,5,6,23] During the early phase of ischemia (0 to 10 minutes), the release of NE, if present, is exocytotic and depends on the activation of efferent sympathetic neurons. Accumulation of catecholamines in the extracellular space during this early phase is prevented by the highly efficient U1 mechanism and by presynaptic inhibition by adenosine, which accumulates in cardiac tissue during this phase of ischemia. The latter has been shown to be of particular importance in the rat, because adenosine concentrations are considerably higher than those in other species.[24] During the second phase of ischemia (10 to 40 minutes), the release of NE becomes nonexocytotic and is thought to involve the U1 mechanism in the carrier-mediated efflux of NE in reverse of its normal transport direction.[5,6] During the third phase (>40 minutes ischemia), the release of NE is no longer attenuated by U1 inhibitors, which is explained by the occurrence of structural changes in the neuronal membrane of the myocardial neurons.[2]

In the present study, a rapid and pronounced increase in MIF concentrations of all 3 catecholamines was observed shortly after occlusion of the LAD. Because released NE is avidly taken up by the cardiac U1 mechanism, we expected a larger rise in NE_{MIF} in the presence of the U1 inhibitor DMI than without U1 inhibition. However, during this first 10 minutes of ischemia, the increment of NEMIF with U1 blockade (5.8 to 8.5 nmol/L) was similar to the increment without U1 blockade (0.9 to 2.7 nmol/L). Possibly, in the parasympathetically dominant porcine heart, U1 carrier–mediated nonexocytotic NE efflux already occurred within the initial 10 minutes of ischemia, so in the presence of DMI, any decrease in U1-mediated clearance was compensated for by a decrease in the ischemia-induced U1 carrier–mediated NE efflux. As mentioned, myocardial release of NE in first 10 minutes of

ischemia is not an invariable finding. For example, stimulation-evoked NE release has shown to be suppressed in rat hearts and human atrial tissue but to be facilitated in guinea pig hearts.[25]

Throughout the ischemic period, MIF catecholamine concentrations rose progressively in the ischemic area. Concentrations of catecholamines did not change in either the nonischemic LCx area or the systemic circulation. The reversal of the U1 mechanism continued to contribute to the catecholamine release during the entire period of ischemia. Thus, U1 blockade attenuated the release of all catecholamines by .50%, indicating that despite infarction of 83% of the area at risk, U1 was operative after 60 minutes of ischemia. Our findings vary from those obtained in the ischemic myocardium of the isolated rat heart, where the reversed U1 mechanism no longer contributes to the release of catecholamines 40 minutes after the induction of ischemia. This difference may be explained by the differences in experimental conditions (eg, *in vivo* versus *in vitro* studies) and the species investigated.[2]

On reperfusion, MIF catecholamine concentrations rapidly declined in the postischemic myocardium. Washout probably was the predominant factor in the clearance of catechol-amines in this early phase of reperfusion. However, the decline in the first 10 minutes of reperfusion was substantially greater without inhibition of the U1 mechanism, indicating that the U1 mechanism also contributed significantly to the clearance of NE during early reperfusion. Although it should be noted that in contrast to techniques used in *in vitro* studies,[2,25] the time resolution of the MD technique as presently used does not allow conclusions to be made regarding minute-to-minute changes in catecholamine concentrations. Compatible with previous findings that E and DA are less avidly taken up by U1 than NE,[26] the decline in E_{MIF} and DA_{MIF} during reperfusion was not affected by U1 blockade.

Origin of Myocardial Interstitial E

An interesting finding was the ischemia-induced increase in E_{MIF}, albeit small compared with the increase in NE_{MIF}. Because the concentrations of E_{art} and E_{MIF} in the nonischemic LCx region did not change during ischemia, this increase must have originated from the heart. It is currently unclear whether this source is neuronal or extraneuronal. Evidence favoring extraneuronal synthesis and release of E is the presence of the enzyme phenylethanolamine N-methyltransferase in extraneuronal myocardial tissue.[10,27] Furthermore, an intrinsic cardiac adrenergic cell type outside the sympathetic nervous system, capable of releasing E and NE, has been identified in the human heart.[10] Finally, enhanced cardiac E spillover into the coronary circulation of patients with heart failure during sympathetic stimulation was disproportionate to the spillover of NE, suggesting that E may in part be derived from sources other than chromaffin cells or sympathetic nerves.[9,11] On the other hand, it is known that sympathetic neurons can take up E from the circulation and release it upon stimulation.[28] In the present study, the pattern of release and clearance of E during ischemia and reperfusion was similar to that of NE and DA. Furthermore, inhibition of the neuronal U1 mechanism

attenuated the ischemia-induced release of all catecholamines to a similar degree, suggesting a common source, thus favoring a neuronal origin.

Functional Integrity of Sympathetic Nerve Endings

The effects of U1 blockade on the NE_{MIF} responses to ischemia and reperfusion suggested that U1 mechanism of the sympathetic nerves was still functioning during and after the 60-minute LAD occlusion. This is further substantiated by the NE_{MIF} response to tyramine at the end of reperfusion. Similar to NE, tyramine is taken up by neurons through U1, where it displaces NE from the nerve terminals because of its higher affinity for the neuronal storage proteins. Consequently, the tyramine-induced NE release reflects both neuronal NE content and the efficacy of U1.[12,29] The increase in NE_{MIF} in the postischemic LAD region was very similar to that in the nonischemic control hearts (Figure 2).[12] These findings are in line with those reported by Shindo et al,[4] who studied the tyramine-induced NE release in nonischemic and postischemic areas in feline hearts after 40 minutes of reperfusion after 40 minutes of ischemia. In addition, in the present study, the attenuation of the tyramine-induced NE release by U1 inhibition in both postischemic and nonischemic groups also was similar (Figure 2). Although the present experimental setup does not allow for any predictions concerning the long-term survival of sympathetic nerves, the present findings indicate that sympathetic nerve terminals remained functionally intact at least during the first few hours after reperfusion. However, functional alterations of the somata of the sympathetic nerves cannot be entirely excluded. Thus, in a canine model of tachycardia-induced heart failure, impairment of the myocardial contractile response to electrical or chemical stimulation of sympathetic somata was observed at a time when the contractile response to tyramine was completely preserved.[30]

Implications

Although the pathophysiological significance of the massive accumulation of catecholamines in the ischemic myocardial issue was not investigated in the present study, there is evidence from experimental as well as clinical studies that high catecholamine concentrations are deleterious to the heart.[31-34] Several studies have demonstrated NE-dependent antiarrhythmic effects of U1 inhibition during ischemia with either tricyclic antidepressant agents like desipramine and imipramine or structurally unrelated U1 inhibitors like cocaine and nisoxetine.[7,35] This study provides a possible explanation for this beneficial effect by demonstrating that the reversed U1 mechanism contributes substantially to the release of catecholamines during ischemia.

Acknowledgements

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References

1. Akiyama T, Yamazaki T, Ninomiya I. Differential regional responses of myocardial interstitial noradrenaline levels to coronary occlusion. *Cardiovasc Res.* 1993;27:817– 822.
2. Schomig A, Dart AM, Dietz R, et al. Release of endogenous catecholamines in the ischemic myocardium of the rat, part A: locally mediated release. *Circ Res.* 1984;55:689 –701.
3. Shindo T, Akiyama T, Yamazaki T, et al. Increase in myocardial interstitial norepinephrine during a short period of coronary occlusion. *J Auton Nerv Syst.* 1994;48:91–96.
4. Shindo T, Akiyama T, Yamazaki T, et al. Regional myocardial interstitial norepinephrine kinetics during coronary occlusion and reperfusion. *Am J Physiol.* 1996;270:H245–H251.
5. Kranzhofer R, Haass M, Kurz T, et al. Effect of digitalis glycosides on norepinephrine release in the heart: dual mechanism of action. *Circ Res.* 1991;68:1628 –1637.
6. Kurz T, Richardt G, Hagl S, et al. Two different mechanisms of noradrenaline release during normoxia and simulated ischemia in human cardiac tissue. *J Mol Cell Cardiol.* 1995;27:1161–1172.
7. Kurz T, Offner B, Schreieck J, et al. Nonexocytotic noradrenaline release and ventricular fibrillation in ischemic rat hearts. *Naunyn-Schmiedebergs Arch Pharmacol.* 1995;352:491– 496.
8. Esler M, Eisenhofer G, Dart A, et al. Adrenaline release by the human heart. *Clin Exp Pharmacol Physiol.* 1991;18:67–70.
9. Esler M, Kaye D, Thompson J, et al. Effects of aging on epinephrine secretion and regional release of epinephrine from the human heart. *J Clin Endocrinol Metab.* 1995;80:435– 442.
10. Huang MH, Friend DS, Sunday ME, et al. An intrinsic adrenergic system in mammalian heart. *J Clin Invest.* 1996;98:1298 –1303.
11. Kaye DM, Lefkovits J, Cox H, et al. Regional epinephrine kinetics in human heart failure: evidence for extra-adrenal, nonneural release. *Am J Physiol.* 1995;269:H182–H188.
12. Lameris TW, van Den Meiracker AH, Boomsma F, et al. Catecholamine handling in the porcine heart: a microdialysis approach. *Am J Physiol.* 1999;277:H1562–H1569.
13. Yamazaki T, Akiyama T. Effects of locally administered desipramine on myocardial interstitial norepinephrine levels. *J Auton Nerv Syst.* 1996;61:264–268.
14. Koning MM, Gho BC, van Klaarwater E, et al. Rapid ventricular pacing produces myocardial protection by nonischemic activation of K+ATP channels. *Circulation.* 1996;93:178 –186.
15. Bier J, Sharaf B, Gewirtz H. Origin of anterior interventricular vein blood in domestic swine. *Am J Physiol.* 1991;260:H1732–H1736.
16. Boomsma F, Alberts G, van Eijk L, et al. Optimal collection and storage conditions for catecholamine measurements in human plasma and urine. *Clin Chem.* 1993;39:2503–2508.
17. Alberts G, Lameris T, van den Meiracker AH, et al. Sensitive and specific method for the simultaneous determination of natural and synthetic catecholamines and 3,4-dihydroxyphenylglycol in microdialysis samples. *J Chromatogr B Biomed Sci Appl.* 1999;730:213–219.
18. Verdouw PD, Schamhardt HC, Remme WJ, et al. Antiarrhythmic, metabolic and hemodynamic effects of Org 6001 (3alpha-amino-5alpha-androstan-2beta-ol-17-one-hydrochloride) after coronary flow reduction in pigs. *J Pharmacol Exp Ther.* 1978;204:634 –644.
19. van der Hoorn FA, Boomsma F, Man in 't Veld AJ, et al. Determination of catecholamines in human plasma by high-performance liquid chroma-tography: comparison between a new method with fluorescence detection and an established method with electrochemical detection. *J Chromatogr.* 1989;487:17–28.
20. Akiyama T, Yamazaki T, Ninomiya I. *In vivo* monitoring of myocardial interstitial norepinephrine by dialysis technique. *Am J Physiol.* 1991;261: H1643–H1647.
21. Sunn N, Harris PJ, Bell C. Effects on renal sympathetic axons in dog of acute 6-hydroxydopamine treatment in combination with selective neuronal uptake inhibitors. *Br J Pharmacol.* 1990;99:655– 660.
22. Amara SG, Kuhar MJ. Neurotransmitter transporters: recent progress. *Annu Rev Neurosci.* 1993;16:73–93.
23. Schomig A. Adrenergic mechanisms in myocardial infarction: cardiac and systemic catecholamine release. *J Cardiovasc Pharmacol.* 1988; 12(suppl 1):S1–S7.

24. Headrick JP. Ischemic preconditioning: bioenergetic and metabolic changes and the role of endogenous adenosine. *J Mol Cell Cardiol.* 1996;28:1227–1240.
25. Seyfarth M, Feng Y, Hagl S, et al. Effect of myocardial ischemia on stimulation-evoked noradrenaline release: modulated neurotransmission in rat, guinea pig, and human cardiac tissue. *Circ Res.* 1993;73:496 –502.
26. Eisenhofer G, Esler MD, Cox HS, et al. Differences in the neuronal removal of circulating epinephrine and norepinephrine. *J Clin Endocrinol Metab.* 1990;70:1710 –1720.
27. Kennedy B, Ziegler MG. Cardiac epinephrine synthesis: regulation by a glucocorticoid. *Circulation.* 1991;84:891– 895.
28. Thompson JM, Wallin BG, Lambert GW, et al. Human muscle sympathetic activity and cardiac catecholamine spillover: no support for augmented sympathetic noradrenaline release by adrenaline co-transmission. *Clin Sci (Colch).* 1998;94:383–393.
29. Yamazaki T, Akiyama T, Kitagawa H, et al. A new, concise dialysis approach to assessment of cardiac sympathetic nerve terminal abnor-malities. *Am J Physiol.* 1997;272:H1182–H1187.
30. Cardinal R, Nadeau R, Novak V. Reduced capacity of cardiac efferent sympathetic neurons to release norepinephrine and modify cardiac function in tachycardia-induced canine heart failure. *Can J Physiol Pharmacol.* 1996;74:1070 –1078.
31. Rona G. Catecholamine cardiotoxicity. *J Mol Cell Cardiol.* 1985;17: 291–306.
32. Cruickshank JM, Neil-Dwyer G, Degaute JP, et al. Reduction of stress/catecholamine-induced cardiac necrosis by beta 1-selective blockade. *Lancet.* 1987;2:585–589.
33. Dhalla KS, Rupp H, Beamish RE, et al. Mechanisms of alterations in cardiac membrane Ca^{2+} transport due to excess catecholamines. *Car-diovasc Drugs Ther.* 1996;10(suppl 1):231–238.
34. Rump AF, Klaus W. Evidence for norepinephrine cardiotoxicity mediated by superoxide anion radicals in isolated rabbit hearts. *Naunyn-Schmiedebergs Arch Pharmacol.* 1994;349:295–300.
35. Du XJ, Woodcock EA, Little PJ, et al. Protection of neuronal uptake-1 inhibitors in ischemic and anoxic hearts by norepinephrine-dependent and -independent mechanisms. *J Cardiovasc Pharmacol.* 1998;32:621– 628.

Cardioprotection in pigs by exogenous norepinephrine but not by cerebral ischemia-induced release of endogenous norepinephrine

9

Cardioprotection in Pigs by Exogenous Norepinephrine, but not by Cerebral Ischemia-Induced Release of Endogenous Norepinephrine

Sandra de Zeeuw, Thomas W Lameris, Dirk J Duncker, Djo Hasan, Frans Boomsma, Anton H van den Meiracker, Pieter D Verdouw

Background Endogenous norepinephrine release induced by cerebral ischemia may lead to small areas of necrosis in normal hearts. Conversely, norepinephrine may be one of the mediators that limit myocardial infarct size (IS) by ischemic preconditioning. Because brief ischemia in kidneys, gut or skeletal muscle limits IS produced by a coronary artery occlusion, we investigated whether cardiac norepinephrine release during transient cerebral ischemia is also capable of eliciting remote myocardial preconditioning.

Methods and Results In 10 anesthetized control pigs, IS after a 60-min coronary occlusion and 120 min of reperfusion was 84±3% (mean±SEM) of the area at risk. Intracoronary infusion of 0.03 nmol/kg·min^{-1} norepinephrine for 10 min before coronary occlusion did not affect IS (80±3%, n=6), whereas infusion of 0.12 nmol/kg·min^{-1} limited IS (65±2%, n=7, $P<0.05$). Neither a 10-min (n=5) nor a 30-min (n=6) period of cerebral ischemia produced by elevation of intracranial pressure before coronary occlusion affected IS (83±4% and 82±3%, respectively). Myocardial interstitial norepinephrine levels tripled during cerebral ischemia and during low dose norepinephrine, but increased ten-fold during high dose norepinephrine. Norepinephrine levels increased progressively up to 500-fold in the area at risk during the 60-min coronary occlusion, independent of the pretreatment, while norepinephrine levels remained unchanged in adjacent non-ischemic myocardium and arterial plasma.

Conclusions Cerebral ischemia preceding a coronary occlusion did not modify IS, which is likely related to the modest increase in myocardial norepinephrine levels during cerebral ischemia. The IS limitation by high dose exogenous norepinephrine is not associated with blunting of the ischemia-induced increase in myocardial interstitial norepinephrine levels.
(Stroke, revision submitted)

Introduction

Ischemic preconditioning, originally described for the myocardium[1] is not organ specific as it also occurs in kidney,[2,3] skeletal muscle,[4,5] lung[6] and brain.[7,8] Przyklenk et al.[9] have shown that brief regional myocardial ischemia protects not only the jeopardized myocardium from infarction during a subsequent coronary artery occlusion, but also the adjacent "virgin" myocardium. Several groups of investigators have extended this observation and have shown that brief ischemia in remote organs such as kidney,[10-12] small intestines[10] and skeletal muscle,[13] is capable of limiting myocardial infarct size produced by a prolonged coronary artery occlusion. Norepinephrine is one of the mediators involved in the signaling pathway leading to ischemic preconditioning,[14-16] and because cerebral ischemia has been reported to cause a profound release of norepinephrine from sympathetic nerve endings in normal myocardium,[17] this raises the question whether transient cerebral ischemia prior to a coronary artery occlusion may also be cardioprotective. In addition, exogenous administration of norepinephrine before a coronary artery occlusion has been shown to elicit cardioprotection in rabbits[18] and rats.[19] The aim of the present study was therefore to study the effect of cerebral ischemia on myocardial infarct size produced by a coronary artery occlusion in pigs. Because the cardioprotective effect of norepinephrine has not been established in pigs, we first studied whether intracoronary infusions of norepinephrine are capable of limiting myocardial infarct size. A further aim was, using the microdialysis technique,[17,20] to quantitate the increase in myocardial norepinephrine concentrations during cerebral ischemia and exogenous norepinephrine infusions, and to determine whether limitation of infarct size by these interventions is mediated by attenuation of myocardial interstitial norepinephrine levels during the infarct-producing coronary artery occlusion.[21]

Methods

The present experiments conformed with the *"Guide for Care and Use of Laboratory Animals"* published by the US National Institutes of Health (NIH publication No. 85-23, revised 1996) and under the regulations of the Erasmus University Rotterdam.

Experimental Groups

Fourty one crossbred Landrace x Yorkshire pigs of either sex (34±1 kg) were assigned to one of 7 experimental groups of which in 6 groups myocardial infarct size was determined at the end of the protocol (Figure 1). Ten of these animals (Control) underwent only a 60-min occlusion of the left anterior descending coronary artery (LAD) followed by 120 min of reperfusion, while in 13 animals the 60-min LAD occlusion-reperfusion protocol was preceded by a 10-min infusion of norepinephrine into the LAD at a rate of either 0.03 nmol/kg·min^{-1} (NE_{LOW}, n=6) or 0.12 nmol/kg·min^{-1} (NE_{HIGH}, n=7). In 3 additional animals, the effects of a 10-min infusion of the high dose of norepinephrine ($NE_{HIGH\ SHAM}$) on regional myocardial function

and metabolism was evaluated in order to assess whether this dose produced myocardial ischemia and asynchrony of contraction. Infarct size was not determined in these animals. In 5 animals a 10-min period of global cerebral ischemia (CI_{10}) preceded the 60-min LAD-occlusion by 20 min, while in 6 animals the LAD-occlusion was preceded by a 30-min period of cerebral ischemia (CI_{30}) and 30 min of reperfusion. Finally, in 4 animals, we studied whether 30 min of global cerebral ischemia *per se* ($CI_{30\ SHAM}$) produced damage of normal myocardium. Cerebral ischemia was achieved by rapid infusion of artificial cerebrospinal fluid,[22] such that intracranial pressure increased to approximately 250 mmHg, which was invariably above the systolic arterial pressure. In all groups, a 120-min stabilization period followed the surgical procedures, after which baseline measurements of cardiovascular performance were made. Microdialysis was performed in the Control, NE_{LOW}, NE_{HIGH}, and CI_{30} groups. Dialysate samples were collected over 10-min periods for determination of myocardial interstitial norepinephrine concentrations starting 90 min into the stabilization period, a time at which norepinephrine concentrations have reached stable levels.[20,21] During the subsequent 30 min baseline dialysate samples were collected. Plasma samples were obtained halfway through each 10-min dialysate collection period Animals which encountered ventricular fibrillation during the LAD-occlusion or upon reperfusion, but in which sinus rhythm was successfully restored by DC-countershock within 2 min, were allowed to complete the experimental protocol.

Surgery

Overnight fasted pigs were sedated with ketamine (20-25 mg/kg i.m., Apharmo BV, Arnhem, The Netherlands), anesthetized with sodium pentobarbital (20 mg/kg i.v., Apharmo), intubated, and connected to a respirator for intermittent positive pressure ventilation with 30% oxygenated room air. Respiratory rate and tidal volume were set to keep arterial blood gases within the normal range. Catheters were inserted into the superior caval vein for infusion of sodium pentobarbital (10-15 $mg/kg \cdot h^{-1}$) to maintain a constant depth of anesthesia and saline for replacement of blood withdrawn during sampling. A fluid-filled catheter was placed in the descending aorta for the measurement of aortic blood pressure and collection of arterial blood samples, while a micromanometer-tipped catheter was inserted into the left carotid artery and advanced into the left ventricle for the measurement of left ventricular pressure (LVP) and its first derivative (LVdP/dt). After administration of pancuronium bromide (4 mg, Organon Teknika BV, Boxtel, The Netherlands), a midsternal thoracotomy was performed, and the heart was suspended in a pericardial cradle. An electromagnetic flow probe (Skalar, Delft, The Netherlands) was placed around the ascending aorta for the measurement of cardiac output, while the segment of the LAD between the first and the second diagonal branch was dissected free for placement of a Doppler flow probe (Triton Technology Inc., San Diego, CA, U.S.A.) and a microvascular clamp. In the 16 animals which received intracoronary infusions of

norepinephrine (NE_{LOW}, NE_{HIGH}, and $NE_{HIGH\ SHAM}$), a small cannula was inserted into the LAD distal to the flow probe.

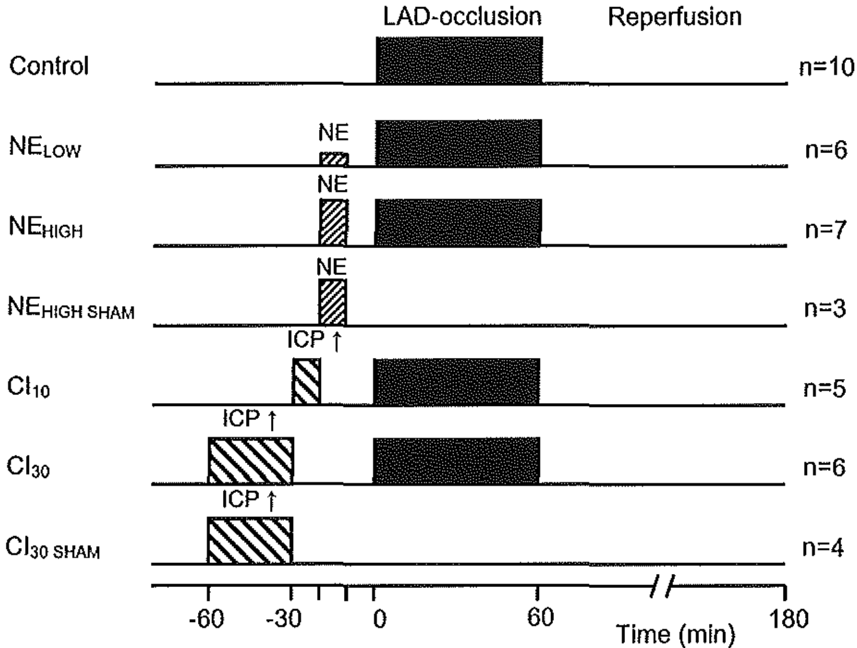

Figure 1 Diagram showing the 7 experimental groups. Norepinephrine (NE) was infused at a rate of either 0.03 (NE_{LOW}) or 0.12 nmol/kg·min^{-1} (NE_{HIGH} and $NE_{HIGH\ SHAM}$) into the left arterial descending coronary artery (LAD). Global cerebral ischemia (CI) produced by elevating intracranial pressure (ICP) to 250 mmHg, was maintained for either 10 min (CI_{10}) or 30 min (CI_{30} and $CI_{30\ SHAM}$). Infarct size was determined at the end of 120 min of reperfusion in all groups except in $NE_{HIGH\ SHAM}$.

Microdialysis probes were implanted in the left ventricular myocardium using a steel guiding needle and split plastic tubing: one in the area perfused by the LAD and one in the area perfused by the left circumflex coronary artery (LCX). Finally, in the CI_{30} group a third microdialysis probe was placed in the cortex of the brain. Perfusion of the probes started immediately after insertion.[20,21]

In the $CI_{30\ SHAM}$ and $NE_{HIGH\ SHAM}$ groups ultrasound crystals were implanted to assess the effect on regional myocardial wall function using sonomicrometry (Triton Technology Inc., San Diego, CA). Each pair of crystals was implanted in the midmyocardial layer approximately 10 mm apart and parallel to the fiber direction. In these animals also the great cardiac vein accompanying the LAD was cannulated for collection of coronary venous blood samples. Finally, in the $NE_{HIGH\ SHAM}$ group, also the left atrium was cannulated for injection of

radioactive microspheres (^{113}Sn or ^{141}Ce, 15±1 (SD) μm) to determine the distribution of myocardial blood flow at baseline and at the end of the norepinephrine infusion.[1]

In order to produce cerebral ischemia, two catheters were inserted into the left and right cerebral lateral ventricles through bore holes.[23] A fluid-filled catheter was used for infusion of the artificial cerebrospinal fluid to elevate intracranial pressure, which was monitored with a micromanometer-tipped catheter.

Microdialysis Methodology

The polycarbonate dialysis membrane of the microdialysis probes (CMA/20, Carnegie Medicine AB, Sweden) has a cut-off value of 20 kD, a length of 10 mm and a diameter of 0.5 mm. Cardiac probes were perfused with an isotonic Ringer's solution and the cerebral probe with the artificial cerebrospinal fluid, at a rate of 2 μl/min using a CMA/100 microinjection pump. Dialysate volumes of 20 μl (sampling time 10 min) were collected in microvials containing 20 μl of a solution of 2% ($^{w}/_{v}$) EDTA and 30 nM l-erythro-alpha-methyl-norepinephrine (AMN) as internal standard in 0.08 N acetic acid. Plasma samples were drawn into chilled heparinized tubes containing 12 mg glutathione. All samples were stored at –80°C, until analysis within the next five days.[20,21,24]

In vivo probe recovery of norepinephrine (52±1%) has previously been determined by retrodialysis, using AMN as a calibrator and by direct comparison of hemo-microdialysis and plasma samples.[20,21,25]

Infarct Size Determination

At the end of the 120-min reperfusion period, the LAD was reoccluded and the area at risk was determined by intra-atrial infusion of 20 ml of 5% ($^{w}/_{w}$) fluorescein sodium.[26] Ventricular fibrillation was then induced using a 9V battery and the heart was excised. Subsequently, the left ventricle was isolated and cut parallel to the atrioventricular groove into five slices of equal thickness. After the area at risk was demarcated on an acetate sheet under ultraviolet light, slices were incubated in 0.125 g para-nitrobluetetrazolium (Sigma Chemicals Co., St Louis, U.S.A.) per liter of phosphatebuffer (pH 7.4) at 37°C for 30 min, after which the non-stained pale infarcted area was also traced onto the sheet. The area at risk and the infarct area were determined by summation of the areas at risk and infarct areas of the individual rings. Myocardial infarct size was defined as the ratio of the infarct area and area at risk.

Regional Myocardial Function and Perfusion

From the segment length tracings percent systolic shortening (SS) was calculated as the difference in segment length at end-diastole and the minimal segment length during systole divided by the segment length at end-diastole. The development of asynchrony during local

infusion of norepinephrine was assessed by determining the time interval between the occurrence of minimal segment length (L_{min}) in the areas perfused by the LAD and LCX.

Myocardial O_2-extraction (%) was calculated as the ratio of the arterio – coronary venous O_2-content difference and the arterial O_2-content. At the end of the experiment, the heart was excised, the LAD and LCX perfused areas were separated and divided into three layers of equal thickness. The subendocardial (inner layer) and subepicardial (outer layer) blood flows and their ratios were determined using standard techniques.[1]

Statistical Analysis

All data have been expressed as mean±SEM. Statistical significance ($P<0.05$) for the changes in hemodynamics and norepinephrine concentrations was determined by two-way ANOVA and one-way ANOVA for repeated measures, followed by Dunnett's multiple comparison test. Statistical significance ($P<0.05$) for differences in infarct size was determined using one-way ANOVA without replication followed by Student's *t* test.

Results

Hemodynamics

Norepinephrine infusions. Intracoronary norepinephrine infusion in the NE_{LOW} group produced an increase in $LVdP/dt_{max}$, reflecting an increase in regional contractility as the other cardiovascular variables remained unaffected (Table 1). During the 10-min washout period $LVdP/dt_{max}$ returned to baseline. During norepinephrine infusion in the NE_{HIGH} + $NE_{HIGH\ SHAM}$ animals, mean arterial pressure decreased rapidly from 91±2 to 74±6 mmHg at 5 min followed by a gradual recovery (Table 1). The decrease in cardiac output was responsible for the fall in arterial blood pressure as systemic vascular resistance remained unchanged. Cardiac output decreased, because the increase in heart rate was insufficient to compensate for the decrease in stroke volume. The latter occurred despite the increase in $LVdP/dt_{max}$, and was likely due to asynchrony of contraction (see below). At the end of the 10-min norepinephrine infusion mean arterial pressure was still slightly depressed (10%), while heart rate (10%) and $LVdP/dt_{max}$ (65%) remained elevated. All parameters recovered during the 10-min washout period that preceded the 60-min LAD-occlusion.

After 2 min of norepinephrine infusion in the $NE_{HIGH\ SHAM}$ group SS in the LAD perfused myocardium increased from 27±2% at baseline to 34±4%, while SS in the LCX area decreased from 18±1% to 13±1% (both $P<0.05$). SS in either area did not change further during the remainder of the infusion period. These changes were accompanied by asynchrony of contraction between the LAD and LCX areas. Thus, whereas under baseline conditions L_{min} of both areas occurred at the end of global left ventricular systole, during norepinephrine the occurrence of L_{min} in the LAD area preceded L_{min} in the LCX area by 119±2 ms ($P<0.05$). The latter was due to L_{min} in the LAD area occurring 56±15 ms before and L_{min} in the LCX area

Table 1 Cardiovascular Hemodynamics During Norepinephrine Infusion or Cerebral Ischemia

	Treatment	Baseline	Δ abs from Baseline				
			2 min	5 min	10 min	30 min	Recovery
MAP, mmHg	NE_{LOW}	93±3	0±3	2±2	3±3	-	-1±2
	NE_{HIGH} + $NE_{HIGH\ SHAM}$	91±2	-15±6*	-17±5*	-8±4	-	-2±2
	CI_{10}	92±5	80±10*	53±13*	-11±5	-	-16±12
	CI_{30} + $CI_{30\ SHAM}$	90±4	83±6*	67±8*	-19±4*	-17±5*	-20±4*
CO, l·min^{-1}	NE_{LOW}	2.9±0.2	0.0±0.1	0.0±0.1	0.0±0.1	-	-0.1±0.1
	NE_{HIGH} + $NE_{HIGH\ SHAM}$	2.5±0.2	-0.4±0.2*	-0.5±0.2*	-0.2±0.1	-	-0.1±0.1
	CI_{10}	3.8±0.5	0.7±0.2*	2.1±0.4*	0.7±0.3	-	-0.3±0.6
	CI_{30} + $CI_{30\ SHAM}$	3.1±0.2	0.7±0.2*	2.2±0.3*	0.4±0.1*	0.9±0.2*	-0.2±0.4
SVR, mmHg/ l·min^{-1}	NE_{LOW}	33±3	0.2±0.8	0.4±0.7	1.1±0.7	-	1.1±1.1
	NE_{HIGH} + $NE_{HIGH\ SHAM}$	37±3	-0.6±1.4	0.7±0.9	0.6±1.0	-	1.2±0.9
	CI_{10}	26±3	15.9±4.6*	1.4±4.4	-7.4±1.9*	-	-3.6±1.0*
	CI_{30} + $CI_{30\ SHAM}$	30±2	18.8±4.2*	1.8±3.2	-8.8±1.3*	-11.0±1.5*	-9.0±2.1*
HR, bpm	NE_{LOW}	107±7	1±1	1±2	1±2	-	1±3
	NE_{HIGH} + $NE_{HIGH\ SHAM}$	117±6	13±4*	15±5*	12±5*	-	2±2
	CI_{10}	112±5	37±8*	68±12*	19±13	-	17±10
	CI_{30} + $CI_{30\ SHAM}$	101±3	46±5*	79±6*	22±4*	2±3	9±7
SV, ml	NE_{LOW}	27±4	1.1±1.5	1.5±1.4	1.2±1.2	-	0.5±1.1
	NE_{HIGH} + $NE_{HIGH\ SHAM}$	22±2	-5.4±1.9*	-6.4±1.6*	-3.8±1.7	-	-1.1±0.7
	CI_{10}	33±3	-2.6±2.2	0.2±3.8	1.4±3.0	-	-5.8±3.0
	CI_{30} + $CI_{30\ SHAM}$	30±2	-4.4±1.8*	-0.8±1.5	-1.8±1.2	8.0±1.6*	-4.2±3.2
LVdP/dt$_{max}$, mmHg·s^{-1}	NE_{LOW}	1560±110	760±100*	790±90*	850±60*	-	-60±70
	NE_{HIGH} + $NE_{HIGH\ SHAM}$	2030±200	860±210*	890±160*	1270±120*	-	-150±70
	CI_{10}	1790±170	2810±660*	4650±910*	-310±410	-	410±560
	CI_{30} + $CI_{30\ SHAM}$	1640±120	2570±440*	5010±340*	160±230	-90±170	50±220
LVEDP, mmHg	NE_{LOW}	8±2	1.2±0.5	1.3±0.6	1.9±0.8	-	1.1±0.6
	NE_{HIGH} + $NE_{HIGH\ SHAM}$	8±1	-1.7±0.8	-2.0±0.8*	-1.4±0.6	-	0.7±0.6
	CI_{10}	9±1	8.5±1.7*	1.5±1.2	0.2±2.4	-	-2.5±1.8
	CI_{30} + $CI_{30\ SHAM}$	7±1	10.6±2.5*	0.9±1.7	-1.2±1.2	1.8±1.5	0.0±0.9
CBF, ml/ming^{-1}	NE_{low}	1.7±0.2	0.3±0.2	0.3±0.2	0.3±0.1	-	0.0±0.2
	NE_{HIGH} + $NE_{HIGH\ SHAM}$	1.0±0.1	0.0±0.1	0.1±0.1	0.3±0.1	-	0.0±0.1
	CI_{10}	1.0±0.2	1.0±0.2*	0.7±0.1*	0.0±0.1	-	-0.1±0.1
	CI_{30} + $CI_{30\ SHAM}$	1.3±0.1	1.3±0.2*	1.4±0.3*	-0.1±0.1	0.2±0.2	-0.1±0.1

MAP, mean arterial blood pressure; CO, cardiac output; SVR, systemic vascular resistance; HR, heart rate; SV, stroke volume; LVdP/dtmax, maximal rate of rise in left ventricular pressure; LVEDP, left ventricular end-diastolic pressure; CBF,coronary blood flow. NE_{low} and NE_{high} refer to the infusion rates of norepinephrine (0.03 and 0.12 nmol/kg·min^{-1}, respectively); CI_{10} and CI_{30} refer to the durations of cerebral ischemia (CI) (10 and 30 min, respectively); The $NE_{HIGH\ SHAM}$ and $CI_{30\ SHAM}$ animals did not undergo the 60-min LAD-occlusion and 120-min reperfusion protocol LVdP/dt$_{max}$, maximal rate of rise of left ventricular pressure. Data are mean±SEM; n=6 (NE_{LOW}), n=10 (NE_{HIGH} + $NE_{HIGH\ SHAM}$), n=5 (CI_{10}), n=10 (CI_{30} + $CI_{30\ SHAM}$); *P<0.05 vs Baseline.

occurring 63±7 ms after closure of the aortic valves (both $P<0.05$ vs their respective baseline values). During washout all wall function parameters returned to baseline values.

In the LAD area of the $NE_{HIGH\ SHAM}$ group, O_2-extraction decreased slightly from 64±7% at baseline to 54±7% during norepinephrine infusion, indicating that O_2-delivery increased slightly in excess of the increase in myocardial O_2-demand. In agreement with this finding, myocardial proton release was not increased, as reflected by the maintained arterio – coronary venous pH difference (0.06±0.01 at baseline and 0.06±0.01 at the end of infusion). Moreover, the subendocardial to subepicardial blood flow ratio remained unchanged in both the LAD area (1.14±0.25 at baseline and 1.30±0.17 at the end of infusion) and the LCX area (1.21±0.07 and 1.24±0.05, respectively). Finally, SS in the LAD and LCX areas returned to baseline values immediately during the recovery period (24±3% and 17±1%, respectively), indicating that the infusion of norepinephrine did not produce myocardial ischemia and stunning.

Cerebral ischemia. The elevation of intracranial pressure from 12±2 mmHg at baseline to 250 mmHg produced an immediate increase in mean aortic pressure in both the CI_{10} and CI_{30} + $CI_{30\ SHAM}$ animals, which was initially the consequence of increases in both cardiac output and systemic vascular resistance (Table 1). However, after 5 min the tachycardia-mediated increase in cardiac output was exclusively responsible for the elevated arterial blood pressure. Despite the dramatic increase in afterload, stroke volume was maintained most likely due to enhanced myocardial contractility as $LVdP/dt_{max}$ increased up to four times its baseline value. The increase in coronary blood flow paralleled the increase in myocardial oxygen demand, reflected by the 150% increase in double product (heart rate · systolic arterial pressure).

Similar to earlier observations in dogs[27] and pigs,[17] the transient hyperdynamic phase was followed by a fall in mean arterial pressure below baseline levels at 10 min of cerebral ischemia, which was the result of a decrease in systemic vascular resistance. Except heart rate, which remained slightly elevated, all other variables had recovered at 10 min. In the CI_{30} + $CI_{30\ SHAM}$ animals, mean arterial pressure, cardiac output and systemic vascular resistance did not change further during the remainder of the 30 min period. Although cardiac output did not change, heart rate returned to baseline levels, and stroke volume increased. During the recovery period, intracranial pressure returned to baseline values (12±2 mmHg), and except for mean arterial pressure and systemic vascular resistance, all other variables had also recovered (Table 1).

The elevation of the intracranial pressure produced a small decrease in myocardial O_2-extraction from 64±5% at baseline to 58±6% at 5 min and no change in myocardial proton release (i.e. no widening of the arterio – coronary venous pH difference: 0.04±0.01 both at baseline and at 5 min), indicating the absence of myocardial ischemia. The elevation of the intracranial pressure decreased SS from 24±1% to 16±2% at 2 min, but at 5 min SS had already recovered to 23±1% and to 26±1% at 10 min, with no evidence of depressed regional

Table 2 Cardiovascular Hemodynamics During Coronary Occlusion and Reperfusion

	Treatment	Pre-occlusion	End-occlusion	End-reperfusion
MAP, mmHg	Control	90±2	75±2*	76±6*
	NE_{LOW}	93±4	72±6*	70±5*
	NE_{HIGH}	87±4	77±5	77±4
	CI_{10}	75±9	74±5	65±5
	CI_{30}	65±4	61±5	66±4
CO, l·min^{-1}	Control	2.6±0.2	2.1±0.1*	2.1±0.2*
	NE_{LOW}	2.8±0.2	2.3±0.2*	1.9±0.1*
	NE_{HIGH}	2.2±0.2	1.9±0.2*	1.8±0.2*
	CI_{10}	3.5±0.4	2.9±0.3	2.5±0.4
	CI_{30}	2.9±0.3	2.2±0.2*	2.0±0.1*
SVR, mmHg/l·min^{-1}	Control	38±3	37±3	38±4
	NE_{LOW}	34±4	32±3	38±4
	NE_{HIGH}	41±4	43±4	45±5
	CI_{10}	22±3	27±4	28±4*
	CI_{30}	24±2	28±2*	33±2*
HR, bpm	Control	115±4	123±7	139±8*
	NE_{LOW}	108±9	112±9	119±13
	NE_{HIGH}	124±6	133±11	136±11
	CI_{10}	129±7	119±9	120±7
	CI_{30}	112±6	112±5	115±6
SV, ml	Control	22±2	18±1*	16±1*
	NE_{LOW}	27±4	22±3	17±2
	NE_{HIGH}	18±2	14±2*	13±1*
	CI_{10}	28±4	25±4	21±3
	CI_{30}	25±2	20±2*	18±1*
LVdP/dt$_{max}$, mmHg·s^{-1}	Control	1610±100	1480±100	1640±260
	NE_{LOW}	1500±110	1350±110	1250±100
	NE_{HIGH}	1980±280	2060±350	2050±340
	CI_{10}	2200±410	1630±140	1350±140
	CI_{30}	1710±320	1250±160	1170±100
LVEDP, mmHg	Control	7±1	9±1	10±1*
	NE_{LOW}	9±2	10±1	10±1
	NE_{HIGH}	8±2	10±2	12±1
	CI_{10}	7±3	13±3	13±1
	CI_{30}	6±1	9±2*	12±1*

MAP, mean arterial blood pressure; CO, cardiac output; SVR, systemic vascular resistance; HR, heart rate; SV, stroke volume; LVdP/dt$_{max}$, maximal rate of rise in left ventricular pressure; LVEDP, left ventricular end-diastolic pressure. Pre-occlusion values of the Norepinephrine and Cerebral Ischemia groups correspond to the recovery values in Table 1; NE low and NE high refer to the infusion rates of norepinephrine (0.03 and 0.12 nmol/kg·min^{-1}, respectively); CI_{10} and CI_{30} refer to the durations of cerebral ischemia (CI) (10 and 30 min, respectively); LVdP/dt$_{max}$, maximal rate of rise of left ventricular pressure.
Data are mean±SEM; n=10 (Control), n=6 (NE_{LOW}), n=7 (NE_{HIGH}), n=5 (CI_{10}), n=6 (CI_{30}); *P<0.05 vs Pre-occlusion.

wall function during the remainder of the 30 min period (27±1%) or the subsequent recovery phase (23±3%).

LAD-occlusion and reperfusion. Mean arterial pressure in the control group decreased secondary to the decrease in cardiac output during the 60-min LAD-occlusion and did not change further during reperfusion (Table 2). Heart rate increased slightly, but insufficiently to compensate for the decrease in stroke volume.

Pretreatment with norepinephrine had no effect on the hemodynamic responses during the subsequent LAD-occlusion and reperfusion, in either the NE_{LOW} or NE_{HIGH} group. In the animals subjected to cerebral ischemia prior to the LAD-occlusion, mean aortic pressure did not further decrease during the LAD-occlusion most likely because systemic vascular resistance, which was still below baseline levels at the onset of the LAD-occlusion, recovered.

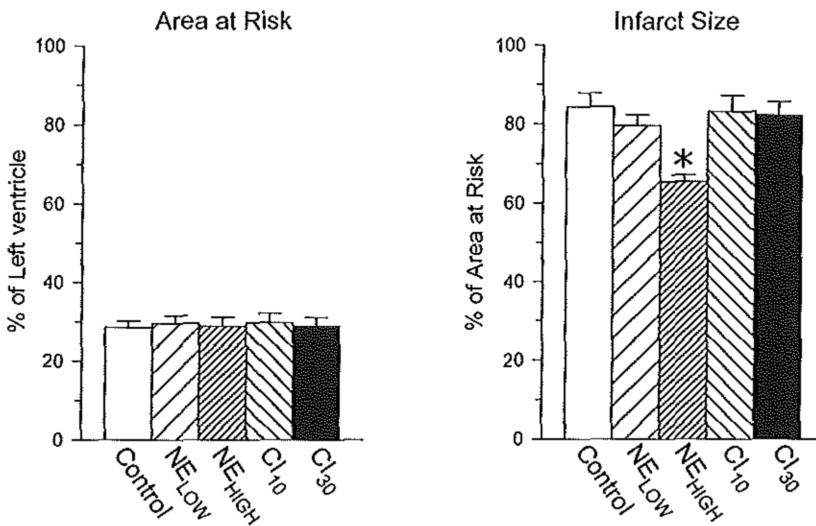

*Figure 2 Area at risk and infarct size for the 5 experimental groups in which the LAD was occluded for 60 min and reperfused for 120 min. Global cerebral ischemia alone did not cause irreversible myocardial damage (not shown). *P<0.05 vs Control. For further details see Figure 1.*

Myocardial Infarct Size

The area at risk of the Control group and of the groups pretreated with norepinephrine or cerebral ischemia prior to the 60-min LAD-occlusion were identical (Figure 2). Infarct size was 84±3%, in the Control group and 80±3% in the NE_{LOW} group, but was only 65±2% in the NE_{HIGH} group ($P<0.05$). Cerebral ischemia had no effect on infarct size development during the 60-min LAD occlusion as in the CI_{10} and CI_{30} groups infarct size was 83±4% and 82±3%,

respectively. Cerebral ischemia *per se* did not cause irreversible damage as in none of the $CI_{30\,SHAM}$ animals infarct tissue was detected.

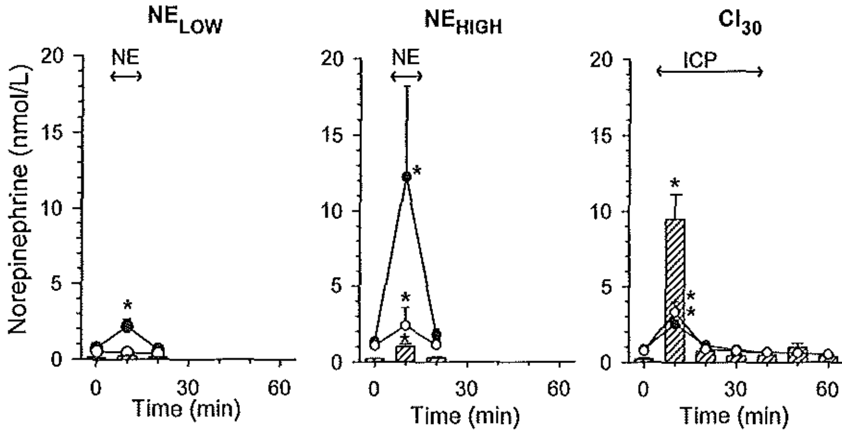

*Figure 3 Norepinephrine levels in plasma (hatched bars) and in the interstitium of left ventricular myocardium perfused by the LAD (full circles) and LCX (open circles) during the 10-min norepinephrine infusions and during 30 min of cerebral ischemia. *P<0.05 vs Baseline. For further details see Figure 1.*

Myocardial Interstitial Norepinephrine Concentrations

The myocardial interstitial norepinephrine levels in the LAD perfused area increased from 0.8±0.2 nmol/l to 2.2±0.5 nmol/l during norepinephrine infusion in the group NE_{LOW}, and to 12.2±5.9 nmol/l in the NE_{HIGH} group (both $P<0.05$, Figure 3). Despite the intracoronary route of administration, there was some spillover in the NE_{HIGH} animals as evidenced by small transient increments of norepinephrine in plasma from 0.2±0.1 to 1.0±0.2 nmol/l and in the interstitium of the LCX-perfused myocardium from 1.1±0.3 to 2.4±1.2 nmol/l (both $P<0.05$, Figure 3).

In the CI_{30} group, cerebral interstitial norepinephrine levels increased from 0.9±0.4 nmol/l at baseline to 6.1±1.9 nmol/l at 10 min of intracranial pressure elevation and increased gradually up to 8.3±1.8 nmol/l at 30 min (not shown in Figure 3). Upon cerebral reperfusion, interstitial levels initially increased further to 12.3±2.3 nmol/l but returned to baseline during the 30-min recovery period. Cerebral ischemia resulted in a transient tripling of interstitial norepinephrine levels in both the LAD- and LCX-perfused myocardium, and a 20-fold increase in plasma norepinephrine levels (Figure 3).

In the Control, NE_{LOW}, NE_{HIGH}, and CI_{30} groups, norepinephrine levels increased progressively during the LAD-occlusion by up to approximately 500-fold, and decreased to baseline levels during reperfusion, independent of the preceding intervention (Figure 4). There was no correlation (r = 0.03) between the maximum interstitial norepinephrine levels during the LAD-occlusion and myocardial infarct size.

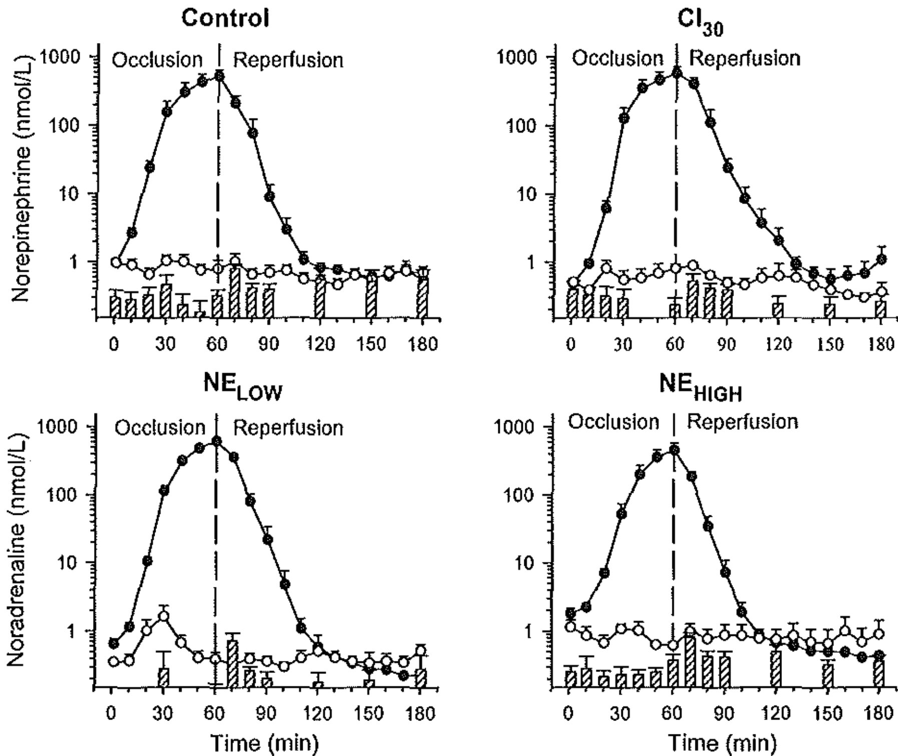

Figure 4 Norepinephrine levels in plasma (hatched bars) and in the interstitium of left ventricular myocardium perfused by the LAD (full circles) and LCX (open circles) during 60-min LAD occlusion and 120 min of reperfusion. Notice that the levels in the LAD-perfused myocardium increased 500-fold during LAD-occlusion independent of the preceding intervention, while the levels in the LCX-perfused myocardium and plasma remained unchanged.

Discussion

The major findings of the present study in swine are: (*i*) global cerebral ischemia, produced by either a 10-min or a 30-min elevation of intracranial pressure, which by itself produced no irreversible myocardial damage, had no effect on myocardial infarct size produced by a 60-min coronary artery occlusion; (*ii*) intracoronary infusion of 0.03 $nmol/kg{\cdot}min^{-1}$ norepinephrine produced similar increases in myocardial interstitial levels as cerebral ischemia, and did also not limit myocardial infarct size; (*iii*) conversely, intracoronary infusion of 0.12 $nmol/kg{\cdot}min^{-1}$ of norepinephrine that resulted in five times higher myocardial interstitial norepinephrine levels than cerebral ischemia and low dose norepinephrine was capable of limiting myocardial infarct size; (*iv*) the cardioprotection by exogenous norepinephrine was not caused by *ischemic* preconditioning and (*v*) this protection was not

associated with a blunting of the progressive increase in myocardial interstitial norepinephrine levels during the coronary artery occlusion.

Catecholamines and myocardial injury

The relation between catecholamines and myocardial injury was first established by Rona and co-workers,[28-30] who showed some 40 years ago that administration of high systemic doses of isoproterenol produced focal necrotic lesions in normal rat hearts.[29]

Elevation of intracranial pressure is well recognized as a cause for myocardial dysfunction and injury. Brain death caused by increased intracranial pressure has been shown to produce echocardiographic alterations, hemodynamic instability, and contraction band necrosis, all of which have been suggested to be the result of massive neuronal depolarization and release of catecholamines.[31-35] These clinical observations initiated a large number of experimental investigations in which deleterious effects of brain death on function and integrity of normal myocardium were found, but generally no or only minimal focal myocardial necrosis could be demonstrated.[36-40]

In view of the massive release of norepinephrine in the myocardium during coronary artery occlusion,[21,41] it could be hypothesized that catecholamines may contribute to the development of irreversible injury during a coronary artery occlusion. Several,[42-45] though certainly not all,[46-48] studies have reported that β-adrenoceptor blockade slows the development of myocardial infarction produced by a coronary artery ligation. In contrast, depletion of cardiac norepinephrine stores by reserpination, did not limit myocardial infarct size in rabbits[14] and dogs,[16] suggesting that endogenous catecholamines do not contribute to irreversible damage.

In contrast to the potentially deleterious effects of norepinephrine on normal and ischemic myocardium, this catecholamine has also been implicated in mediating cardioprotection by ischemic preconditioning. Thus, Toombs et al.[14] have shown that in rabbits the protection by ischemic preconditioning was abolished when catecholamine stores in sympathetic nerve endings were depleted by prior administration of reserpine. Furthermore, Thornton et al.[18] demonstrated in the same species that tyramine-induced norepinephrine release 10 min before a 30-min coronary artery occlusion also protected the myocardium. This cardioprotective action of catecholamines has been confirmed in other species such as the rat[19] and the dog.[15,16] We now show that a high dose of norepinephrine can also protect the porcine myocardium against a sustained ischemic epidose.

Our data on wall function, (distribution of) myocardial blood flow, O_2-extraction and proton release indicate that the high dose of norepinephrine did not produce myocardial ischemia and did therefore not protect the myocardium by ischemic preconditioning. In support of this observation, the degree of protection afforded by norepinephrine was less than what we previously reported for ischemic preconditioning, but similar to that produced by

other non-ischemic stimuli, like ventricular pacing[49] and pharmacological agents such as the K^+_{ATP} channel openers bimakalim[50] and aprikalim.[51] Since all these stimuli have in common that they ultimately activate K^+_{ATP} channels, it is tempting to speculate that norepinephrine also protected via α_1-adrenoceptor-mediated protein kinase C activation and subsequent opening of (mitochondrial) K^+_{ATP} channels.[52] Another mechanism by which norepinephrine might protect the myocardium is via a blunted release in catecholamines during the sustained ischemic episode as has been suggested for ischemic preconditioning.[53] However, pretreatment with norepinephrine did not modify the release of cardiac norepinephrine during sustained myocardial ischemia, in the present study, implying that the norepinephrine-mediated cardioprotection is not related to a blunting of the ischemia-induced increase in norepinephrine levels.

Finally, the present study clarifies another issue on the role of norepinephrine in cardioprotection. Przyklenk et al.[9] demonstrated that myocardial ischemia could also elicit cardioprotection in adjacent virgin myocardium and speculated that this cardioprotective effect might have been triggered by a substantial release of catecholamines in that adjacent region. However, the present study shows that norepinephrine levels in the normal (LCX-perfused) myocardium remained unaltered during and after the 60-min LAD-occlusion (Figure 4), even though the interstitial norepinephrine levels in the LAD-perfused myocardium were 100-fold higher than the value observed after 10 min of ischemia, corresponding to the period used by Przyklenk et al.[9] to produce preconditioning of the adjacent virgin myocardium.

Cerebral ischemia as a stimulus for cardioprotection

It is well documented that transient ischemia in small intestines, kidneys and skeletal muscle prior to a coronary artery occlusion can be cardioprotective.[10-13] We therefore hypothesized that cerebral ischemia might similarly protect the myocardium, especially because cerebral ischemia is associated with substantial norepinephrine release, one of the mediators involved in cardioprotection by ischemic preconditioning. However, in the present study transient cerebral ischemia did not reduce myocardial infarct size. The explanation for the lack of protection might be two-fold. Firstly, 30 min of cerebral ischemia did not produce myocardial ischemia and could not protect the myocardium via ischemic preconditioning. Secondly, although myocardial norepinephrine levels increased during 30 min of cerebral ischemia the failure to elicit cardioprotection appears to be related to the magnitude of the rise in myocardial interstitial norepinephrine levels, as the high dose of norepinephrine, which yielded 5 times higher interstitial concentrations than cerebral ischemia elicited cardioprotection. This is further corroborated by the findings with the low dose of norepinephrine, which produced similar interstitial myocardial norepinephrine levels as cerebral ischemia, and was also ineffective in protecting the heart.

It could be argued that even the 30-min global cerebral ischemia (CI_{30}) was too short to elicit cardioprotection. However, there is ample evidence that the intensity of the preconditioning stimulus is more important than its duration.[54,55] Moreover, because the elevation of myocardial interstitial norepinephrine levels occurred exclusively during the first 10 min of cerebral ischemia, it is unlikely that extending the period of cerebral ischemia would produce cardioprotection. On the contrary, it might be argued that the duration of the intracranial pressure elevation and recovery phase lasted *too long* as the maximum myocardial interstitial norepinephrine levels reached their peak during the first 10 min, so that a potential effect of that stimulus was lost by the time (50 min later) the LAD was occluded for 60 min. This is supported by observations that the memory for cardioprotection is shorter when stimuli are used that do not cause myocardial ischemia.[49,56,57] However, when cerebral ischemia was maintained for only 10 min, and the cerebral reperfusion was shortened to 20 min (CI_{10}), infarct size after the 60-min coronary artery occlusion was also not different from control.

Finally, it can be excluded that a protective effect of transient global cerebral ischemia exerted during the coronary artery occlusion was masked by irreversible myocardial damage produced by transient global cerebral ischemia prior to the coronary artery occlusion, as in the animals which were subjected to only the 30 min of cerebral ischemia ($CI_{30\ SHAM}$), irreversible damage could not be detected. This observation is in agreement with most experimental studies that have generally reported minimal or no focal myocardial necrosis following cerebral ischemia.[36-38,58]

Conclusions

In conclusion, global cerebral ischemia preceding a coronary artery occlusion did not modify myocardial infarct size, which is likely related to the modest increase in myocardial norepinephrine levels during cerebral ischemia. The infarct size limitation by the high dose of norepinephrine is not associated with a blunting of the increase in myocardial interstitial norepinephrine levels during the coronary occlusion.

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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;74:1124-1136.
2. Cochrane J, Williams BT, Banerjee A, Harken AH, Burke TJ, Cairns CB, Shapiro JI. Ischemic preconditioning attenuates functional, metabolic, and morphologic injury from ischemic acute renal failure in the rat. *Ren Fail*. 1999;21:135-145.

3. Toosy N, McMorris EL, Grace PA, Mathie RT. Ischaemic preconditioning protects the rat kidney from reperfusion injury. *BJU Int.* 1999;84:489-494.
4. Pang CY, Neligan P, Zhong A, He W, Xu H, Forrest CR. Effector mechanism of adenosine in acute ischemic preconditioning of skeletal muscle against infarction. *Am J Physiol.* 1997;273:R887-R895.
5. Whetzel TP, Stevenson TR, Sharman RB, Carlsen RC. The effect of ischemic preconditioning on the recovery of skeletal muscle following tourniquet ischemia. *Plast Reconstr Surg.* 1997;100:1767-1775.
6. Li G, Chen S, Lu E, Hu T. Protective effects of ischemic preconditioning on lung ischemia reperfusion injury: an in-vivo rabbit study. *Thorac Cardiovasc Surg.* 1999;47:38-41.
7. Perez-Pinzon MA, Alonso O, Kraydieh S, Dietrich WD. Induction of tolerance against traumatic brain injury by ischemic preconditioning. *Neuroreport.* 1999;10:2951-2954.
8. Stagliano NE, Perez-Pinzon MA, Moskowitz MA, Huang PL. Focal ischemic preconditioning induces rapid tolerance to middle cerebral artery occlusion in mice. *J Cereb Blood Flow Metab.* 1999;19:757-761.
9. 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.
10. 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.
11. Pell TJ, Baxter GF, Yellon DM, Drew GM. Renal ischemia preconditions myocardium: role of adenosine receptors and ATP-sensitive potassium channels. *Am J Physiol.* 1998;275:H1542-H1547.
12. Takaoka A, Nakae I, Mitsunami K, Yabe T, Morikawa S, Inubushi T, Kinoshita M. Renal ischemia/reperfusion remotely improves myocardial energy metabolism during myocardial ischemia via adenosine receptors in rabbits: effects of "remote preconditioning". *J Am Coll Cardiol.* 1999;33:556-564.
13. Birnbaum Y, Hale SL, Kloner RA. Ischemic preconditioning at a distance: reduction of myocardial infarct size by partial reduction of blood supply combined with rapid stimulation of the gastrocnemius muscle in the rabbit. *Circulation.* 1997;96:1641-1646.
14. Toombs CF, Wiltse AL, Shebuski RJ. Ischemic preconditioning fails to limit infarct size in reserpinized rabbit myocardium. Implication of norepinephrine release in the preconditioning effect. *Circulation.* 1993;88:2351-2358.
15. Kitakaze M, Hori M, Morioka T, Minamino T, Takashima S, Sato H, Shinozaki Y, Chujo M, Mori H, Inoue M, et al. Alpha 1-adrenoceptor activation mediates the infarct size-limiting effect of ischemic preconditioning through augmentation of 5'- nucleotidase activity. *J Clin Invest.* 1994;93:2197-2205.
16. Vander Heide RS, Schwartz LM, Jennings RB, Reimer KA. Effect of catecholamine depletion on myocardial infarct size in dogs: role of catecholamines in ischemic preconditioning. *Cardiovasc Res.* 1995;30:656-662.
17. Mertes PM, Burtin P, Carteaux JP, Jaboin Y, Dopff C, Pinelli G, Villemot JP, Burlet C, Boulange M. Brain death and myocardial injury: role of cardiac sympathetic innervation evaluated by *in vivo* interstitial microdialysis. *Transplant Proc.* 1994;26:231-232.
18. Thornton JD, Daly JF, Cohen MV, Yang XM, Downey JM. Catecholamines can induce adenosine receptor-mediated protection of the myocardium but do not participate in ischemic preconditioning in the rabbit. *Circ Res.* 1993;73:649-655.
19. Asimakis GK, Inners-McBride K, Conti VR, Yang CJ. Transient beta adrenergic stimulation can precondition the rat heart against postischaemic contractile dysfunction. *Cardiovasc Res.* 1994;28:1726-1734.
20. Lameris TW, Van den Meiracker AH, Boomsma F, Alberts G, de Zeeuw S, Duncker DJ, Verdouw PD, Man in 't Veld AJ. Catecholamine handling in the porcine heart: a microdialysis approach. *Am J Physiol.* 1999;277:H1562-H1569.

21. Lameris TW, De Zeeuw S, Alberts G, Boomsma F, Duncker DJ, Verdouw PD, Man in 't Veld AJ, Van den Meiracker AH. Time course and mechanism of myocardial catecholamine release during transient ischemia *in vivo*. *Circulation*. 2000;101:2645-2650.
22. Davson H. *Physiology of the cerebrospinal fluid*. Eds. J. Churchill and A. Churchill, London; 1967.
23. Ulatowski JA, Kirsch JR, Traystman RJ. Hypoxic reperfusion after ischemia in swine does not improve acute brain recovery. *Am J Physiol*. 1994;267:H1880-H1887.
24. Boomsma F, Alberts G, van Eijk L, Man in 't Veld AJ, Schalekamp MA. Optimal collection and storage conditions for catecholamine measurements in human plasma and urine. *Clin Chem*. 1993;39:2503-2508.
25. Alberts G, Lameris TW, Van den Meiracker AH, Man in 't Veld AJ, Boomsma F. Sensitive and specific method for the simultaneous determination of natural and synthetic catecholamines and 3,4-dihydroxyphenylglycol in microdialysis samples. *J Chromatogr B Biomed Sci Appl*. 1999;730:213-219.
26. 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.
27. Sebening C, Hagl C, Szabo G, Tochtermann U, Strobel G, Schnabel P, Amann K, Vahl CF, Hagl S. Cardiocirculatory effects of acutely increased intracranial pressure and subsequent brain death. *Eur J Cardiothorac Surg*. 1995;9:360-372.
28. Chappel CI, Rona G, Balazs T, Gaudry R. Comparison of cardiotoxic actions of certain sympathomimetic amines. *Canad J Biochem*. 1959;37:35-42.
29. Rona G, Boutet M, Huttner I, Peters H. Pathogenesis of isoproterenol-induced myocardial alterations. Functional and morphological correlates. In: Dhalla N, ed. *Recent advances in studies on cardiac structure and metabolism. Myocardial Metabolism*. Baltimore: University Park Press; 1975:507-525.
30. Rona G. Catecholamine cardiotoxicity. *J Mol Cell Cardiol*. 1985;17:291-306.
31. Hugenholtz PG. Electrographic abnormalities in cerebral disorders: report of six cases and review of the literature. *Am Heart J*. 1962;63:451-461.
32. DePasquale NP, Burch GE. How normal is the donor heart? *Am Heart J*. 1969;77:719-720.
33. Kolin A, Norris JW. Myocardial damage from acute cerebral lesions. *Stroke*. 1984;15:990-993.
34. Cooper DK, Novitzky D, Wicomb WN. The pathophysiological effects of brain death on potential donor organs, with particular reference to the heart. *Ann Royal Coll Surg Engl*. 1989;71:261-266.
35. Talman WT. Cardiovascular regulation and lesions of the central nervous system. *Ann Neurol*. 1985;18:1-13.
36. Herijgers P, Borgers M, Flameng W. The effect of brain death on cardiovascular function in rats. Part I. Is the heart damaged? *Cardiovasc Res*. 1998;38:98-106.
37. Bruinsma GJ, Nederhoff MG, Geertman HJ, van Huffelen AC, Slootweg PJ, Ferrari R, Galinanes M, Hearse DJ, Bredee JJ, Ruigrok TJ. Acute increase of myocardial workload, hemodynamic instability, and myocardial histological changes induced by brain death in the cat. *J Surg Res*. 1997;68:7-15.
38. Shanlin RJ, Sole MJ, Rahimifar M, Tator CH, Factor SM. Increased intracranial pressure elicits hypertension, increased sympathetic activity, electrocardiographic abnormalities and myocardial damage in rats. *J Am Coll Cardiol*. 1988;12:727-736.
39. Shivalkar B, Van Loon J, Wieland W, Tjandra-Maga TB, Borgers M, Plets C, Flameng W. Variable effects of explosive or gradual increase of intracranial pressure on myocardial structure and function. *Circulation*. 1993;87:230-239.
40. Murphy DA, O'Blenes S, Nassar BA, Armour JA. Effects of acutely raising intracranial pressure on cardiac sympathetic efferent neuron function. *Cardiovasc Res*. 1995;30:716-724.
41. Schömig A. Catecholamines in myocardial ischemia. Systemic and cardiac release. *Circulation*. 1990;82:II13-II22.
42. Ku DD, Lucchesi BR. Effects of dimethyl propranolol (UM-272; SC-27761) on myocardial ischemic injury in the canine heart after temporary coronary artery occlusion. *Circulation*. 1978;57:541-548.

43. Miura M, Thomas R, Ganz W, Sokol T, Shell WE, Toshimitsu T, Kwan AC, Singh BN. The effect of delay in propranolol administration on reduction of myocardial infarct size after experimental coronary artery occlusion in dogs. *Circulation.* 1979;59:1148-1157.
44. Jang IK, Van de Werf F, Vanhaecke J, De Geest H. Coronary reperfusion by thrombolysis and early beta-adrenergic blockade in acute experimental myocardial infarction. *J Am Coll Cardiol.* 1989;14:1816-1823.
45. Feuerstein GZ, Hamburger SA, Smith EFd, Bril A, Ruffolo Jr. RR. Myocardial protection with carvedilol. *J Cardiovasc Pharmacol.* 1992;19:S138-S141.
46. Torr S, Drake-Holland AJ, Main M, Hynd J, Isted K, Noble MI. Effects on infarct size of reperfusion and pretreatment with beta- blockade and calcium antagonists. *Basic Res Cardiol.* 1989;84:564-582.
47. Drake-Holland AJ, Belcher PR, Hynd J, Noble MI. Infarct size in rabbits: a modified method illustrated by the effects of propranolol and trimetazidine. *Basic Res Cardiol.* 1993;88:250-258.
48. Genth K, Hofmann M, Schaper W. The effect of beta-adrenergic blockade on infarct size following experimental coronary occlusion. *Basic Res Cardiol.* 1981;76:144-151.
49. Koning MMG, Gho BCG, van Klaarwater E, Opstal RL, Duncker DJ, Verdouw PD. Rapid ventricular pacing produces myocardial protection by nonischemic activation of K^{+}_{ATP} channels. *Circulation.* 1996;93:178-186.
50. 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.
51. Gross GJ, Auchampach JA. Blockade of ATP-sensitive potassium channels prevents myocardial preconditioning in dogs. *Circ Res.* 1992;70:223-233.
52. Przyklenk K, Kloner RA. Ischemic preconditioning: exploring the paradox. *Prog Cardiovasc Dis.* 1998;40:517-547.
53. Feng J, Yamaguchi N, Foucart S, Chahine R, Lamontagne D, Nadeau R. Transient ischemia inhibits nonexocytotic release of norepinephrine following sustained ischemia in rat heart: is bradykinin involved? *Can J Physiol Pharmacol.* 1997;75:665-670.
54. 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.
55. 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.
56. Yao Z, Mizumura T, Mei DA, Gross GJ. K^{+}_{ATP} channels and memory of ischemic preconditioning in dogs: synergism between adenosine and K^{+}_{ATP} channels. *Am J Physiol.* 1997;272:H334-H342.
57. Duncker DJ, Verdouw PD. Role of K^{+}_{ATP} channels in ischemic preconditioning and cardioprotection. *Cardiovasc Drugs Ther.* 2000;14:7-16.
58. Bittner HB, Chen EP, Milano CA, Kendall SW, Jennings RB, Sabiston DC, Jr., Van Trigt P. Myocardial beta-adrenergic receptor function and high-energy phosphates in brain death-related cardiac dysfunction. *Circulation.* 1995;92:II472-II478.

Discussion and summary

10

Myocardial ischemia

Coronary artery disease, one of the most prominent health problems in modern society, may lead to myocardial ischemia (i.e. the energy demand cannot be met by the energy supply). The resultant damage of myocardial ischemia is dependent on the duration and severity of the myocardial ischemia and diverges from no damage, reversible damage (contractile dysfunction), to irreversible damage (myocardial infarction). During the last 25 years both experimental and clinical observations have led to the recognition of three ischemic syndromes: (1) *Myocardial stunning*, the delayed recovery of regional myocardial contractile function upon reperfusion of myocardial ischemia, despite the absence of irreversible damage[1], (2) *Ischemic preconditioning*, the cardioprotective effect of short periods of ischemia preceding a period of prolonged ischemia[2], and (3) *Hibernation*, the chronic left ventricular dysfunction in the absence of ongoing metabolic evidence of ischemia by a self-protecting downregulation in myocardial function and metabolism to abort evolution toward cell death despite chronic hypoperfusion[3] *(chapter 1)*.

The general aim of this thesis was to study some remaining questions on the effects of brief periods of myocardial ischemia on the heart muscle, focusing on stunning and ischemic preconditioning. For these studies, an *in vivo* porcine model was used because of the extensive possibilities to determine regional contractile function. The size of the heart allows implantation of ultrasonic crystals in stunned and normal myocardium, for determination of segment shortening and pressure-segment length/area loops, but also implantation of microdialysis probes, for continuous measurement of interstitial levels of norepinephrine. In addition, both arterial and coronary venous samples can be taken at several time points throughout the whole experiment, for determination of myocardial oxygen consumption. In the ischemic preconditioning studies in which infarct size is the most important parameter and regional myocardial function measurements were not mandatory, the rat was used as experimental animal. In these studies it is important that transmural flow is not influenced by the presence of coronary collaterals. Both pigs and rats have the advantage that they possess a negligible amount of collaterals, which provide less variability in the severity of myocardial ischemia, and thus less variability in the degree of stunning and myocardial infarct size (see *chapter 2*).

Myocardial stunning

Established myocardial stunning can be treated with administration of positive inotropic agents. Ca^{2+} sensitizers may pose an advantage over classic positive inotropic agents, because the former can increase force production with no (or negligible) increments in energy costs.[4-6] However, a serious concern of increased Ca^{2+} sensitivity is that it can prolong the duration of contraction and delay the onset of relaxation, thereby impairing diastolic function.[7,8] Therefore, we studied in *chapter 3* the effects of the Ca^{2+} sensitizer EMD 60263 on diastolic

function. The results show that a low dose could fully restore systolic function without any adverse effect on diastolic function. However, diastolic function was slightly impaired when the dose of EMD 60263 was doubled. Because EMD 60263 is not a pure Ca^{2+} sensitizer, but also possesses inward rectifier K^+ current properties, we also used the enantiomer EMD 60264, which only possesses inward rectifier K^+ current properties. The study with the enantiomer showed that the diastolic impairment at high dose of EMD 60263 was not caused by the inward rectifier K^+ current properties, but by the Ca^{2+} sensitizing properties, as EMD 60264 did not produce such a delay in onset of relaxation.

In view of the narrow therapeutic window of Ca^{2+} sensitizers, as is the case with EMD 60263, Ca^{2+} sensitizers with additional phosphodieasterase III (PDE-III) properties may be advantageous over pure Ca^{2+} sensitizers. In *chapter 4* we therefore used EMD 57033, which has no effect on the delayed inward rectifier K^+ current, but exerts in addition to its Ca^{2+} sensitizing properties also minor PDE-III inhibitory actions.[8] This Ca^{2+} sensitizer is different from pimobendan and levosimendan, which have already been used in clinic trials,[9,10] as the latter mainly exert their cardiovascular actions by PDE-III inhibition and a relatively minor Ca^{2+} sensitization. The results showed that in an *in vivo* porcine model of regionally stunned myocardium EMD 57033 had no adverse effect on global and regional diastolic function in a dose that increased regional systolic function far beyond baseline levels. To assess the contribution of PDE-III inhibition to the effects of EMD 57033, the actions of the PDE-III inhibition were minimized by adrenergic blockade. This blockade, however, did not influence the effect of EMD 57033 on stunned or non-stunned myocardium, and consequently at the dose used in this study the positive inotropic actions of EMD 57033 cannot be ascribed to PDE-III inhibition.

In an earlier study by our laboratory,[11] it has been suggested that the inotropic response to Ca^{2+} sensitizers may differ between stunned and normal myocardium. For instance, it has been shown that after infusion of EMD 60263 the increase in regional systolic segment shortening in stunned myocardium of anesthetized pigs was much more pronounced than that in normal myocardium. In accordance with this finding, in *chapters 3 and 4* we observed that the Ca^{2+} sensitizers fully restored segment shortening in the stunned region, but only slightly increased segment shortening in the control area. Regional systolic segment shortening is, however, a load-dependent parameter for regional contractile function. Therefore, we also used the left ventricular end systolic pressure-segment length relation, in analogy to the time-varying elastance concept. This parameter showed that infusion of EMD 57033 induced similar increases in end-systolic elastance in both stunned and normal myocardium. The same observations are made when dobutamine is used, which suggests that the difference in response of the stunned and normal myocardium using load-dependent or load-independent parameters results predominantly from a greater sensitivity of regions with a lower end-systolic elastance for positive inotropic interventions.

Several investigators have questioned, despite *in vitro* evidence, whether Ca^{2+} sensitizers exert their cardiovascular effects *in vivo* by Ca^{2+} sensitization. Thus, although infusion of Ca^{2+} in *in vivo* experiments can increase myocardial contractility reflected by $LVdP/dt_{max}$,[12-15] segment shortening,[12] wall thickening[13,14] and external work,[15] it has not been adressed in any study whether the responsiveness to Ca^{2+} was enhanced when Ca^{2+} sensitizers were administered. Moreover, *in vivo* evidence for a decreased Ca^{2+} responsiveness in stunned myocardium is lacking. In *chapter 5* we therefore studied the effect of EMD 57033 on the responsiveness of normal and stunned myocardium to intracoronary Ca^{2+} infusions *in vivo*. Intracoronary infusion of Ca^{2+} increased global and regional contractility of normal myocardium and this effect was markedly enhanced by EMD 57033. Conversely, stunned myocardium was characterized by a loss of the inotropic response to Ca^{2+} and this response was restored after infusion of EMD 57033. These *in vivo* findings support the concept, based on *in vitro* observations, that EMD 57033 increases myocardial contractility via an increase in myofilament Ca^{2+}-responsiveness, and that the myofilament Ca^{2+}-responsiveness of stunned myocardium is decreased.

Ischemic preconditioning

Brief periods of myocardial ischemia preceding a prolonged period of ischemia limit myocardial infarct size (ischemic preconditioning).[2] In addition, several studies have now shown that brief ischemia in the adjacent myocardium[16] or in other organs, like the kidney[17] or small intestines,[17] can also induce cardioprotection (remote preconditioning, *chapter 6*). Another group of stimuli, which do not lead to myocardial ischemia, such as ventricular pacing,[18] myocardial stretch[19] or pharmacological preconditioning[20-22] can also limit infarct size. The cardioprotection by remote preconditioning and non-ischemic stimuli is, however, less than the cardioprotection induced by ischemic preconditioning.

Adenosine as a mediator of cardioprotection

During myocardial ischemia, several substances such as adenosine and norepinephrine are released and accumulate in the interstitium. Adenosine is the breakdown product of ATP and is transported into the interstitium via the nucleoside transporter and activates adenosine A_1 and A_3 receptors on the cardiomyocytes and adenosine A_2 receptors in the vasculature. The role of adenosine in the pathway leading to ischemic preconditioning, has been confirmed in several species, although the subtypes of adenosine receptor that contribute to ischemic preconditioning remain to be elucidated. In the rat, the role of adenosine in ischemic preconditioning is controversial, as several studies failed to block ischemic preconditioning with adenosine receptor blockade.[23,24] However, because interstitial adenosine levels are much higher in rats than in other species,[25] blockade of adenosine receptors in these studies could have been inadequate. These considerations led us to study the role of adenosine receptors in

ischemic preconditioning in the *in vivo* rat heart. The results in *chapter 7* show that the non-selective adenosine blocker 8-sulphophenyl-theophylline (8-SPT) dose dependently inhibited ischemic preconditioning, with a complete abolition of protection after a high dose, demonstrating the importance of adenosine in ischemic preconditioning in the rat heart. As cardioprotection induced by the adenosine A_3 receptor agonist IB-MECA was not affected by the low dose of 8-SPT, but was blunted by the high dose of 8-SPT, these results suggest that both adenosine A_1 and A_3 receptor subtypes are involved in ischemic preconditioning in the rat heart.

Norepinephrine as a mediator of cardioprotection

Norepinephrine, released by the cardiac sympathetic nerve endings during myocardial ischemia, is another mediator involved in the signaling pathway leading to ischemic preconditioning. Using the microdialysis technique, we first studied in *chapter 8* the mechanism by which norepinephrine is released during ischemia and the effects of ischemia on the functional integrity of nerve endings. The role of the uptake 1 mechanism was studied using the uptake 1 blocker desipramine and the results showed that during the first 10 min of ischemia norepinephrine was released by local exocytotic release, selectively in the ischemic area. However, in contrast to previous studies in rats,[26-29] nonexocytotic uptake via the uptake 1 mechanism was not enhanced. After 10 min the release involved non-exocytotic release via reversal of the carrier-mediated uptake 1 mechanism. Upon reperfusion the uptake 1 mechanism contributed to the clearance of norepinephrine. This observation together with the finding that tyramine could induce release of norepinephrine after 60 min myocardial ischemia suggests that the functional integrity of the nerve endings is still intact, at a time when massive cardiomyocyte necrosis has occurred (84% of the area at risk infracted).

The effect of norepinephrine, released during ischemia, on cardiomyocytes is still under debate. It has been shown that norepinephrine can be cardiotoxic and cause focal myocardial necrosis,[30] but norepinephrine is also known as one of the mediators in ischemic preconditioning.[31-33] Cerebral ischemia has been reported to cause an excessive release of norepinephrine from sympathetic nerve endings in normal myocardium.[34] Therefore in *chapter 9* we tested the hypothesis whether cerebral ischemia-induced norepinephrine release could protect the heart during sustained myocardial ischemia via remote preconditioning. Global cerebral ischemia, induced by increasing the intracranial pressure above systolic blood pressure, caused a tripling of the interstitial norepinephrine levels. Cerebral ischemia by itself did not cause myocardial necrosis, but also did not protect the heart against a prolonged period of myocardial ischemia. Exogenous infusion of a low dose of norepinephrine, which produced similar increases in interstitial norepinephrine levels as cerebral ischemia, was also unable to induce cardioprotection. On the other hand, a high dose of norepinephrine, which resulted in a 10-fold increase in interstitial norepinephrine levels decreased infarct size after prolonged

myocardial ischemia. The cardioprotection by exogenous norepinephrine was not caused by ischemic preconditioning or associated with blunting of norepinephrine release during sustained ischemia. These findings suggest that the lack of cardioprotection by cerebral ischemia could be related to the modest rise in myocardial interstitial norepinephrine levels.

Future studies

One of the most interesting remaining questions regarding myocardial stunning is the molecular mechanism underlying the decrease in Ca^{2+}-responsiveness of the myofilaments. It has been proposed, based on *in vitro* studies, that troponin I proteolysis by proteases such as calpaïns, and the consequent alteration in Ca^{2+}-troponin C interaction, contribute to the loss of Ca^{2+}-responsiveness. Future studies, using inhibitors of calpaïns are required to establish whether this mechanism contributes to myocardial stunning *in vivo*.

Treatment of contractile dysfunction with positive intropic agents such as β-agonists and pure phosphodiesterase inhibitors has been limited by the increased mortality as a result of increased incidence of arrhythmias, which can be ascribed to the effects of an increase in cAMP. Experimental studies in genetically modified mice suggest that a positive inotropic intervention that does not modify cAMP and sarcolemmal function can improve left ventricular contractile function without a concommittant increase in mortality (Rockman and Chien unpublished data). Positive inotropic intervention that acts via the sarcomplasmic reticulum (up-regulation of Ca^{2+} ATPase or down-regulation of phospholamban) or the myofilaments (increasing the Ca^{2+} sensitivity) might become useful in the therapy of contractile dysfunction of the human heart. Gene therapy (i.e. phospholamban knock-out or SERCA overexpression) is certainly of interest, but still in an early experimental phase, and further study in transgenic animals is required.

For the treatment of contractile dysfunction, Ca^{2+} sensitizers appear promising, due to their favorable cardiac energetic effects compared to traditional positive inotropic agents, such as ß-agonists and PDE-inhibitors. However, a concern has been their potentially detrimental effects on diastolic function. The Ca^{2+} sensitizer EMD 57033 exerted minimal effects on diastolic function and promising results in animal models of heart failure warrant further studies in patients with heart failure.

In this thesis the role of adenosine A_3 receptor in rats was studied using the non-selective blocker 8-SPT. Our data that are suggestive, but not definitive prove of a role for A_3 receptors in ischemic preconditioning. The use of novel selective adenosine A_3 receptor antagonists, such as MRS 1191, is mandatory to proof the role of adenosine A_3 receptors in ischemic preconditioning. In addition, the use of microdialysis will enable us to determine the role of exaggerated increases in interstitial adenosine levels during myocardial ischemia *in vivo*, in overcoming the non-selective adenosine receptor blockade as was proposed by Headrick.[25]

The experiments on the effect of cerebral ischemia on the development of myocardial ischemia, showed that global ischemia could not induce cardioprotection. These experiments were done in pigs anesthetized with pentobarbital, of which is known to decrease cerebral blood flow to one-third of cerebral blood flow measured in conscious pigs. To exclude the influence of the anesthesia on the lack of induction of cardioprotection, these experiments should be repeated with an anesthetic regoment that has less influence on cerebral blood flow. The model of global cerebral ischemia used in our study resembles that of brain death. It is possible that regional cerebral ischemia could induce cardioprotection. For example, subarachnoidal hemorrhage, of which it is known that it causes myocardial stunning in patients, could be an interesting regional cerebral model.[35,36]

Similar to stunning, the exact mechanism of ischemic preconditioning is also not yet known, and future studies should be directed at unraveling this mechanism. Although there is consensus that K^{+}_{ATP} channels are the end-effector of cardioprotection, there is still some debate whether this concerns the mitochondrial or also the sarcolemmal K^{+}_{ATP} channels. This can now be further studied using mitochondrial and sarcomlemmal selective K^{+}_{ATP} channel openers and blockers.

Another issue that is often raised with respect to ischemic preconditioning is its clinical relevance. In recent years, the clinical relevance of ischemic preconditioning, which was originally a laboratory observation, has become accepted. Clinical application of the cardioprotective strategies could include the use of K^{+}_{ATP} channel openers, such as nicorandil, in patients with unstable angina pectoris. A question that needs to be addressed is whether tolerance to its cardioprotective actions occurs. However, many patients do not experience unstable angina pectoris prior to a myocardial infarction and treatment can only be given as part of reperfusion-therapy. Thus, treatment should also be focused on reperfusion injury, with for example Na^{+}/H^{+} exchange inhibitors, because they can decrease Ca^{2+} overload at the onset of reperfusion. In a recent clinical trial it has been demonstrated that the Na^{+}/H^{+} exchange inhibitor HOE 642 attenuates reperfusion injury and thereby improve the recovery from left ventricular dysfunction after myocardial infarction.[37] In addition, the combination of percutaneous transluminal angioplasty (PTCA) and a cardioprotective agent, such as adenosine, has already been successfully applied to primary PTCA.[38]

References

1. Braunwald E, Kloner RA. The stunned myocardium: prolonged, postischemic ventricular dysfunction. *Circulation*. 1982;66:1146-1149.
2. Murry CE, Jennings RB, Reimer KA. Preconditioning with ischemia: a delay of lethal cell injury in ischemic myocardium. *Circulation*. 1986;74:1124-1136.
3. Rahimtoola SH. The hibernating myocardium. *Am Heart J.* 1989;117:211-221.
4. de Tombe PP, Burkhoff D, Hunter WC. Effects of calcium and EMD-53998 on oxygen consumption in isolated canine hearts. *Circulation*. 1992;86:1945-1954.

5. Hata K, Goto Y, Futaki S, Ohgoshi Y, Yaku H, Kawaguchi O, Takasago T, Saeki A, Taylor TW, Nishioka T, et al. Mechanoenergetic effects of pimobendan in canine left ventricles. Comparison with dobutamine. *Circulation*. 1992;86:1291-1301.
6. Gross T, Lues I, Daut J. A new cardiotonic drug reduces the energy cost of active tension in cardiac muscle. *J Mol Cell Cardiol*. 1993;25:239-244.
7. Solaro RJ, Gambassi G, Warshaw DM, Keller MR, Spurgeon HA, Beier N, Lakatta EG. Stereoselective actions of thiadiazinones on canine cardiac myocytes and myofilaments. *Circ Res*. 1993;73:981-990.
8. Ravens U, Himmel HM, Fluss M, Davia K, Harding SE. Phosphodiesterase inhibition and Ca^{2+} sensitization. *Mol Cell Biochem*. 1996;157:245-249.
9. Kubo SH. Effects of pimobendan on exercise tolerance and quality of life in patients with heart failure. *Cardiology*. 1997;88:21-27.
10. Lehtonen L. Levosimendan: A Promising Agent for the Treatment of Hospitalized Patients with Decompensated Heart Failure. *Curr Cardiol Rep*. 2000;2:233-243.
11. Soei LK, Sassen LM, 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.
12. Crystal GJ, Zhou X. Nitric oxide does not modulate the increases in blood flow, O_2 consumption, or contractility during $CaCl_2$ administration in canine hearts. *Cardiovasc Res*. 1999;42:232-239.
13. Ito BR, Tate H, Kobayashi M, Schaper W. Reversibly injured, postischemic canine myocardium retains normal contractile reserve. *Circ Res*. 1987;61:834-846.
14. Buffington CW, Rothfield KP. Effects of intracoronary calcium chloride on the postischemic heart in pigs. *Ann Thorac Surg*. 1995;59:1448-1455.
15. Heusch G, Rose J, Skyschally A, Post H, Schulz R. Calcium responsiveness in regional myocardial short-term hibernation and stunning in the *in situ* porcine heart. Inotropic responses to postextrasystolic potentiation and intracoronary calcium. *Circulation*. 1996;93:1556-1566.
16. 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.
17. Gho BC, Schoemaker RG, van den Doel MA, Duncker DJ, Verdouw PD. Myocardial protection by brief ischemia in noncardiac tissue. *Circulation*. 1996;94:2193-2200.
18. Koning MM, Gho BC, van Klaarwater E, Opstal RL, Duncker DJ, Verdouw PD. Rapid ventricular pacing produces myocardial protection by nonischemic activation of K^{+}_{ATP} channels. *Circulation*. 1996;93:178-186.
19. Ovize M, Kloner RA, Przyklenk K. Stretch preconditions canine myocardium. *Am J Physiol*. 1994;266:H137-H146.
20. Ytrehus K, Liu Y, Downey JM. Preconditioning protects ischemic rabbit heart by protein kinase C activation. *Am J Physiol*. 1994;266:H1145-H1152.
21. Thornton JD, Daly JF, Cohen MV, Yang XM, Downey JM. Catecholamines can induce adenosine receptor-mediated protection of the myocardium but do not participate in ischemic preconditioning in the rabbit. *Circ Res*. 1993;73:649-655.
22. Toombs CF, McGee S, Johnston WE, Vinten-Johansen J. Myocardial protective effects of adenosine. Infarct size reduction with pretreatment and continued receptor stimulation during ischemia. *Circulation*. 1992;86:986-994.
23. Li Y, Kloner RA. The cardioprotective effects of ischemic 'preconditioning' are not mediated by adenosine receptors in rat hearts. *Circulation*. 1993;87:1642-1648.
24. Bugge E, Ytrehus K. Ischaemic preconditioning is protein kinase C dependent but not through stimulation of alpha adrenergic or adenosine receptors in the isolated rat heart. *Cardiovasc Res*. 1995;29:401-406.
25. Headrick JP. Ischemic preconditioning: bioenergetic and metabolic changes and the role of endogenous adenosine. *J Mol Cell Cardiol*. 1996;28:1227-1240.
26. Schomig A, Dart AM, Dietz R, Mayer E, Kubler W. Release of endogenous catecholamines in the ischemic myocardium of the rat. Part A: Locally mediated release. *Circ Res*. 1984;55:689-701.
27. Kranzhofer R, Haass M, Kurz T, Richardt G, Schomig A. Effect of digitalis glycosides on norepinephrine release in the heart. Dual mechanism of action. *Circ Res*. 1991;68:1628-1637.
28. Kurz T, Richardt G, Hagl S, Seyfarth M, Schomig A. Two different mechanisms of noradrenaline release during normoxia and simulated ischemia in human cardiac tissue. *J Mol Cell Cardiol*. 1995;27:1161-1172.
29. Schomig A. Adrenergic mechanisms in myocardial infarction: cardiac and systemic catecholamine release. *J Cardiovasc Pharmacol*. 1988;12:S1-S7.
30. Rona G. Catecholamine cardiotoxicity. *J Mol Cell Cardiol*. 1985;17:291-306.

31. Toombs CF, Wiltse AL, Shebuski RJ. Ischemic preconditioning fails to limit infarct size in reserpinized rabbit myocardium. Implication of norepinephrine release in the preconditioning effect. *Circulation.* 1993;88:2351-2358.
32. Kitakaze M, Hori M, Morioka T, Minamino T, Takashima S, Sato H, Shinozaki Y, Chujo M, Mori H, Inoue M, et al. Alpha 1-adrenoceptor activation mediates the infarct size-limiting effect of ischemic preconditioning through augmentation of 5'- nucleotidase activity. *J Clin Invest.* 1994;93:2197-2205.
33. Vander Heide RS, Schwartz LM, Jennings RB, Reimer KA. Effect of catecholamine depletion on myocardial infarct size in dogs: role of catecholamines in ischemic preconditioning. *Cardiovasc Res.* 1995;30:656-662.
34. Mertes PM, Burtin P, Carteaux JP, Jaboin Y, Dopff C, Pinelli G, Villemot JP, Burlet C, Boulange M. Brain death and myocardial injury: role of cardiac sympathetic innervation evaluated by in vivo interstitial microdialysis. *Transplant Proc.* 1994;26:231-232.
35. Handlin LR, Kindred LH, Beauchamp GD, Vacek JL, Rowe SK. Reversible left ventricular dysfunction after subarachnoid hemorrhage. *Am Heart J.* 1993;126:235-240.
36. Ohtsuka T, Hamada M, Kodama K, Sasaki O, Suzuki M, Hara Y, Shigematsu Y, Hiwada K. Images in Cardiovascular Medicine. Neurogenic stunned myocardium. *Circulation.* 2000;101:2122-2124.
37. Rupprecht HJ, vom Dahl J, Terres W, Seyfarth KM, Richardt G, Schultheibeta HP, Buerke M, Sheehan FH, Drexler H. Cardioprotective effects of the Na^+/H^+ exchange inhibitor cariporide in patients with acute anterior myocardial infarction undergoing direct PTCA. *Circulation.* 2000;101:2902-2908.
38. Marzilli M, Orsini E, Marraccini P, Testa R. Beneficial effects of intracoronary adenosine as an adjunct to primary angioplasty in acute myocardial infarction. *Circulation.* 2000;101:2154-2159.

Myocard ischemia

Coronair vaatlijden, één van de meest voorkomende gezondheidsproblemen van deze tijd, kan uiteindelijk leiden tot myocard ischemie, wanneer de energie voorziening via het bloed niet voldoet aan de energie behoefte van de hartspier. De uiteindelijke schade als gevolg van myocard ischemie is afhankelijk van de duur en de ernst van de ischemie en varieert van géén schade, reversibele schade (contractiele dysfunctie), tot irreversibele schade (hartinfarct). Gedurende de afgelopen 25 jaar hebben zowel experimentele als klinische studies geleid tot onderscheid van 3 ischemische syndromen: (1) *Myocardiale stunning*, het vertraagde herstel van regionale contractiele hartfunctie na volledig herstel van perfusie van het ischemische hartweefsel, ondanks de afwezigheid van irreversibele schade, (2) *Ischemische preconditionering*, het cardioprotectieve effect van korte perioden van ischemie voorafgaand aan een lange periode van ischemie, en (3) *Hibernation*, de chronische linker ventrikel dysfunctie in de afwezigheid van metabole aanwijzingen voor ischemie door een zelfbeschermende down-regulatie van de hartspierfunctie en metabolisme om ontwikkeling tot celdood te voorkomen ondanks chronische hypoperfusie *(hoofdstuk 1)*.

Het doel van dit proefschrift was het bestuderen van een aantal openstaande vragen betreffende stunning en ischemische preconditionering. In deze studies werd een *in vivo* varkensmodel gebruikt vanwege de uitgebreide mogelijkheden om de regionale hartspierfunctie te meten. Door de grootte van het hart is het mogelijk om ultrasone kristallen in gestunned en normaal hartweefsel te plaatsen voor de bepaling van segment lengte verkorting en druk-segment lengte/oppervlakte relatie. Ook is het mogelijk microdialyse probes te implanteren voor de continue meting van noradrenaline concentraties tussen de cellen (interstitiële concentraties). Naast deze metingen kunnen ook arteriële en coronair veneuze bloedmonsters worden genomen op verschillende tijdstippen gedurende het gehele experiment. Voor studies naar ischemische preconditionering waarbij vooral de bepaling van de infarctgrootte belangrijk is en die van de hartspierfunctie niet vereist is, zijn ratten gebruikt. In studies naar de effecten van ischemie van de hartspier is het belangrijk dat de transmurale doorbloeding van het hart niet beïnvloed wordt door de aanwezigheid van collateralen. Zowel in varkens als in ratten zijn nauwelijks collateralen aanwezig. Hierdoor is er bij een coronair afsluiting minder variabiliteit in de ernst van ischemie en dus ook minder variabiliteit in de mate van stunning en infarct ontwikkeling (*hoofstuk 2*).

Myocardiale stunning

Stunning van de hartspier kan worden behandeld door toediening van positief inotrope stoffen. Calcium sensitizers zouden voordeliger kunnen zijn dan klassieke positief inotrope stoffen, omdat zij de contractiekracht kunnen verbeteren zonder of met verwaarloosbare toename in energieverbruik. Echter, een probleem van een toename in de calcium gevoeligheid kan zijn dat de duur van de contractie wordt verlengd Dit kan leiden tot een vertraging van de

start van de relaxatie van de ventrikel, hetgeen de vulling van het hart ongunstig kan beïnvloeden. Daarom is in *hoofdstuk 3* het effect van de calcium sensitizer EMD 60263 op de relaxatie bestudeerd. De resultaten laten zien dat een lage dosis van deze calcium sensitizer de contractiele functie volledig kan herstellen zonder nadelige effecten op de relaxatie. Echter, de relaxatie was wel gedeeltelijk verminderd wanneer de dosis werd verdubbeld. Omdat EMD 60263 geen zuivere calcium sensitizer is, maar ook invloed heeft op de kalium kanalen in de celmembraan hebben we ook de effecten van de enantiomeer EMD 60264 bestudeerd. Deze enantiomeer die geen calcium gevoeligheid-verhogende eigenschappen heeft, maar alleen een effect heeft op de kalium kanalen, liet zien dat de relaxatie vermindering bij de hoge dosering van EMD 60263 veroorzaakt werd door de calcium gevoeligheid-verhogende eigenschappen, maar niet door de effecten op de kalium kanalen.

Om problemen met de relaxatie te voorkomen, kan het voordelig zijn een calcium sensitizer te hebben die zowel calcium gevoeligheid-verhogende als fosfodiesterase-III remmende eigenschappen heeft. In *hoofdstuk 4* hebben we daarom de effecten van EMD 57033 bestudeerd, een stof die geen effect heeft op de kalium kanalen in de celmembraan, maar naast calcium gevoeligheid-verhogende eigenschappen ook enige fosfodiesterase-III remmende eigenschappen bezit. EMD 57033 is verschillend van pimobendan en levosimendan, die beide al gebruikt zijn in klinische studies, in dat de laatstgenoemden meer uitgesproken fosfodiesterase-III remmende eigenschappen hebben dan calcium gevoeligheid-verhogende eigenschappen. De resultaten laten zien, dat in een *in vivo* varkensmodel van regional gestunned hartweefsel EMD 57033 geen negatief effect heeft op globale en regionale relaxatie zelfs in een dosis die de regionale contractiele functie herstelde tot ver boven uitgangswaarden. Om de bijdrage van de fosfodiesterase-III remmende eigenschappen van EMD 57033 te bepalen, werden de fosfodiesterase-III remmende effecten geminimalizeerd door de adrenerge receptoren te blokkeren. Deze blokkade had echter geen invloed op de effecten van EMD 57033 op gestunned en normaal hartweefsel en dus kunnen de positief contractiele effecten van EMD 57033 in de dosis gebruikt in deze studie niet worden toegeschreven aan fosfodiesterase-III remming.

In een eerdere studie door ons laboratorium uitgevoerd, werd gesuggereerd dat de positief inotrope respons op calcium sensitizers in gestunned en normaal hartweefsel verschillend zou zijn. Deze studie liet zien dat na infusie van EMD 60263 in genarcotiseerde varkens de toename in systolische segment lengte verkorting groter was in gestunned dan in normaal hartweefsel. In overeenstemming met deze bevinding hebben we in *hoofdstuk 3 en 4* gevonden dat de calcium sensitizers de segment lengte verkorting volledig herstelde in gestunned hartweefsel en maar een kleine toename liet zien in normaal hartweefsel. Regionale segment lengte verkorting is echter een load-afhankelijke parameter voor regionale contractiele functie. We hebben daarom ook gebruik gemaakt van de linker ventrikel eind-systolische druk-segment lengte relatie, analoog aan het concept van de tijdsafhankelijke

variatie in elastantie. Deze parameter liet zien dat infusie van EMD 57033 dezelfde toename in eind-systolische elastantie induceerde in zowel gestunned als normaal hartweefsel. Dezelfde resultaten worden gevonden wanneer dobutamine wordt gebruikt. Deze studies suggereren dat parameters die niet gecorrigeerd zijn voor load-afhankelijkheid ongeschikt zijn voor de bepaling van regionale contractiliteit.

Ondanks de aanwijzingen *in vitro* twijfelen verschillende onderzoekers of calcium sensitizers hun cardiovasculaire effect *in vivo* ook uitoefenen door verhoging van de calcium gevoeligheid. *In vivo* experimenten hebben laten zien dat infusie van calcium de contractiliteit kan verhogen, weergegeven door een toename in de $LVdP/dt_{max}$, segment verkorting, wandverdikking en externe arbeid. Er zijn echter geen studies die hebben aangetoond dat de calcium respons is toegenomen na toediening van een calcium sensitizer. Bovendien is het *in vivo* bewijs voor een afgenomen calcium gevoeligheid in gestunned hartweefsel nog niet geleverd. In *hoofdstuk 5* hebben we daarom de effecten van EMD 57033 op de respons van normaal en gestunned hartweefsel op intracoronaire calcium infusies *in vivo* bestudeerd. Intracoronair calcium verhoogde zowel de globale als de regionale contractiele functie van normaal hartweefsel en de respons op calcium was duidelijk toegenomen na toediening van EMD 57033. Anderzijds was er in gestunned myocard een verlies van de inotrope respons op calcium, die echter was hersteld na toediening van EMD 57033. Deze *in vivo* bevindingen ondersteunen het concept, gebaseerd op *in vitro* studies, dat EMD 57033 de contractiele functie doet verhogen via een toename in de calcium respons van de myofilamenten, en dat de calcium respons van de myofilamenten in gestunned hartweefsel is verminderd.

Ischemische preconditionering

Korte perioden van ischemie voorafgaand aan een langdurige periode van ischemie kan de infarct ontwikkeling vertragen (ischemische preconditionering). Tevens laten verschillende studies zien dat een korte periode van ischemie in het naastgelegen hartweefsel of in andere organen, zoals de nieren en de darmen, ook het hart kunnen beschermen ('remote' preconditionering, oftewel bescherming op afstand). Prikkels die niet tot ischemie leiden, zoals ventrikel pacen, myocardiale stretch of activatie van (bijvoorbeeld adenosine) receptoren mogelijk betrokken bij ischemische preconditionering kunnen ook de infarctontwikkeling vertragen. De bescherming van de hartspier door preconditionering op afstand en niet-ischemische prikkels is echter minder dan de bescherming via ischemische preconditionering (*hoofdstuk 6*).

Adenosine als een mediator van ischemische preconditionering

Tijdens ischemie van de hartspier, worden verschillende stoffen, zoals adenosine en noradrenaline, uitgescheiden en hopen op tussen de cellen (interstitium). Adenosine is een afbraakprodukt van ATP en wordt via de nucleoside transporter in het interstitium

uitgescheiden, waar het de adenosine A_1 en A_3 receptoren op de hartspiercellen en adenosine A_2 receptoren in de vaten activeert. De rol van adenosine in het mechanisme dat leidt tot ischemische preconditionering is in verschillende diersoorten bevestigd, maar in de rat is de rol van adenosine controversieel omdat in verschillende studies ischemische preconditionering niet kon worden geblokkeerd met adenosine receptor blokkers. In de rat zijn interstitiële adenosine concentraties tijdens ischemie echter veel hoger dan in andere diersoorten en dus kan blokkade van de adenosine receptoren in de hiervoorgenoemde studies niet voldoende zijn geweest. Deze hypothese heeft ertoe geleid dat we in *hoofdstuk 7* de rol van de adenosine receptoren in ischemische preconditionering in ratten *in vivo* hebben bestudeerd. De resultaten laten zien dat de non-selectieve adenosine receptor blocker 8-sulphophenyl theophylline (8-SPT) ischemische preconditionering dosis afhankelijk kon remmen, met een complete blokkade van de bescherming na de hoge dosering (50mg/kg). Omdat cardioprotectie opgewekt door de adenosine A_3 agonist IB-MECA niet werd beïnvloed door de lage dosering 8-SPT, maar gedeeltelijk verminderd was door de hoge dosering 8-SPT, lijkt het dat zowel adenosine A_1 als A_3 receptoren betrokken zijn bij ischemische preconditionering in de rat.

Noradrenaline als mediator voor ischemische preconditionering

Noradrenaline, tijdens ischemie van de hartspier uitgescheiden door de sympatische zenuwuiteinden in het hart, is ook betrokken bij het mechanisme van ischemische preconditionering. Gebruik makend van microdialyse, hebben we in *hoofdstuk 8* eerst bestudeerd wat het uitscheidingsmechanisme van noradrenaline is tijdens ischemie van de hartspier en wat de effecten van ischemie op de functionele integriteit van de zenuwuiteinden zijn. De rol van het presynaptische opname kanaal (Uptake 1) werd bestudeerd door de Uptake 1 blokker desipramine te gebruiken. De studie laat zien dat tijdens de eerste 10 minuten van ischemie noradrenaline selectief wordt uitgescheiden in het ischemische gebied via lokale exocytose. In tegenstelling tot bevindingen in eerdere studies in ratten, was de opname van noradrenaline via het uptake 1 mechanisme niet verhoogd. Na 10 minuten ischemie werd noradrenaline ook uitgescheiden via omkering van het presynaptische uptake 1 mechanisme. Tijdens reperfusie is de uptake 1 weer hersteld en zorgt mede voor de klaring van noradrenaline. Deze resultaten en de bevindingen dat tyramine de uitstoot van noradrenaline na 60 minuten ischemie kon stimuleren laat zien dat de functionele integriteit van de zenuwuiteinden nog intact is, op een tijdstip dat een groot deel van de hartspier cellen reeds irreversible beschadigd is (84% van het risicogebied is afgestorven).

Het effect van noradrenaline uitgescheiden tijdens ischemie op hartspiercellen is nog steeds een punt van discussie. Hoewel studies hebben aangetoond dat noradrenaline is betrokken bij ischemische preconditionering zijn er ook studies die hebben uitgewezen dat noradrenaline toxisch kan zijn voor het hart en kleine infarcten kan veroorzaken. Van hersen ischemie is bekend dat het enorme uitstoot van noradrenaline induceert in normaal

hartweefsel. Daarom hebben we in *hoofdstuk 9* de hypothese getest dat uitscheiding van noradrenaline in het hart als gevolg van hersen ischemie het hart kan beschermen via remote preconditionering. Globale hersen ischemie, geinduceerd door de intracraniele druk te verhogen boven de systolische bloeddruk, veroorzaakte een verdrievoudiging van de interstitiële noradrenaline concentraties. Hersen ischemie zelf veroorzaakte geen necrose in het normale hartweefsel, maar kon ook het hart niet beschermen tijdens een daaropvolgende lange periode van ischemie van de hartspier. Exogene toediening van een lage dosis noradrenaline, die een vergelijkbare toename in de interstitiële noradrenaline spiegels induceerde als hersen ischemie, gaf ook geen cardioprotectie. Echter, na toediening van een hoge dosis noradrenaline, die de interstitiële noradrenaline spiegels vertienvoudigde, was de infarct grootte na 60 minuten ischemie van de hartspier wel kleiner. Aanvullende experimenten toonden aan dat de cardioprotectie via exogene noradrenaline niet werd veroorzaakt door ischemische preconditionering. Hoewel het hartinfarct kleiner was in de experimenten waarin noradrenaline exogeen werd toegediend was er géén vermindering van de uitstoot van noradrenaline tijdens de daaropvolgende 60 minuten ischemie. Deze studie suggereert dat hersen ischemie geen cardioprotectie kon opwekken omdat de interstitiële noradrenaline spiegels in het hart niet hoog genoeg kwamen.

Toekomstige studies

Eén van de meest interessante vragen die nog open staat betreffende stunning is het moleculaire mechanisme dat ten grondslag ligt aan de afname in calcium respons van de myofilamenten. Er is verondersteld, gebaseerd op *in vitro* studies, dat troponin I proteolyse door proteasen zoals calpaine, en de daaropvolgende veranderingen in calcium-troponin C interactie bijdragen aan het verlies van calcium respons. Studies die gebruik maken van calcpaine remmers kunnen uitwijzen of dit mechanisme inderdaad bijdraagt aan myocardiale stunning *in vivo*.

Behandeling van contractiele dysfunctie met positief inotrope stoffen zoals β-agonisten en pure fosfodiesterase-III remmers, is gecontraindiceerd door de verhoogde kans op plotse dood ten gevolge van een toename van het aantal ventriculaire aritmieen, toegeschreven aan de verhoogde concentratie cAMP. Studies in genetisch gemodificeerde muizen hebben laten zien dat een positief inotrope interventie die geen invloed heeft op de cAMP concentratie of op de functie van het celmembraan, contractiele functie van de linker ventrikel kan verbeteren zonder een toename in sterfte. Positief inotrope interventie die via het sarcoplasmatische reticulum (up-regulatie van Ca^{2+} ATPase of down-regulatie van phospholamban) of de myofilamenten (toename van de calcium gevoeligheid) werkt, kan belangrijk zijn in de behandeling van contractiele dysfunctie van het humane hart. Gentherapie (bijv. phospholamban knock-out of SERCA overexpressie) is zeker interessant, maar nog steeds in de experimentele fase.

Voor de behandeling van contractiele dysfunctie lijken calcium sensitizers een veelbelovende groep positief inotropica, vanwege het gunstige energie verbruik van het hart in vergelijking met traditionele positief inotrope stoffen, zoals β–agonisten en fosfodiesterase-III remmers. Een belangrijk probleem kan echter het effect op de linker ventrikel relaxatie zijn. EMD 57033 heeft minimale effecten op de relaxatie en de veelbelovende resultaten in diermodellen van hartfalen (ventrikel pacen en hartinfarct) kunnen aanleiding zijn tot het uitvoeren van studies in patienten met hartfalen.

In dit proefschrift is de rol van adenosine A_1 en A_3 in ischemische preconditionering in de rat bestudeerd door gebruik van de non-selectieve adenosine receptor blokker 8-SPT. Onze data zijn echter suggestief omdat we door gebruik te maken van non-selectieve blockers geen direct bewijs hebben geleverd. Het gebruik van nieuwe selectieve adenosine A_3 receptor blokkers, zoals MRS 1191, is nodig om de rol van adenosine A_3 receptor in ischemische preconditionering aan te tonen. Daarnaast zal het gebruik van microdialyse het mogelijk maken om de rol te bepalen van de enorme toename in interstitiële adenosine concentraties tijdens myocard ischemie die de non-selectieve adenosine blokkade doorbreken, zoals in eerdere studies.

De studies naar de effecten van hersen ischemie op de ontwikkeling van het hartinfarct in dit proefschrift liet zien dat globale ischemie geen cardioprotectie opwekte. Deze experimenten zijn uitgevoerd in varkens genarcotiseerd met pentobarbital, waardoor de bloeddoorstroming van de hersenen maar 30% was vergeleken met wakkere varkens. Om de invloed van het narcose middel op het onvermogen om cardioprotectie op te wekken uit te sluiten, zouden deze experimenten herhaald moeten worden in varkens met een narcose middel dat minder effect heeft op de bloeddoorstroming van de hersenen. Het in onze studie gebruikte model van globale hersen ischemie, komt overeen met een model voor hersendood. Het is echter heel goed mogelijk dat een model van regionale hersen ischemie wel cardioprotectie zou opwekken. Een voorbeeld van regionale hersen ischemie zou een model met subarachnoidale bloedingen kunnen zijn. Van subarachnoidale bloedingen is bekend dat het stunning in patienten kan veroorzaken en dus misschien ook wel cardioprotectie.

Evenals bij stunning is ook het exacte mechanisme van ischemische preconditionering nog niet bekend en in de toekomst zouden studies zich moeten richten op het ontrafelen van dit mechanisme. Hoewel het algemeen geaccepteerd is dat ATP gevoelige kalium kanalen de eind-effector zijn van ischemische preconditionering, is er nog steeds de vraag of dit de mitochondriale dan wel de sarcolemmale kalium kanalen betreft. Dit zou nu verder onderzocht kunnen worden met behulp van selectieve openers en blokkers van mitochondriale en sarcolemmale kalium kanalen.

Een ander belangrijk punt dat vaak naar voren wordt gebracht met betrekking tot ischemische preconditionering is de klinische relevantie. In de afgelopen jaren is de klinische relevantie van ischemische preconditionering, dat oorspronkelijk een laboratorium bevinding

was, algemeen geaccepteerd. Klinische toepassing van cardioprotectieve strategieën zouden het gebruik van ATP gevoelige kalium kanaal openers, zoals nicorandil, in patienten met instabiele angina pectoris kunnen inhouden. Een vraag die dan opkomt is of patienten tolerantie tegen de cardioprotectieve effecten van deze stoffen zouden ontwikkelen. Echter veel patienten hebben last van instabiele angina pectoris voordat het hartinfarct ontstaat en behandeling kan alleen gegeven worden als onderdeel van de reperfusie therapie. Dus behandeling zou ook gericht moeten zijn op beperking van de reperfusie schade, zoals door natrium-waterstof exchange remmers die de calcium overload verminderen bij aanvang van de reperfusie. Een recente klinische studie heeft laten zien dat de natrium-waterstof exchange remmer HOE 642 de reperfusie schade vermindert en daarmee het herstel van de linker ventrikel dysfunctie na een hartinfarct verbetert. De combinatie van percutane transluminale angioplastie (PTCA, "dotteren") en een cardioprotectieve stof zoals adenosine wordt inmiddels succesvol toegepast bij primaire PTCA.

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Sandra

Curriculum Vitae

Petronella Alexandra de Zeeuw werd geboren op 6 april 1971 in Dordrecht. In 1989 behaalde zij haar V.W.O. diploma op scholengemeenschap Noordendijk in Dordrecht, waarna ze aan de Hogere Laboratoriumschool in Delft begon met de studie Zoölogie (medische biologie). In haar laatste jaar deed ze haar afstudeerstage op de afdeling Experimentele Cardiologie (Thoraxcentrum) op de Erasmus Universiteit Rotterdam onder begeleiding van Prof Dr PD Verdouw en Dr MMG Koning en onderzocht de beschermingsduur van ischemische preconditionering in het hart in een *in vivo* varkensmodel. Aansluitend na het behalen van haar diploma in 1993, begon ze een verkorte opleiding Biologie in Leiden, met als specialisatie Medische Biologie. Tijdens deze studie werd in 1994 negen maanden onderzoek gedaan aan de Universiteit van Leiden bij Prof Dr AC Gittenberger-de Groot op de afdeling Anatomie en Embryologie, waar de histopathologie van links hypoplastische harten werd onderzocht. In 1995 werd een andere stage van zes maanden gelopen op de afdeling Dierfysiologie aan de Universiteit van Leiden bij Dr GEEJM van den Thillart en Dr GJ Vianen-de Mooy naar het glucosemetabolisme bij karpers. In januari 1996 studeerde zij af en keerde terug naar de Experimentele Cardiologie op de Erasmus Universiteit Rotterdam om promotieonderzoek te beginnen. De afgelopen jaren heeft zij zich bezig gehouden met stunning en ischemische preconditionering, 2 verschillende effecten van korte perioden van ischemie op het myocard, in *in vivo* varkens en rattenmodellen.

List of publications

Full papers

1. Monique MG Koning, **Sandra de Zeeuw**, Selma Nieukoop, Jan Willem de Jong, Pieter D Verdouw. In myocardial infarct size limitation by ischemic preconditioning an "all or nothing" phenomenon? Ann N Y Acad Sci 1994;723:333-336.

2. Monique MG Koning, Laurens AJ Simonis, **Sandra de Zeeuw**, Selma Nieukoop, Sandor Post, Pieter D Verdouw. Ischaemic preconditioning by partial occlusion without intermittent reperfusion. Cardiovasc Res 1994;28:1146-1151.

3. Ben C Gho, Yvonne EG Eskildsen-Helmond, **Sandra de Zeeuw**, Jos MJ Lamers, Pieter D Verdouw. Does protein kinase C play a pivotal role in the mechanisms of ischemic preconditioning. Cardiovasc Drugs Ther 1996;10:775-786.

4. Pieter D Verdouw, Mirella A van den Doel, **Sandra de Zeeuw**, Dirk J. Duncker. On the relevance of ischemic preconditioning in humans. Basic Res Cardiol 1997;92(Suppl 2):51-53.

5. Ben C. Gho, Monique MG Koning, Mirella A van den Doel, Stanley Y Duval, **Sandra de Zeeuw**, Regien Schoemaker, Dirk J Duncker, Pieter D Verdouw. Myocardial adaptation in response to ischemic and nonischemic stress. Exp Clin Cardiol 1997;2:85-92.

6. Pieter D Verdouw, Mirella A van den Doel, **Sandra de Zeeuw**, Dirk J Duncker. Animal models in the study of myocardial ischaemia and ischaemic syndromes. Cardiovasc Res 1998;39:121-135.

7. Adriana C Gittenberger-de Groot, **Sandra de Zeeuw**, Petra W Oosthoek, Ad JJC Bogers. Microvascular patterning in the right ventricle pre-and post-surgery in hypoplastic left heart. Proceedings of the second world congress of pediatric cardiology and cardiac surgery, 1998;543-546.

8. **Sandra de Zeeuw**, Mirella A van den Doel, Dirk J Duncker, Pieter D Verdouw. New insights into cardioprotection by ischemic preconditioning and other forms of stress. Ann N Y Acad Sci 1999;874:178-191.

9. Thomas W Lameris, Anton H van den Meiracker, Frans Boomsma, Gooitzen Alberts, **Sandra de Zeeuw**, Dirk J Duncker, Pieter D Verdouw, Arie J Man in 't Veld. Catecholamine handling in the porcine heart: a microdialysis approach. Am J Physiol 1999;277:H1562-H1569.

10. Loe Kie Soei, **Sandra de Zeeuw**, Rob Krams, Dirk J Duncker, Pieter D Verdouw. Ca^{2+} sensitization and diastolic function of normal and stunned porcine myocardium. Eur J Pharmacol 1999;386:55-67.

11. Martin P Schuijt, Jorge P van Kats, **Sandra de Zeeuw**, Dirk J Duncker, Pieter D Verdouw, Maarten ADH Schalekamp, AH Jan Danser. Cardiac interstitial fluid levels of angiotensin I and II in the pig. J Hypertens 1999;17:1885-1891.

12. **Sandra de Zeeuw**, Serge AIP Trines, Rob Krams, Pieter D Verdouw, Dirk J Duncker. Cardiovascular profile of the calcium sensitizer EMD57033 in open-chest anaestetized pigs with regionally stunned myocardium. Br J Pharmacol 2000;129:1413-1422.

13. Thomas W Lameris, **Sandra de Zeeuw**, Gooitzen Alberts, Frans Boomsmsa, Dirk J Duncker, Pieter D Verdouw Arie J Man in 't Veld, Anton H van den Meiracker. Time course and mechanism of myocardial catecholamine release during transient ischemia *in vivo*. Circulation 2000;101:2645-2650.

14. **Sandra de Zeeuw**, Serge AIP Trines, Rob Krams, Dirk J Duncker, Pieter D Verdouw. *In vivo* evidence that EMD 57033 restores myocardial responsiveness to intracoronary Ca^{2+} in stunned myocardium. Eur J Pharmacol 2000; 403:99-109.

15. **Sandra de Zeeuw**, Thomas W Lameris, Dirk J Duncker, Djo Hasan, Frans Boomsma, Anton H van den Meiracker, Pieter D Verdouw. Cardioprotection in pigs by exogenous norepinephrine, but not by cerebral ischemia-induced release of endogenous norepinephrine. Stroke, revision submitted.

Abstracts

1. **Sandra de Zeeuw**, Loe Kie Soei, Jan R van Meegen, Serge AIP Trines, Rob Krams, Dirk J Duncker, Pieter D Verdouw. Recovery of systolic function of stunned myocardium by the Ca^{2+} sensitizer EMD 57033 is not accompanied by impairment of diastolic function. Circulation 1997;96 Suppl I:I-200.

2. Thomas W Lameris, Anton H van den Meiracker, Frans Boomsma, Gooitzen Alberts, **Sandra de Zeeuw**, Dirk J Duncker, Pieter D Verdouw, Arie J Man in 't Veld, Maarten ADH Schalekamp. Norepinephrine handling in the porcine heart as assessed by microdialysis. Circulation 1998;98 Suppl I:I-748.

3. **Sandra de Zeeuw**, Loe Kie Soei, Jan R van Meegen, Serge AIP Trines, Rob Krams, Dirk J Duncker, Pieter D Verdouw. EMD 57033 increases calcium responsiveness of normal and stunned myocardium *in vivo*. Cardiovasc Drugs Ther 1999;13:8.

4. Thomas W Lameris, **Sandra de Zeeuw**, Anton H van den Meiracker, Gooitzen Alberts, Frans Boomsma, Dirk J Duncker, Pieter D Verdouw, Arie J Man in 't Veld.

Catecholamine release in the porcine myocardial interstitium during severe transient ischemia. Circulation 1999;100 Suppl I:I-124.

5. **Sandra de Zeeuw**, Mirella A van den Doel, Denis Susa, Dirk J Duncker, Pieter D Verdouw. Adenosine A_1 and A_3 receptors are involved in ischemic preconditioning in rats. FASEB 2000;14:A684.

6. Thomas W Lameris, **Sandra de Zeeuw**, Wouter Tietge, Gooitzen Alberts, Frans Boomsma, Dirk J Duncker, Pieter D Verdouw, Anton H van den Meiracker. Angiotensin II does not enhance interstitial norepinephrine release in the porcine heart. Circulation 2000; in press.

Awards

1998 Price for best poster awarded by Cardiovascular Research School COEUR at the 'AIO-dagen' in Lunteren, the Netherlands.