THERAPEUTICAL MODULATION

OF

CARDIOVASCULAR DISEASE

THERAPEUTICAL MODULATION OF CARDIOVASCULAR DISEASE

THERAPEUTISCHE MOGELIJKHEDEN VOOR HART- EN VAATZIEKTEN

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Aan mijn ouders

Contents

Chapter 1

Introduction and Aims of the Thesis

With aging all tissues are subjected to degenerative processes. For the vascular bed this process is called atherosclerosis and involves thickening and induration of one or more layers of the vessel wall, of mainly the arteries. Atherosclerosis of the coronary arteries starts at a young age, but remains symptomless for many years. During this period atherosclerotic lesions mature from fatty streaks to fibrous lesions and lipid laden plaques with calcifications and ulceration of the endothelial surface. This maturing process is accompanied by a narrowing or stenosis of the diseased coronary arteries. Eventually, the stenosis may become severe and restrict blood flow to the distribution territory of the diseased coronary artery.

The heart is an aerobic organ and relies on oxygen to maintain its contractile function and viability. Under normal conditions oxygen is already nearly maximally extracted from the blood and an increase in oxygen demand will primarily be met by an increase in coronary blood flow. Even under conditions of strenuous exercise healthy coronary arteries can meet the increased energy demand of the myocardium. However, in the presence of a coronary artery stenosis, the energy demand may not be met and an imbalance develops between demand and supply, leading to regional myocardial ischemia. The latter is characterized by anaerobicmetabolism (e.g. lactate extraction changes to lactate production), loss of regional contractile function and potentially lethal arrhythmias.

The ultimate fate of ischemic myocardium depends on the duration and the severity of the coronary artery occlusion, and the ability to supply the ischemic myocardium via coronary collaterals. Complete coronary occlusions lasting less than 2 minutes have no persisting effects on the myocardium, as upon reperfusion there is an almost immediate recovery of contractile function.[1] On the other hand when severe myocardial ischemia persists longer than 20 minutes myocardial cells start to die and contractile function will never fully recover after institution of reperfusion.[2] With ischemic episodes lasting between 2 and 20 min myocardial cells remain viable, but when perfusion of the ischemic myocardium is restored there will be no immediate recovery of contractile function as this may remain depressed for hours to days. This phenomenon of prolonged contractile dysfunction of reperfused viable myocardium has first been described by Heyndrickx et al[3] and is called myocardial stunning.

When a region of the myocardium is stunned, there is no need for treatment when the remainder of the myocardium is functioning normally and global left ventricular function is not compromised. However, when stunning is so severe that global left ventricular function becomes impaired, stunning may require treatment. Chapter 2 presents an overview of the different pharmacological modalities that have been used to prevent and treat myocardial stunning.

Stunning is usually examined in the left ventricle. Little is known about the development of stunning in the right ventricle and its response to pharmacological interventions. Because filling of the left ventricle is dependent on the cardiac output of the right ventricle, loss of right ventricular contractile function may also compromise global left ventricular function.[4] Thus, the response of the stunned right ventricle to inotropic stimulation could play an important role in

the restoration of global left ventricular function. In chapter 3 the effect of a brief occlusion of the left anterior descending coronary artery on both left and right myocardial contractile function has been examined and the responses of stunned left and right myocardium to chronotropic and inotropic stimulation were studied.

The mechanisms behind the sustained contractile dysfunction of stunned myocardium are still not very well understood. Bolli reviewed many of the proposed hypotheses and concluded that an action of oxygen-derived free radicals and a disturbance of the intracellular calcium handling are the most likely mechanisms leading to stunning.[5] The intracellular location of this disturbance, however, has been a major point of discussion (Figure 1). Krause et al proposed that a decreased calcium uptake by the sarcoplasmic reticulum and subsequent decrease in calcium transient underlies the prolonged contractile dysfunction of post-ischemic myocardium.[6] This hypothesis has been challenged by Lamers et al who found that the maximal calcium uptake by the sarcoplasmic reticulum isolated from stunned myocardium had not decreased.[7] Kusuoka and Marban, who had found that in stunned myocardium calcium transients were increased,[8] also challenged the hypothesis of Krause et al[6] and advocated a decrease in the sensitivity of the myofilaments to calcium as a major factor underlying myocardial stunning.[8] Current view holds that the formation of free radicals during ischemia and early reperfusion and the subsequent decrease in the sensitivity of the myofilaments to calcium are likely mechanisms underlying myocardium stunning.[5,9] Thus, a rational therapy for stunned myocardium should be either to inhibit the formation oxygen-derived free radicals or to restore the calcium sensitivity of the myofilaments. The role of oxygen-derived free radicals in the genesis of myocardial stunning and its prevention by free radical scavengers has been studied and reviewed extensively (see ref 5) and the role of free radical scavengers in ameliorating stunning has therefore not been reviewed in chapter 2.

Until recently *in vivo* evidence for a decreased calcium sensitivity of the myofibrils as a cause of stunning could not be obtained, because all available calcium sensitizing agents also possessed phosphodiesterase inhibitory properties, and could therefore enhance myocardial contractile function by a mechanism different from calcium sensitization of the myofibrils. In this thesis the effects of a novel calcium sensitizer EMD 60263, which is devoid of phosphodiesterase inhibiting properties, on contractile function of normal and stunned myocardium have been investigated (Chapter 4). There is some scepticism about the therapeutic use of calcium sensitizers to restore myocardial contractility.[10] In stunned myocardium levels of intracellular calcium are increased and enhancing the calcium sensitivity of the myofilaments may impair diastolic relaxation and myocardial filling. We have addressed this issue in chapter 5 by studying the effect of EMD 60263 on diastolic function of normal and stunned myocardium.

To investigate whether EMD 60263 indeed increased myocardial contractile function by enhancing the myofibrillar calcium sensitivity, we have also tested the effect of this agent on isolated purified sarcoplasmic reticulum vesicles and myofibrils (chapter 6). In figure 1 is shown how these two cell organelles are involved in normal intracellular handling of calcium during the

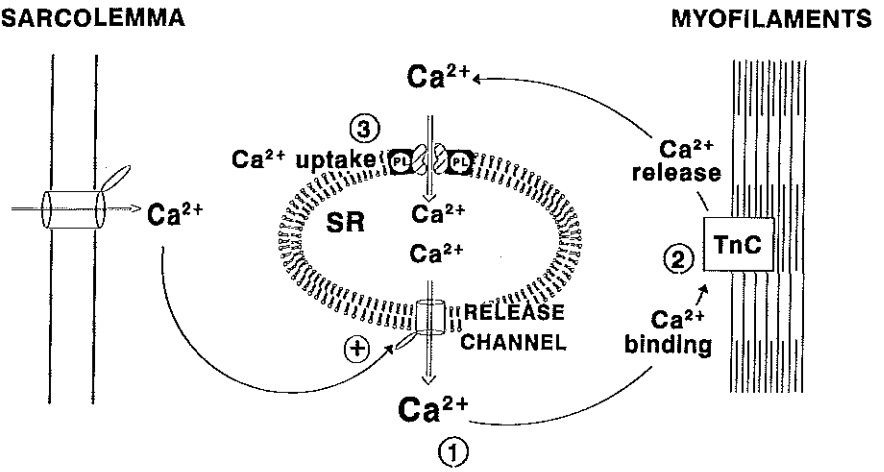

Fig 1. Schematic presentation of the intracellular calcium handling of the myocardium. The numbers 1, 2 and 3 depict possible locations of a disturbance in the calcium handling. A decrease in available activator calcium (1) caused by a decreased calcium uptake by the sarcoplasmic reticulum (3) has been proposed as underlying the prolonged post-ischemic myocardial dysfunction. However, current view holds that formation of oxygen-derived free radicals causes a decrease in the myofibrillar sensitivity to calcium (2). Ca^{2+}=calcium ion; SR=sarcoplasmic reticulum, TnC=troponin C.

excitation-contraction coupling. From a homogenate of stunned and not-stunned porcine myocardium sarcoplasmic reticular vesicles and myofibrils are purified and subsequently biochemically characterized before their response to EMD 60263 was tested.

In the second part of this thesis the modulation of atherosclerosis in the veno-arterial bypass grafts was studied. Atherosclerosis in the coronary vasculature can become so severe that the viability of the myocardium becomes at risk because of impaired blood supply and therefore the need for coronary revascularization develops. Coronary bypass grafting and percutaneous transluminal angioplasty with or without stenting are the most often used approaches to restore normal myocardial perfusion. The ultimate success of these interventions, however, is hampered by an ensuing accelerated development of atherosclerosis.

Bang et al have reported, that in the Inuit population (Eskimos on the west coast of Greenland) the mortality from cardiovascular disease was lower than in a comparable population of Danes.[11] This observation has been correlated with a high intake of fish oil. These epidemiological findings were followed by a large number of experimental studies showing that n-3 fatty acid component of fish oil has potential beneficial effects on the risk factors of atherosclerosis such as plasma lipoprotein levels, thrombosis, hypertension, endothelial dysfunction and inflammatory response. These aspects are reviewed in chapter 7. The experimental studies primarily concern arterial atherosclerosis, however, as knowledge on the effects of fish oil in veno-arterial bypass grafts is limited. In the subsequent chapters it was therefore investigated whether fish oil can modulate not only the progression (chapter 8), but also the regression of atherosclerosis (chapter 9) in vein grafts.

In saphenous vein grafts the benefits of fish oil may stretch from reduction in platelet aggregation to inhibition of fibrointimal hyperplasia. Fish oil, administered prior to coronary bypass surgery, may in the early post-operative phase decrease the incidence of thrombosis. This will not only decrease early failure, but, as thrombosis is also implicated in the development of fibrointimal hyperplasia (due to release of growth factors from the activated platelets), improve the long-term patency rates of vein grafts. The prevention of intimal hyperplasia in both vein grafts and aorta by fish oil is discussed in chapter 8. Once intimal hyperplasia has developed in the vein graft fish oil may still exert a favorable effect as it may alter the chemical composition of the lipids present in the lesion. The latter can facilitate the removal of the lipids from the vessel wall. Furthermore, the action of several cellular components in the intima is modified by fish oil, and thus fish oil may reduce the release of growth factors. Therefore, in chapter 9 not the preventive effect, but the regressive effect of fish oil on intimal hyperplasia in saphenous vein grafts is the focus of attention.

Aims of the thesis

In chapters 2 to 6 the focus of attention is the prolonged post-ischemic myocardial contractile dysfunction, myocardial stunning and the various pharmaceutical modalities of treating this phenomenon. A decrease in myofibrillar calcium sensitivity is likely to underlie this phenomenon and current inotropic drugs overcome this decrease in myofibrillar calcium sensitivity by increasing the calcium transients. A more rational therapy is to restore the myofibrillar calcium sensitivity with calcium sensitizers. We have therefore investigated the effects of myofibrillar calcium sensitization with EMD 60263 on systolic and diastolic function.

Chapters 7 to 9 focus on the problems concerning revascularisation of the myocardium with venous coronary artery bypass grafting. In vein grafts atherosclerosis occurs in an accelerated fashion and is responsible for late graft failures. Although fish oil has been shown to inhibit intimal thickening of the graft, the role of lipoproteins is still unclear. We have therefore investigated the role of modulation of plasma lipoproteins by fish oil on progression and regression of vein graft atherosclerosis.

Experimental model

Studies have been performed in pigs, not only because of the extensive experience that exists with this species in the laboratory of Experimental Cardiology, but also because the pig is particularly suited for cardiovascular studies. The latter has been doubted for a long time although Leonardo da Vinci already used pigs to demonstrate the motion of the heart nearly five

centuries ago. Among those who questioned the usefulness of pigs in biomedical research was the Russian physiologist Pavlov, who after he brought a pig into his laboratory found its shrieking so disruptive that he banned pigs from his laboratory with the declaration that all swine are hysterical.[12,13]

In the last 20 years the pig has gained an increasing popularity in biomedical research. This has not only been the consequence of scientific considerations as many laboratories replaced dogs as their animal model by pigs, but also because of factors such as costs and adverse pressure on the use of dogs in biomedical research. A number of considerations which makes the porcine model so useful for the studies described in this thesis are listed below.

Compared to the dog the pig has a coronary arterial anatomy more similar to that of man.[14] Post mortem studies of the porcine heart also revealed similarities between man and pigs in intramural branching patterns, supply to papillary muscles and nodal conduction tissue. Rather striking differences exist between the presence and function of coronary collateral vessels in normal hearts of dogs and pigs. Dog hearts usually have many subepicardial and intraseptal anastomoses, the number of which may vary, whereas in pig hearts only few subendocardial anastomoses are found.[14] Because of the wide range in the number of coronary collaterals that can be found in dogs a coronary artery occlusion in this species may result in different degrees of myocardial ischemia and mortality rates whereas in pigs an acute coronary artery occlusion invariably results in severe ischemia. Furthermore, complete coronary artery occlusion in pigs has a higher mortality from ventricular fibrillation than in dogs.[15] In the pig the innate collateral circulation in the distribution territory of left anterior descending and left circumflex coronary artery is approximately 1.0 and 5.5 ml/min/100g, respectively of a total blood flow of 100-130 ml/min/100g.[16] Pigs can develop coronary collaterals, however. Twelve weeks after implantation of an ameroid constrictor around the left circumflex coronary artery, collateral blood flow to the myocardium supplied by the left circumflex coronary artery can be as high as 80% of left anterior descending coronary artery blood flow at rest and approximately 50% during exercise. Because of the potential contribution of the collateral blood flow to myocardial perfusion in normal dogs, myocardial perfusion must be determined (e.g. with radioactive microspheres) after a native coronary artery has been occluded in order to determine the perfusion deficit. In normal pigs there is usually no need for such additional measurements as collateral perfusion will invariably be less than 5% of baseline.

Domestic swine develop atherosclerosis spontaneously as they grow older and early lesions and their location closely resemble those in man.[17,18] But just as in man atherosclerosis is a slow process. However, the development of atherosclerotic lesions can be accelerated by either hypercholesterolemia or endothelial denudation or a combination of both interventions.[19] In contrast, dogs rarely develop atherosclerosis and are more resilient to hypercholesterolemia than pigs. The atherosclerosis process in dogs also has a higher media involvement than in humans, monkeys or pigs.[20,21] Furthermore, the response to injury in the coronary arteries are different for pigs and dogs. Schwartz et al damaged coronary arteries in pigs and dogs using tantalum stents

on oversized balloons and observed that, despite similar injury scores, dogs have significantly less neointimal thickening and area stenoses than pigs.[22] Their results also suggested that there was no relation between injury and neointimal thickness in dogs and therefore the authors proposed the pig as a more appropriate model to study the development of neointima. Other characteristics which may promote pigs as a better model to study the development and regression of atherosclerosis than dogs are the lipoprotein metabolism and the fibrinolytic system.[23,24] In contrast to humans dogs have very low levels of triglycerides, VLDL and LDL. Lipoproteins may therefore have a different role in the pathogenesis of atherosclerosis.[23] In contrast serum lipoprotein profile in pigs and humans is quite similar, and from previous studies from our laboratory it appears that in pigs the plasma lipoproteins respond to modulation with dietary n-3 fatty acids.[25-27] Thrombosis is often implicated in the development of atherosclerosis. The higher activity of the fibrinolytic system in dogs warrants caution in the interpretation of intervention studies that may produce thrombosis.[28]

In the atherosclerosis studies of this thesis we have used Göttingen miniature swine, instead of the usual farm pigs (Yorkshire x Landrace), which have been used in the myocardial stunning experiments. Farm pigs grow rapidly and will reach an adult weight approaching or exceeding 300 kg if fed *ad libitum*.[29] This weight gain limits the use of these farm pigs in long lasting experiments. The problem of size can be overcome by using breeds of miniature swine. The Göttingen miniature swine is not a naturally occurring breed of miniature swine, such as the Yucatan, Kangaroo Island, Lee Sung and Assam Pigmy Hog, but is the result of a breeding program with 4 strains of breeding stock at the University of Göttingen in Göttingen, Germany.[29] Miniature swine with their smaller stature and lower rate of growth pose less demands and thus are more economical in care and feeding. An advantage of Göttingen miniature swine is that they respond better to high cholesterol feeding than Yorkshire x Landrace pigs. In a pilot study we have found in Göttingen miniature swine that with a 2% cholesterol diet their plasma cholesterol level increased from 3 mM to 15 mM, prior and after 2 weeks of diet, respectively. In a previous study from our laboratory we had to add bile acids to the diet of farm pigs to increase their plasma cholesterol level from 2 mM to 10 mM.[30] Moreover, Kobari et al have shown that high fat and high cholesterol feeding induced fatty streaks and fibrous lesions in the aorta of Göttingen miniature swine.[31] The same group of investigators also reported that the a cholesterol lowering diet induces regression of fatty streaks in the aorta of Göttingen miniature swine.[31]

In spite of the similarity of the atherosclerotic process in the pig compared to that of man, the cost and duration of the studies, often exceeding a period of one year, limit its general acceptance and has led to the development of other experimental pig-models, which produce results in a shorter period of time. Considerable attention has therefore been paid to the development of lesions in saphenous vein grafts implanted as an end-to-end anastomosis in the carotid artery. The advantage of this model is that the lesions in the grafts, which show a large degree of similarity with lesions in human vein grafts, already develop without a specific dietary intake and within a much shorter period of time (1-2 months). Angelini and Newby proposed that

this model is ideal to test anti-atherosclerotic therapies, because of the analogy between graft and arterial atherosclerosis and because atherosclerosis arises more rapidly and predictably in grafts than in native coronary arteries.[32]

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Chapter 2

Pharmacological Modulation of Myocardial Stunning

Running title: Pharmacological modulation

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Pharmacological Modulation of Myocardial Stunning

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When regional myocardial stunning leads to impairment of global left ventricular function pharmacotherapy may become important. In this chapter we review rational modalities of treatment, based on our current understanding of the mechanisms and pathogenesis of stunning. After stunning has been established, only administration of positive inotropic agents (β-adrenoceptor agonists, calcium, calcium promotors and calcium-sensitizers) results in complete recruitment of contractile function. In view of the role of oxygen-derived free radicals in the pathogenesis of stunning, it is not surprising that only those agents that exhibit at least some free radical scavenging properties (e.g. prostacyclinmimetics, ACE inhibitors, calcium antagonists and ubiquinone) attenuate myocardial stunning. The observations that the degree of stunning is determined by the severity of the preceding period of ischemia implies that recovery of post-ischemic function can also be enhanced by pretreatment (or treatment at the onset of ischemia) with agents that reduce the severity of ischemia or the associated calcium overload (e.g. β-adrenoceptor antagonists, calcium antagonists, adenosine, K^+_{ATP} channel openers and Na^+/H^+ exchange inhibitors). A wide variety of pharmacological interventions are presently available for prevention or treatment of myocardial stunning, but the choice and hence the efficacy of specific treatment depends critically on the timing of the intervention.

(In Vatner SF, Heyndrickx G, Wijns W (eds) : Intrinsic adaptive mechanisms during ischemia and reperfusion, Futura Publishing)

Although contractile function of regionally stunned myocardium ultimately recovers completely,[1,2] it may temporarily produce critical impairment of global left ventricular function. This is especially true for patients who already have a compromised left ventricular function. Therefore, the development of pharmacological strategies for treatment and prevention of myocardial stunning is important.

The first approach to improve post-ischemic dysfunction of stunned myocardium involved the use of positive inotropic agents.[3] A rational therapy should, however, be based on an understanding of the pathogenesis of myocardial stunning. 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 calcium homeostasis.[4-7] At the present time, the release of oxygen-derived free radicals together with calcium overload during the early phase of reperfusion are considered to be key events in the pathogenesis of stunning,[4,5] which may cause a decreased sensitivity of the myofibrils to calcium.[6,7]

Understanding of the limitations of the parameters that are used to describe contractile function and the modulation of the severity of myocardial stunning by the experimental conditions are pivotal for proper assessment of pharmacological interventions aimed at amelioration of stunning. *In vitro* studies usually employ models of global myocardial ischemia and therefore use global cardiac function parameters such as left ventricular developed pressure (isovolumically beating hearts) and cardiac output (working hearts) to assess the degree of stunning. In *in vivo* experiments stunning is produced regionally by temporary occlusion of a coronary artery, requiring measurement of local contractile function parameters such as regional segment shortening or wall thickening. However, all of these parameters are preload- and afterload-dependent and are therefore not a true measure of intrinsic myocardial contractility. Furthermore, afterload-dependency may be further increased in stunned compared to normal myocardium.[8] Consequently, measurements of left ventricular developed pressure, cardiac output and systolic wall thickening and segment shortening should ideally be made under identical loading conditions. Alternatively, changes in left ventricular pressure should be included in the assessment of contractile performance by using parameters derived from the left ventricular pressure-volume or left ventricular pressure-segment length (or wall thickness) relations such as external work and maximal elastance.[8-11]

The degree of stunning is determined in part by the ischemic burden (flow deficit and duration),[12] which suggests that either formation of the amount of oxygen-derived free radicals or the myocardial susceptibility to the damage by oxygen-derived free radicals increases with the severity of the ischemic insult. Consequently, in studies of pharmacological modulation of stunning measurement of residual myocardial perfusion during ischemia is a necessity. This is particularly true for animals which possess an extensive coronary collateral circulation such as

the dog. Labeled microspheres are the first choice of measurement as this technique allows measurement of residual myocardial blood flow and its transmural distribution. Animals such as the pig have negligible collateral blood flow, so that in acute experiments measurement of residual myocardial perfusion during total coronary artery occlusion is less mandatory.

Studies of myocardial stunning have been performed in a large variety of *in vitro* (often non-blood perfused) and *in vivo* models and stunning has thus been produced under very different experimental conditions. *In vitro* heart preparations not only suffer from limited hemodynamic stability but also from progressive edema formation, allowing only a short period in which recovery of post-ischemic function can be studied. Furthermore, durations of ischemia that do not result in myocardial necrosis under *in vivo* conditions, may already produce irreversible damage in isolated perfused hearts. Thus Borgers et al[13] observed that even a 15-minute period of global ischemia in isolated rabbit hearts irreversibly damaged 8% of the myocytes. In *in vivo* studies the importance of the experimental conditions is illustrated by observations that post-ischemic contractile dysfunction is more pronounced in pentobarbital-anesthetized open-chest than in awake dogs.[14] An explanation might be that pentobarbital anesthesia, in part via altering hemodynamic conditions, results in higher oxygen demand at the onset of ischemia.[14] The effect of anesthesia on myocardial oxygen demand and its importance for the degree of stunning is also suggested by other studies. Thus, halothane[15] or isoflurane[16] anesthesia, which were associated with significantly lower levels of myocardial work than fentanyl[15] or morphine/α-chloralose/urethane anesthesia,[16] resulted in better recovery of contractile function following a 15-minute coronary artery occlusion in dogs. Unfortunately, in these studies post-ischemic recovery was not determined at similar afterloads which precludes a definite conclusion regarding the role of anesthesia as a determinant of the degree of myocardial stunning. Another variable that can influence functional recovery of stunned myocardium is temperature. Open-chest animal preparations are more susceptible to temperature variations than awake animals. Triana et al showed that a decrease in body core temperature by as much as 2°C markedly improved function of the stunned region with minimal effect on function of remote normal myocardium.[14] Consequently, a rigorous control of body temperature is required in open-chest studies of myocardial stunning. Finally, recovery of post-ischemic contractile function has been examined in models which employed periods of ischemia that may have produced a mixture of both reversible and irreversible myocardial injury. In these studies recovery of regional function will in part depend on the amount of irreversibly damaged myocardium. Only those studies that have employed periods of ischemia which exclusively produce reversible injury will be discussed.

In this chapter we will first address studies in which pharmacological agents were administered after myocardial stunning had been produced. Subsequently we will review studies of agents that act primarily by decreasing the severity of myocardial ischemia and/or prevent the development of stunning by interfering with formation of oxygen-derived free radicals or calcium overload during early reperfusion. For an extensive discussion of the therapeutic

potential of scavengers of oxygen-derived free radicals, such as super oxide dismutase and catalase, the reader is referred to reference 5 in which the role of oxygen-derived free radicals in the pathogenesis of stunning has been discussed in great detail.

Positive inotropic drugs

Adrenergic receptor agonists

Little was known about the origin of its functional abnormalities when the first studies were undertaken to stimulate contractile function of stunned myocardium. Initially, it was believed that the deterioration of contractile function after its initial recovery following a brief coronary artery occlusion was due to reperfusion damage. To investigate the nature of this reperfusion damage, Smith[17] subjected anesthetized dogs to a 10-minute left anterior descending (LAD) coronary artery occlusion followed by 15 minutes of reperfusion resulting in an 85% decrease of velocity of early systolic shortening. The post-ischemic dysfunction was associated with a depressed blood flow and oxygen consumption. However, oxygen extraction was not altered and lactate production remained positive, indicating that stunned myocardium was distinctly different from ischemic myocardium. An intracoronary infusion of isoprenaline (0.1 μg/min for 10 minutes) restored velocity of shortening and increased regional myocardial oxygen consumption but did not result in a change in lactate extraction, which would have been expected if stunned myocardium contained residually ischemic myocardium. An intracoronary bolus injection of isoprenaline (0.1μg) also improved early systolic shortening of stunned myocardium, reaching a maximum 30 seconds after injection. The effect disappeared within 10 minutes after the bolus injection, suggesting that isoprenaline increased contractility without modifying the mechanism that underlies myocardial stunning. Similarly, Bolli et al[18] reported that after 3 hours of reperfusion following a 15-minute LAD coronary artery occlusion systolic wall thickening of the akinetic segment could be restored to pre-ischemia levels with isoprenaline (0.1 μg/kg/min, iv) for up to 30 minutes, but that function returned to pre-infusion levels after the infusion was stopped. Becker et al[19] demonstrated that the inotropic response to intravenous infusions of epinephrine could be sustained for 1 hour when myocardium stunned by 12 sequences of 5-minute LAD coronary artery occlusion and 10 minutes of reflow, was stimulated after 1-hour of reperfusion. After termination of the infusion contractile function declined to similar values as observed after the 1 hour reperfusion period. These early studies indicated that β-agonists are capable of recruiting contractile reserve of stunned myocardium without deleterious effects. Nonetheless, in stunned myocardium oxygen consumption was reported to be abnormally high with respect to the work performed, implying a reduced mechanical efficiency.[20,21] Therefore, we studied the effect of inotropic stimulation on mechanical efficiency of stunned porcine myocardium.[11,22] Two sequences of 10-minute coronary artery occlusion and 30 minutes of reperfusion decreased systolic segment shortening from 18±2% to 7±2%, while external work (represented by the area enclosed by the left ventricular pressure- segment length relation)

decreased to 50% of baseline. Since myocardial oxygen and lactate consumption decreased to 70% and 15% of baseline, respectively, a significant decrease in mechanical efficiency (defined as the ratio of external work and oxygen consumption) occurred.[22] Intravenous infusion of low-dose dobutamine (2 μg/kg/min) increased segment shortening, external work and oxygen consumption to baseline levels, so that mechanical efficiency was restored. In a subsequent study, using the left ventricular end-systolic pressure-segment relation, we showed that stunning not only decreased maximal elastance (E_{max}) and external work, but also increased the potential energy,[11] suggesting that the impaired mechanical efficiency was the result of a reduction in efficiency of energy transfer from total work to external work. The effect of dobutamine on mechanical efficiency could be explained by the increased efficiency of energy transfer, which resulted from the increase in maximal elastance. Importantly, dobutamine restored mechanical efficiency without evidence of anaerobic metabolism, reflected by the dobutamine-induced increases in oxygen and lactate consumption.[22] Our findings are supported by Kida et al who reported that in porcine myocardium subjected to a 15-minute coronary artery occlusion, ATP loss was not aggravated when swine received a high dose of dobutamine (10 μg/kg/min) throughout the 120-minute reperfusion period.[23] These studies suggest that inotropic stimulation does not aggravate post-ischemic contractile and metabolic abnormalities.

To investigate whether the contractile reserve of stunned myocardium is impaired, Becker et al titrated an intravenous dose of epinephrine to produce a maximal increase in systolic segment shortening. Twelve cycles of a 5-minute coronary occlusion followed by 10 minutes of reperfusion decreased segment shortening from 21.8% to 7.9% at 60 minutes of recovery. Due to occurrence of arrhythmias in response to the same dose of epinephrine during the post-ischemic period, slightly lower doses had to be infused in 4 of the 11 animals, so that the average dose of epinephrine was 24.5 μg/min at baseline and 20.6 μg/min following stunning. Consequently, the post-ischemic maximal response (24.9% shortening) remained somewhat below the maximal response to epinephrine given before ischemia (30.5%), but a similar trend was found in the remote control segment (17.0% vs 22.1%), suggesting that contractile reserve of the stunned region was not different from that of normal myocardium. To minimize the influence of loading conditions we used end-systolic pressure-segment relations to study the effect of a low dose of dobutamine (2 μg/kg/min, iv) on maximal elastance (E_{max}) of stunned and normal myocardium.[24] A 10-minute coronary artery occlusion and 30 minutes of reperfusion in open-chest swine decreased segment shortening and E_{max} to 55% and 40% of baseline, respectively. Dobutamine increased segment shortening and E_{max} to 95% and 170% of baseline in the stunned left ventricular region, respectively. In the normal region dobutamine had no effect on segment shortening but increased E_{max} to 165% of baseline. These studies suggest that stunned myocardium is more sensitive to inotropic stimulation, but that it does not exhibit a decreased contractile reserve compared to normal myocardium.

In conclusion, there is ample evidence that beta-adrenergic receptor agonists are effective in recruiting function of stunned myocardium. Stimulation by these agents does not appear to

produce deleterious effects, and several of these agents are therefore currently used clinically to detect viable but dysfunctional myocardium in patients.[25]

Calcium and calcium promotors

Ito et al infused calcium into the coronary artery of 10 open-chest swine before and 30 minutes after a 15-minute coronary artery occlusion.[26] Under normal conditions, calcium increased segment shortening from 26±2% to 37±2%, whereas calcium increased shortening from 12±3% following stunning to 35±3%, indicating the presence of normal contractile reserve in stunned myocardium. In view of the greater increase in segment shortening produced by calcium in stunned than normal myocardium, the authors concluded that reduced availability of calcium most likely contributed to myocardial stunning.

Smith administered the calcium ionophore A 23187 (2 mg) into the coronary artery of four dogs that had been subjected to a 10-minute coronary artery occlusion followed by 15 minutes of reperfusion.[17] Velocity of early systolic shortening increased, reaching a maximum after 30 seconds, but deteriorated below pre-drug levels 10 minutes after administration. The author concluded that increased calcium influx during established reperfusion aggravated myocardial stunning. However, without data of the effects on A 23187 on systolic wall thickening or hemodynamics, interpretation of this study is difficult. In contrast, Ito et al did not observe deterioration of segment shortening below pre-calcium levels upon withdrawal of calcium infusions.[26]

Continuous infusion of the calcium entry promotor BAY 5959 starting before a 10-minute coronary artery occlusion in anesthetized dogs and continued until 3 hours of reperfusion resulted in complete recovery of systolic wall thickening against approximately 60% in vehicle-treated animals.[27] Diastolic arterial blood pressure was not affected therefore excluding a major contribution of changes in loading conditions. The authors did not allow washout of the drug and could therefore not determine whether stimulation of the myocardium during ischemia and early reperfusion exerted deleterious effects on post-ischemic function.

In conclusion, increases in intracellular levels of calcium can effectively recruit contractile reserve. The weight of evidence suggests that the calcium-mediated inotropic stimulation is without deleterious effects on stunned myocardium.

Calcium sensitizers

Disturbances in calcium homeostasis have been proposed to play an important role in myocardial stunning.[4-7] Abnormalities in calcium transients were initially thought to be responsible,[26,28] however, more recent work has shown that in stunned myocardium the sensitivity of the myofibrils to calcium is decreased,[29,30] while calcium transients may actually be increased.[6,7,31] Consequently, pharmacological restoration of myofibrillar calcium sensitivity appears a rational therapeutic approach.

Heusch et al studied the inotropic response to the proposed calcium-sensitizing agent AR-L

57, and reported that the increase in velocity of systolic thickening produced by this agent did not differ for stunned and normal myocardium in an *in vivo* canine model of stunning produced by a 15-minute coronary occlusion.[32] Korbmacher et al investigated the effects of the thiadiazinone derivative EMD 57033 on stunned myocardium in an isolated rabbit heart model and found that the function of both normal and stunned myocardium was improved.[33] Interpretation of these studies is difficult as these agents (particularly AR-L 57) also increase contractility by phosphodiesterase inhibition.[34,35]

We investigated the effects of the thiadiazinone derivative EMD 60263 (a calcium sensitizer devoid of phosphodiesterase-inhibiting properties[35,36]) in anesthetized pigs in which regional myocardial stunning was produced by two sequences of 10-minute coronary artery occlusion and 30 minutes of reperfusion.[37] EMD 60263 (0.75-1.5 mg/kg, iv) restored systolic segment shortening and mechanical efficiency of the stunned myocardium with minimal effect on segment shortening and mechanical efficiency in normal myocardium (Fig. 1). Phosphodiesterase inhibition and increased calcium sensitivity via α_1-adrenergic receptor activation were excluded as mechanisms of inotropy, as the actions of EMD 60263 were unmitigated in the presence of combined alpha and beta-adrenoceptor blockade. We also demonstrated that the effects of EMD 60264, the enantiomer of EMD 60263 which is devoid of calcium sensitizing properties but shares its rectifier current I_{kr}-mediated bradycardic action, did not enhance post-ischemic segment shortening (Fig. 1).

Increases in calcium sensitivity not only improve systolic performance but may at the same time affect diastolic function adversely.[38] We therefore studied the effect of EMD 60263 on the pattern of regional diastolic segment lengthening in the same porcine model of myocardial stunning.[39] EMD 60263 in a dose of 1.5 mg/kg had no adverse effect on regional diastolic function yet restored regional systolic function. However, when the dose of EMD 60263 was

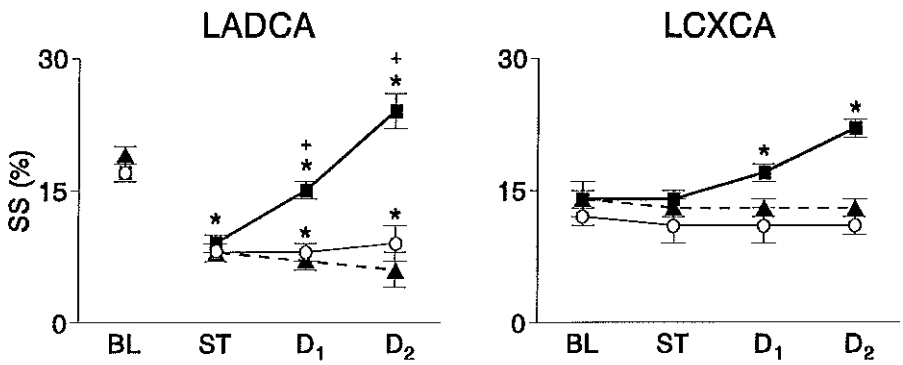

Fig 1. Graphs showing the effect of the calcium sensitizer EMD 60263 and its enantiomer EMD 60264 on both stunned (left panel) and not stunned (right panel) myocardium. LADCA=left anterioar descending coronary artery; LCXCA=left circumflex coronary artery; SS=systolic segment shortening; BL=baseline; ST=stunning; ■=EMD 60263, ▲=EMD 60264, for both D_1=1.5 mg/kg, D_2=3.0 mg/kg.○=saline-treated group, D_1=3 ml, D_2=6 ml; *p<.05 vs baseline and +p<.05 vs stunning.

increased to 3.0 mg/kg relaxation abnormalities emerged, illustrated by a delay in the onset of segment lengthening in both stunned and normal regions. In contrast, the enantiomer EMD 60264 had no effect on either systolic or diastolic function. The effects of EMD 60263 on diastolic function have been compared to those of EMD 57033 in an isolated rabbit heart model.[40] In that study EMD 57033 improved systolic and diastolic function dose-dependently, whereas EMD 60263 had a deleterious effect on diastolic function but only at higher concentrations. The doses used in that study (3 and 10 μM) appear to be rather high, however, as in our *in vivo* experiments plasma concentrations remained below 2 µM after administration of EMD 60263 in a dose of 1.5 mg/kg, iv (unpublished data).

The available data suggest that calcium-sensitizers can effectively recruit function of stunned myocardium. Although at high doses a negative lusitropic effect may become apparent, low doses can fully restore systolic function without an adverse effect on diastolic function. Calcium-sensitizers which display some phosphodiesterase inhibiting activity, particularly at higher doses, may be less likely to cause relaxation abnormalities.

Beta-adrenergic receptor antagonists

The negative chronotropic and inotropic actions of beta-adrenergic receptor antagonists can alleviate regional ischemia by lowering the energy requirements and by improving transmural distribution of myocardial perfusion. Although the anti-ischemic actions of beta-blockers would be expected to enhance recovery of post-ischemic function, results of studies are equivocal. Discrepancies in results could be explained by timing and route of administration and half life of the beta-blocker used. Since bradycardia during reperfusion does not improve function of stunned myocardium,[37] beta-adrenoceptor blockers administered during reperfusion are likely to aggravate post-ischemic dysfunction due to their negative inotropic action. Thus, in order to be most effective beta-blockers should be present before and during ischemia in order to obtain a maximal anti-ischemic effect, but absent during reperfusion in order to avoid further depression of contractile function of stunned myocardium. The most favorable effects can thus be expected from pretreatment with agents such as esmolol which have a short half life. Several studies support this hypothesis. For instance, an intravenous infusion of esmolol (0.10-0.15 mg/kg/min, resulting in 18% reductions in heart rate and LVdP/dtmax) which was started before and continued throughout a 15-minute coronary artery occlusion in anesthetized dogs, resulted in enhanced recovery of function of stunned myocardium.[41] In contrast, an intracoronary infusion of propranolol (0.30 mg/kg + 0.01 mg/kg/hr), which had no effect on heart rate or systolic arterial blood pressure, failed to improve recovery of function in the same canine model of myocardial stunning.[42] Infusion was started prior to occlusion and continued during the first two hours of the 3-hour reperfusion period, but it is unlikely that washout of propranolol (total dose 1.5 mg/kg) was complete within one hour after stopping the infusion. The failure of propranolol to ameliorate stunning is best explained by the persisting negative inotropic actions

of propranol during reperfusion which outweighed the anti-ischemic effects during coronary artery occlusion.[42] In addition, the absence of bradycardia may have attenuated the anti-ischemic effect, which is supported by observations that pretreatment with specific bradycardic agents enhances recovery of function of stunned myocardium.[43]

Przyklenk and Kloner[44] infused esmolol (0.10-0.15 mg/kg/min, iv) for two hours starting after 30 minutes of reperfusion following a 15-minute coronary occlusion to test the hypothesis that stunning is a protective phenomenon which prevents viable tissue to become irreversibly damaged. After 24 hours recovery of wall thickening in the animals treated with esmolol was not different from that of control animals, or animals which underwent the same protocol but received the afterload-reducing agent hydralazine.

The available data indicate that beta-adrenergic receptor antagonists can improve recovery of post-ischemic function by anti-ischemic actions, and should therefore be present during ischemia. To obtain maximum benefit these drugs should be absent during reperfusion.

Calcium antagonists

Calcium antagonists could attenuate myocardial stunning, because of an anti-ischemic action secondary to their effects on the coronary circulation (coronary vasodilation) and systemic hemodynamics (peripheral vasodilation and negative inotropy). The anti-ischemic actions of agents such as verapamil and diltiazem is further enhanced by their bradycardic action, whereas the anti-ischemic effect of the dihydrophyridines may be mitigated by a reflex mediated tachycardia. Another argument in favor of a role for calcium antagonists in myocardial stunning is derived from the two stage model for the role of calcium in the development of stunning proposed by Opie.[46] According to this hypothesis cytosolic calcium increases during ischemia, while at the same time the oscillations in calcium decrease suggesting a reduced ability of the cell to maintain calcium homeostasis. Immediately upon restoration of blood flow, calcium oscillations increase and contractile function transiently increases (first stage). The newly formed oxygen-derived free radicals may increase cytosolic calcium during early reperfusion by a number of actions including release of calcium from the sarcoplasmic reticulum,[47] stimulation of the sodium-calcium exchanger and increased entry of extracellular calcium via voltage-operated calcium channels.[48] The ability of calcium antagonists to prevent calcium overload and their reported oxygen-free radical scavenging properties[49-51] may thus enhance their potential in preventing stunning. After the events during the very early reperfusion period a second stage follows during which contractile function is still decreased, most likely secondary to a decreased sensitivity of the myofilaments to calcium.[6,7] During this second stage an intervention with calcium antagonists does not appear opportune in view of their potential negative inotropic actions.

In spite of its negative inotropic properties, Przyklenk and Kloner[52] reported that verapamil attenuated stunning produced by a 15-minute coronary occlusion in open-chest dogs when

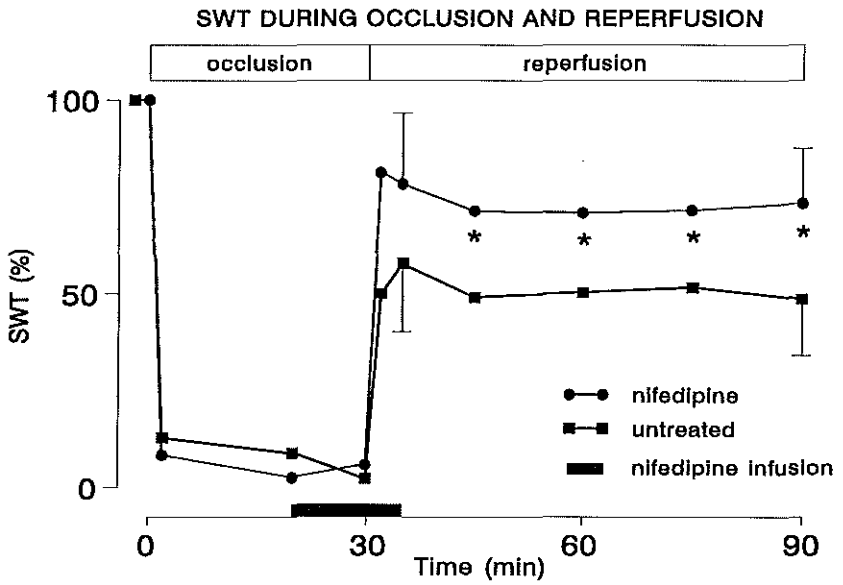

Fig 2. Effect of nifedipine on recovery of systolic wall thickening in anesthetized pigs in which left anterior descending coronary artery blood flow was reduced to 25% of baseline for 30 minutes. Infusion of nifedipine (1 μg/kg per min) started 10 minutes before the stenosis was released and lasted 15 minutes. The dose of nifedipine redistributed transmural blood flow during ischemia in favor of the endocardium. Data (mean ± SD) are expressed as percent of baseline values. *p<.05 vs untreated group. With permission from Eur Heart J (56)

administered after 30 minutes of reperfusion. However, in this study the fall in arterial blood pressure could have contributed to the increase in segment shortening. To avoid systemic hemodynamic effects, the same group of investigators[53] studied nifedipine during intracoronary infusion in the same model of stunning. Nifedipine administration was started after 30 minutes of reperfusion, and was reported to result in improved contractile function. The dose of nifedipine was so low that it had no effect on myocardial blood flow, yet the authors proposed a direct effect of nifedipine on the myocardium, possibly involving an oxygen-derived free radical scavenging action of the calcium antagonist.[49-51] This appears highly unlikely as antioxidant therapy is only effective when started at the onset of reperfusion.[4,5] In keeping with these latter observations Ehring et al observed no effect on recovery of systolic wall thickening when nisoldipine was administered 4 minutes into reperfusion following a 15-minute coronary artery occlusion.[54] The adequacy of the dose of nisoldipine was illustrated by the drug's protection when it was administered prior to ischemia. This study confirmed earlier observations by Van der Giessen et al in a porcine model of coronary thrombosis that addition of nifedipine to porcine plasmin did not enhance recovery of function after reperfusion had been established.[55] The benefit of administration of calcium antagonists during established reperfusion is also

questioned by the observations that contractile function of stunned myocardium can be recruited with substances that increase rather than decrease trans-sarcolemmal calcium fluxes.[26,27,51]

The effectiveness of treatment with calcium-antagonists during ischemia may depend on the timing of administration. When administered just prior to reperfusion calcium-antagonists may protect via prevention of oxygen-derived-free-radical induced calcium overload during early reperfusion.[45-51] When given early after the onset of ischemia calcium antagonists may in addition exert a beneficial effect through an anti-ischemic action.[56-60] In our laboratory an intravenous infusion of nifedipine was started 20 minutes after blood flow in the left anterior descending coronary artery of pigs was reduced to 25% of baseline for 30 minutes and continued until 5 minutes into reperfusion.[56] During the partial coronary artery occlusion there was complete loss of systolic wall thickening which recovered to 75% of baseline in the nifedipine group, but only to 50% of baseline in the vehicle treated group (Fig. 2). The dose of nifedipine (1 μg/kg/min) was sufficient to produce an 8 mmHg decrease in mean aortic pressure, normalize the slightly elevated left ventricular end diastolic blood pressure, modestly increase transmural blood flow of the ischemic myocardium with a redistribution of flow in favor of the subendocardium. In spite of the apparent anti-ischemic effects there was no improvement in function during the ischemic episode, most likely because the improvement in blood flow was insufficient to lead to systolic contraction. The data, however, demonstrate that nifedipine reached the ischemic territory and may have prevented excessive cytosolic calcium concentrations. This may have resulted in a better preservation of the calcium sensitivity during the second stage of recovery. However, it cannot be determined from our study whether the enhanced post-ischemic contractile recovery was due to an anti-ischemic effect or due to modulation of the reperfusion conditions. Administration of verapamil (0.2 μg/kg + 0.6 μg/kg/min, iv) at the onset of reperfusion following a 15 minute coronary occlusion resulted in improved recovery of contractile function, but the drug-induced hypotension could have contributed to this action. In our study nifedipine was discontinued at 5 minutes of reperfusion so that after 60 minutes of reperfusion aortic blood pressure was no longer different from that in the control group, while the improvement in wall thickening persisted. Ehring et al administered nisoldipine (5 μg/kg, iv) 10 minutes after the onset of a 15-minute coronary artery occlusion and found that post-ischemic recovery of stunning was not improved.[54] However, nisoldipine had no effect on collateral blood flow, while the calcium antagonist-induced-hypotension was prevented by inflation of an intraaortic balloon. Therefore, an anti-ischemic effect could only have occurred via a direct myocardial effect. Because pretreatment with nisoldipine did result in a better functional outcome the authors concluded that the beneficial effect of the calcium antagonist was related to an attenuation of calcium overload during the first few minutes of ischemia, rather than during early reperfusion. It cannot be excluded, however, that due to intravenous administration just 5 minutes prior to reperfusion, levels of nisoldipine in the ischemic myocardium may have been too low to prevent calcium overload during early reperfusion.

In *in vitro* studies pretreatment with calcium antagonists has consistently resulted in improved post-ischemic function, but in these studies the drugs were usually present in doses sufficiently high to significantly depress function.[61-63] *In vivo* studies have been performed in anesthetized dogs[54,60,64-68] and have been consistently positive when the calcium antagonists were already present before ischemia was produced (see also ref 69). In most of these studies a decrease in aortic blood pressure may have contributed to the positive outcome, either by decreasing energy demand during ischemia and by decreasing afterload during reperfusion. One study avoided this pitfall as the authors controlled arterial blood pressure with an intra-aortic balloon.[54] Under these controlled conditions nisoldipine improved recovery of contractile function, indicating that calcium antagonists can exert a protective effect independent of an effect on the loading conditions.

Another mechanism of protection by calcium antagonists could be an increase in blood flow to the ischemic myocardium, but in most studies calcium antagonists failed to increase collateral blood flow,[54,59,60,63,66,69] suggesting that an increase in flow cannot be the only mechanism of protection. Improvement in myocardial blood flow can result in an increase in contractile function (Gregg phenomenon) and several studies have suggested that this might be a mechanism by which calcium antagonists improve post-ischemic function.[52,60,64,69,70] However, calcium antagonists also result in an improvement in recovery of function in the absence of an increase in myocardial blood flow.[65,66]

Magnesium is an endogenous calcium antagonist and could ameliorate stunning similar to pharmacological calcium antagonists. This has indeed been shown in isolated perfused rat hearts[63] and in anesthetized open-chest pigs.[71] In the latter study magnesium improved post-ischemic function not only when administered before but also when administered during coronary artery occlusion and the first minutes of reperfusion. Pigs lack a significant coronary collateral circulation and an anti-ischemic effect because of its presence during ischemia can thus be excluded, suggesting that magnesium exerted its protection during reperfusion, possibly by decreasing the oxidative stress.

In summary calcium antagonists can improve recovery of post-ischemic function when administered prior to ischemia. The protection cannot be entirely explained by increases in myocardial blood flow and favorable hemodynamic actions during ischemia. Finally, it also appears that the degree of protection afforded by pretreatment are similar for the different classes of calcium antagonists.[45,46,69]

Eicosanoids

Prostacyclinmimetics

Cycloxygenase converts arachidonic acid into cyclic endoperoxide intermediates PGG_2 and PGH_2, which then form prostaglandins and thromboxane A_2. Prostacylin (PGI) is the main cyclooxygenase product in the vascular endothelium and a potent vasodilator of various vascular

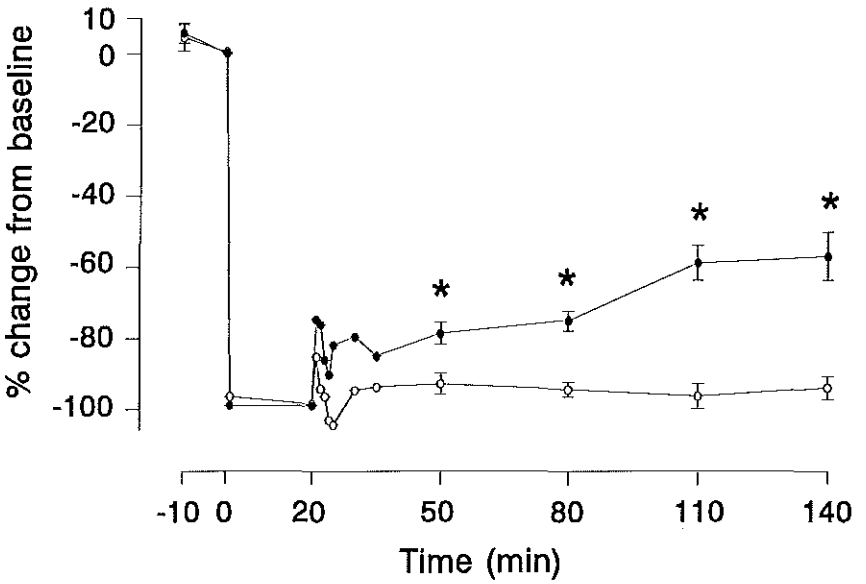

Fig 3. Recovery of systolic wall thickening after 20 minutes of left anterior descending coronary artery occlusion (solid bar) in (●) iloprost (100 ng/kg per min) and (○) solvent-treated pigs. Data are expressed as Δ% from baseline (40 ± 2% for the iloprost and 35 ± 3% for the solvent group). Each point represents the mean, and vertical lines s.e.mean, of the number of animals in parentheses. *p<.01. With permission from Br J Pharmacol (74).

beds including the coronary circulation. In addition, this eicosanoid inhibits platelet aggregation, stabilizes lysosomal membranes and prevents formation of oxygen-derived free radicals by inhibition of neutrophil function, all of which may exert a beneficial effect on myocardial stunning.[72,73] Since prostacyclin has a half life in the order of only a few minutes, stable analogues of prostacyclin (iloprost) or compounds which enhance its endogenous production (defibrotide) have been developed to prolong the duration of action.

In anesthetized pigs, a continuous intravenous infusion of iloprost (100 ng/kg/min) that was started 10 minutes before a 20-minute coronary artery occlusion improved recovery of post-ischemic wall thickening without attenuating the severity of myocardial ischemia, as indicated by the breakdown of ATP.[74] Although contribution of a reduction in afterload to the improved wall thickening could not be excluded, the 8 mmHg decrease in arterial blood pressure was likely too small to entirely explain the improved recovery of function (Fig 3). Farber et al[75] started administration of iloprost to anesthetized dogs either before the coronary artery occlusion or at the onset of reperfusion. Iloprost enhanced recovery of post-ischemic function in both groups of animals, but was most effective when present already prior to and during ischemia. The beneficial effect of iloprost could again not be explained by an effect on systemic

hemodynamics, collateral blood flow or better preservation of higher energy phosphate levels. A decrease in formation of oxygen-derived free radicals by activated neutrophils may have contributed, which would also explain the modest protection when the drug was administered at the onset of reperfusion.[75] Farber et al also showed that prostaglandin E_1 improved systolic wall function in anesthetized dogs after stunning myocardium by a 15-minute coronary occlusion, but the marked hypotension produced by the prostaglandin contributed, at least in part, to the enhanced recovery of post-ischemic function.[76]

Hohlfeld et al[77] reported that defibrotide enhanced recovery of systolic wall thickening that followed a 5-minute coronary artery occlusion in anesthetized pigs. The authors ascribed their findings to both a reduction in ischemia-reperfusion induced platelet aggregation and a free radical scavenging action. Taken together these studies indicate that prostacyclinmimetics attenuate myocardial stunning, which is most effective when these agents are present during the ischemic period, but their mechanism of action is not fully understood. Thus, whereas scavenging of oxygen-derived free radicals may contribute to their salutary effects, it is questionable whether platelet aggregation contributes significantly to contractile abnormalities that follow very brief coronary occlusions.

Thromboxane inhibitors

Thromboxane A_2 (TXA_2) is the main arachidonate metabolite in platelets and may be released from ischemic myocardium. TXA_2 releases catecholamines from terminal nerve endings, mediates vasoconstriction and enhances platelet aggregation, actions that may exacerbate myocardial stunning. The actions of TXA_2 can be antagonized by inhibiting TXA_2 synthesis or blocking TXA_2 receptors. The effects of the TXA_2 receptor antagonist SQ 29,548 on recovery of post-ischemic contractile function were studied in open-chest dogs that underwent a 15-minute coronary artery occlusion followed by 5 hours of reperfusion.[78] Post-ischemic segment shortening was markedly improved irrespective whether drug administration was started prior to occlusion or 1 minute prior to reperfusion. In contrast, pretreatment with BM 13.505, another TXA_2 receptor blocker, failed to ameliorate myocardial stunning produced by 15-minute coronary artery occlusion followed by 3 hours of reperfusion in open-chest dogs.[79] An explanation for these different findings is not readily found, as both compounds were shown to provide adequate TXA_2 receptor blockade and to exert no effect on pre-occlusion hemodynamics or regional myocardial perfusion. It cannot be excluded that the different reperfusion conditions may have played a role, as in the study of Farber and Gross coronary arterial inflow was restricted to match pre-occlusion blood flow values. Interestingly, Farber and Gross, using the same restricted reperfusion model observed that pretreatment with the TXA_2 synthetase inhibitor dazmegrel enhanced post-ischemic segment shortening, and decreased coronary venous TXB_2 levels while increasing 6-keto $PGF_{1\alpha}$ levels (breakdown products of TXA_2 and prostacyclin, respectively) in the coronary venous blood.[79] These findings suggest that dazmegrel produced its beneficial actions via an increase in prostacyclin as a result of

"endoperoxide steal".[80] In support of this hypothesis the authors observed that pretreatment with indomethacin (which itself had no effect on stunning, while decreasing both prostacyclin and TXA_2 production) attenuated the enhanced release of prostacyclin and prevented the beneficial effects of dazmegrel on post-ischemic function. These findings suggested that TXA_2 did not contribute to stunning and that improvement of post-ischemic contractile function by TXA_2 synthetase inhibitors acted via an endoperoxide steal mediated increase in prostacyclin production.[80] The available data do not allow a definitive conclusion regarding the potential usefulness of TXA_2 receptor antagonists in the treatment of myocardial stunning. On the other hand, TXA_2 synthetase inhibitors appear to be protective via an increased prostacyclin production.

ACE-inhibitors

Activity of angiotensin I converting enzyme (ACE) is increased during acute coronary artery occlusion,[81] which results in increased production of angiotensin II and increased breakdown of bradykinin.[82] Since angiotensin II is a potent vasoconstrictor and positive inotropic agent,[75] and bradykinin stimulates prostacyclin production, increased ACE activity during coronary artery occlusion could aggravate myocardial stunning. Consequently ACE inhibitors may attenuate stunning.[83,84] In isolated perfused rat hearts captopril improved recovery of left ventricular developed pressure and $LVdP/dt_{max}$ following a 15-minute period of global ischemia when this ACE inhibitor was present in the perfusate at the time of ischemia.[85,86] Interpretation of these data is complicated by observations that 15-minute of global ischemia in isolated buffer perfused hearts can already result in irreversible damage.[13]

Przyklenk and Kloner infused enalapril for 2.5 hours starting after 45 minutes of reperfusion following a 15-minute coronary artery occlusion in anesthetized dogs.[87] Enalapril (2 mg/kg iv bolus followed by 2 mg/kg per hour) resulted in enhanced recovery of systolic segment shortening to 50% of baseline versus 20% in the control group, but caused a 20 mmHg reduction in arterial pressure which contributed, at least in part, to the improved post-ischemic segment shortening. Westlin and Mullane studied the effects of captopril (5 mg/kg iv) on recovery of function following a 15-minute coronary artery occlusion in open-chest dogs.[88] Administration of captopril prior to ischemia or just before reperfusion resulted in similar improvement of recovery of segment shortening to 50-60% of baseline compared to 0-10% in the control dogs. The mechanism of protection appeared to be the result of scavenging of oxygen derived free radicals as SQ 14,534 (5 mg/kg iv) (a captopril stereoisomer with minimal ACE inhibiting properties but similar scavenging properties) given at the onset of reperfusion produced a degree of protection similar to that by captopril. Furthermore, an equihypotensive dose of enalapril (1.6±0.1 mg/kg iv) (which lacks the free radical scavenging sulfhydril moiety) administered prior to the occlusion had no significant effect on post-ischemic function, which also excludes systemic hemodynamics as a cause for the captopril-induced protection.[88] In

contrast, Przyklenk and Kloner found that administration of enalapril at the end of a 15-minute coronary artery occlusion improved recovery of function to 70-80% of baseline values versus 0-20% in control animals.[89] The differences in the results of the two studies could be due to differences in timing of administration. The finding that in the study by Westlin and Mullane recovery of the enalapril-treated animals was worse than in the control group may point towards more severe ischemia after treatment with enalapril. Collateral blood flow was not measured in that study, but despite the 20 mmHg reduction in mean aortic blood pressure produced by enalapril, the rate pressure product was almost 30% higher during the coronary artery occlusion in the enalapril- treated dogs. On the other hand, in the study by Przyklenk and Kloner[89], collateral blood flow tended to be higher in the enalapril group (~ 0.20 ml/min/g) than in the control group (~ 0.09 ml/min/g) although the small number of animals in each group (n=6) did not yield statistical significance. Interestingly, whereas the doses of enalapril were nearly identical (1.5 mg/kg, iv versus 1.6±0.1 mg/kg, iv) a 20 mmHg decrease was reported in one study but no effect on blood pressure was reported in the other. In accordance with the study by Westlin and Mullane,[88] Przyklenk and Kloner observed that zafenopril (15 mg/kg, iv), another ACE inhibitor with a sulfhydril group, and SQ 14,534 resulted in similar improvement recovery

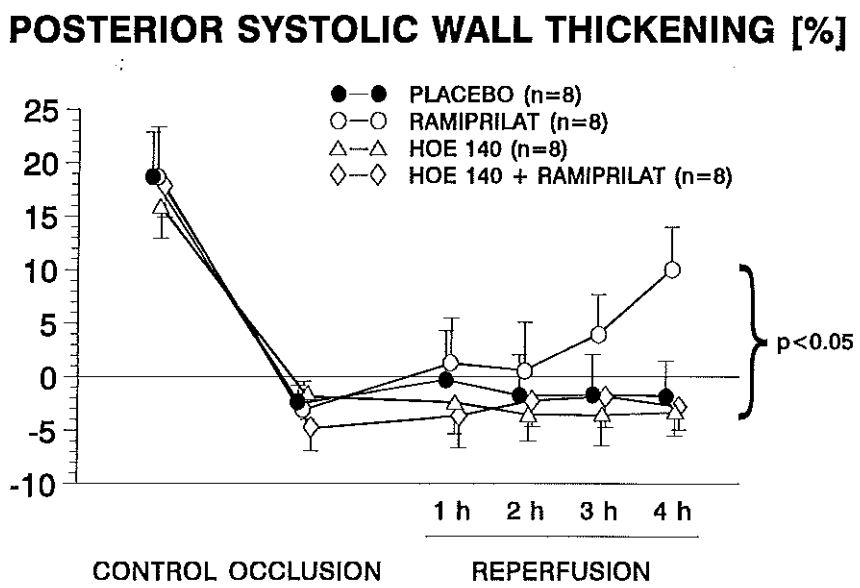

Fig 4. The effect of the ACE inhibitor ramipralat on systolic wall thickening of stunned myocardium. Data are from four groups of animals (see inset of figure) during left circumflex coronary artery occlusion and 4 hours of reperfusion. Only in the dogs that received ramiprilat (O) did systolic wall thickening recover during reperfusion. Data are mean ± SD. With permission from Circulation (90).

of post-ischemic function when administered at the onset of reperfusion. These findings could not be explained by differences in systemic hemodynamics or myocardial blood flow.[89]

Ehring et al[90] studied the role of bradykinin in the beneficial effects of non-sulfhydril ACE inhibitors. For this purpose ramiprilat (20 μg/kg, iv) was administered in the absence and in the presence of the bradykinin B_2 receptor antagonist HOE140 on regional myocardial perfusion and wall thickening, while mean arterial blood pressure was kept constant with an intra-aortic balloon (Fig. 4). This study established that the effect of ramiprilat on recovery of post-ischemic wall thickening was bradykinin mediated. Since bradykinin promotes synthesis of both prostacyclin, which can ameleriorate stunning,[74,75] and nitric oxide, the authors further investigated which of these two pathways was involved in the attenuation of stunning. For this purpose anesthetized dogs received either the cyclo-oxygenase inhibitor indomethacin or a combination of indomethacin and ramiprilat, the synthase inhibitor N^G-nitro-L-arginine methyl ester (L-NAME) or a combination of L-NAME and ramiprilat before they were subjected to a 15-minute coronary artery occlusion and 4 hours of reperfusion. Regional blood flows in the four groups were not different but wall thickening of the stunned myocardium recovered to 47% of baseline during reperfusion in the animals which received L-NAME in combination with ramiprilat. The authors therefore concluded that attenuation of stunning by ramiprilat involves a cascade of bradykinin and prostaglandins but not nitric oxide. The design of the study did not allow to determine when ramipralat exerted its beneficial effect as the drug was only administered prior to ischemia.

Data from the literature indicate that ACE inhibitors, administered prior to ischemia or just before the onset of reperfusion, enhance early recovery of post-ischemic function. The mechanism of protection likely involves scavenging of oxygen-derived free radicals (sulfhydril compounds) or bradykinin-mediated release of prostacyclin.

Adenosine

Adenosine exerts a number of favorable actions during myocardial ischemia and reperfusion, including coronary vasodilation, replenishment of depressed ATP stores, stimulation of glycolysis, inhibition of calcium transport, and inhibition of leukocyte function, lipolysis and free radical generation.[91,92] Since these actions can attenuate stunning,[4] several investigators have examined if increased levels of endogenous adenosine have a salutary effect on stunning.

Kitakaze et al[42] reported that α_1-adrenoceptor stimulation had a beneficial effect on stunning and following their earlier report that release of adenosine was regulated by α_1-adrenoceptor activity,[93] they suggested that attenuation of stunning by α_1-adrenoceptor agonists could be mediated by adenosine. The findings, that the adenosine receptor antagonist 8-phenyltheophylline blocked the beneficial effect of methoxamine and that exogenous adenosine administered before a coronary occlusion ameliorated stunning in the presence of prazosin supported this hypothesis.[42] Attenuation of stunning by α_1-adrenoceptor stimulation had also

been ascribed to an increase in the sensitivity of the myofilaments to calcium, however.[94,95]

Zughaib et al[96] circumvented the potential pitfalls of adrenoceptor stimulation and investigated whether combined inhibition of adenosine deaminase and nucleoside transport improved post-ischemic dysfunction produced by a 15-minute coronary artery occlusion in anesthetized dogs. Intracoronary infusions, which were used to ensure that the effects were independent of changes in systemic hemodynamics, had no effect on loss of systolic wall thickening during ischemia, but improved recovery of function during reperfusion compared with the control group. That enhanced recovery could be secondary to differences in perfusion during occlusion (collateral blood flow) and reperfusion, systemic hemodynamic variables, or myocardial ATP levels was excluded.[97] Compared with control dogs, the levels in adenosine were six times higher in the treated dogs, while the levels in inosine were six times higher in the control animals, suggesting that the increased endogenous adenosine levels attenuated stunning. The results by Zughaib et al[96] are consistent with those reported for acadesine, an other agent that increases endogenous levels of adenosine.[98]

The same group of investigators[99] also examined in the same canine model whether exogenous adenosine alleviated stunning. Anesthetized dogs were therefore again subjected to a 15-minute coronary artery occlusion and 4 hours of reperfusion but received either intracoronary infusions of adenosine or saline starting either 30 minutes before occlusion until 1 hour after onset of reperfusion or 2 minutes before reperfusion until 1 hour after onset of reperfusion.[99] Exogenous adenosine improved recovery of post-ischemic function when present during ischemia, but failed to improve function when infusion started just prior to reperfusion.[99] The beneficial effect of exogenous adenosine could, similar to the study by Zughaib et al,[96] not be explained by an augmented collateral blood flow or more favorable hemodynamic conditions during ischemia. That adenosine had to be present during ischemia in order to be effective suggests that it acted by attenuating ischemic injury. Studies in anesthetized dogs suggest that the responsible adenosine receptor subtype is the A_1 subtype.[100] The latter authors also showed that pretreatment with glibenclamide, in a dose which had no effect on recovery of stunned myocardium itself, abolished the protection by the selective adenosine A_1 agonist cyclopentyladenosine, which suggests that the protection by stimulating adenosine A_1 receptors is, at least in part, mediated by activation of K^+_{ATP} channels.

K^+_{ATP} channels openers

K^+ channels that are sensitive to intracellular levels of ATP (K^+_{ATP} channels) have been identified in a number of tissues, including vascular smooth muscle cells and cardiomyocytes. When ischemia occurs and levels of ATP near the sarcolemma decrease, these K^+ channels are thought to become activated resulting in an outward flux of K^+. K^+_{ATP} channel opening is potentially protective during myocardial ischemia.[101] Thus, coronary vasodilation could promote collateral blood flow while shortening of the cardiomyocyte action potential duration would

result in a decrease of activator calcium influx during phase 2 of the action potential, and reduce calcium overload of the cardiomyocyte.[102,103] The consequent attenuation of ischemia injury would then result in a better recovery of post-ischemic function.

Early studies demonstrated a protective effect of the mixed nitrate-K^+_{ATP} channel activator drug nicorandil on myocardial stunning. Thus, Gross et al observed that a 15-minute total coronary artery occlusion produced a sustained depression of systolic segment shortening to approximately 20% of baseline which lasted throughout the 4 hour reperfusion period; pretreatment with intravenous nicorandil resulted in recovery of segment shortening to 70% of baseline.[104] Nicorandil is not a pure K^+_{ATP} channel activator as it also exhibits nitrate-like properties, but its effects can be blocked by glibenclamide indicating that its K^+_{ATP} channel activator properties are likely responsible for the observed protection.[105] Furthermore, studies with selective K^+_{ATP} channel openers such as cromakalin[106,107] and bimakalim[108] also improve contractile recovery following 15 minutes of regional ischemia in the dog heart, while pure nitrates offer only a slight degree of protection.[104] The observation that intracoronary administration[106] or intravenous administration of non-hypotensive doses[105] of K^+_{ATP} openers enhanced post-ischemic recovery of function indicates that the decrease in afterload is not required for protection. This is further supported by the finding that pretreatment with

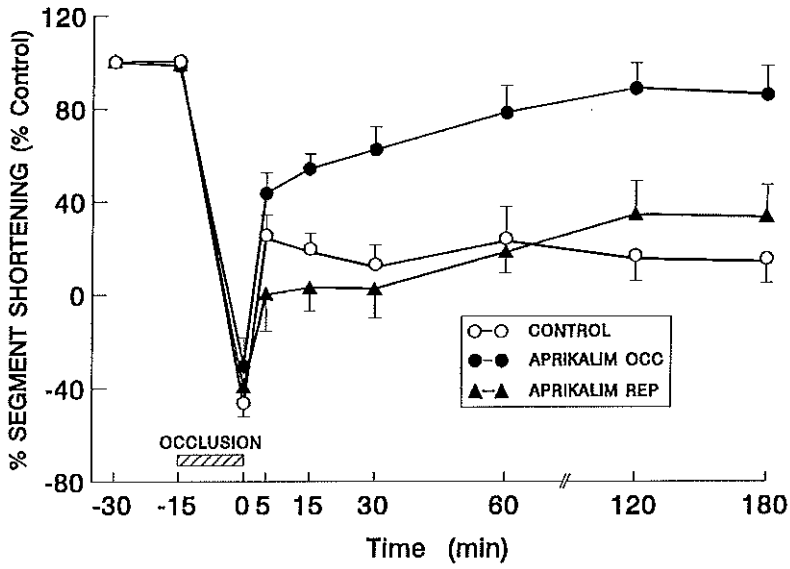

Fig 5. The effect of the K^+_{ATP} channel activator aprikalim on stunned myocardium. Data are from a saline-treated group (control) and two aprikalim-treated groups. Aprikalim was infused at a dose of 10 μg/kg plus 0.1 μg/kg/min beginning either 15 minutes before occlusion (aprikalim occ) or 2 minutes before reperfusion (aprikalim rep). All values are ± SEM. *p<0.05 vs control group. With permission from Circulation (108).

glibenclamide had no effect on the hypotension by the K^+_{ATP} channel openers but abolished the protective effect on function.[108] Increased collateral blood flow or increased perfusion during reperfusion can also not explain the protection by K^+_{ATP} channel openers,[105,108] suggesting that these agents reduce ischemic injury at the level of the cardiomyocyte.

The timing of administration of a K^+_{ATP} channel opener appears to be critical for its beneficial effect on myocardial stunning. In all of the above cited studies administration of the compounds was started at least 10 minutes prior to the onset of ischemia and continued throughout the ischemia and, at least part of, the reperfusion period. Protection was absent when aprikalim was administered intravenously to dogs immediately prior to the 180-minute period of reperfusion that followed the 15-minute coronary artery occlusion (Fig. 5).[108]

In conclusion, there is ample evidence that locally or systemically administered K^+_{ATP} channel openers improve the recovery of post-ischemic contractile recovery of regionally stunned canine myocardium, but only when administered prior to the onset of ischemia. In contrast, protection appears absent when the drugs are administered at the onset of reperfusion. The mechanism by which K^+_{ATP} channel openers attenuate myocardial stunning is not entirely clear. Shortening of the action potential appears currently the most likely explanation for the protective action as the protective effects occurs independent of an increase in collateral blood flow or a reduction in afterload.

Sodium-hydrogen exchange inhibitors

Excessive activation of the plasma membrane sodium-hydrogen (Na^+-H^+) exchanger during ischemia and early reperfusion causes intracellular sodium overload.[109,110] Increased levels of sodium will, because of their linkage with the sodium-calcium (Na^+-Ca^{2+}) exchange, raise intracellular calcium levels, and thereby contribute to the development of calcium overload.[109] Furthermore, activation of the Na^+-H^+ exchanger decreases intracellular acidosis; the latter attenuates the harmful effects of calcium overload by an effect on the Ca^{2+} regulated ATPase. Because an increased Na^+-H^+ exchange activity plays an important role in events leading to cardiac dysfunction during myocardial ischemia and reperfusion it is conceivable that inhibition of the Na^+-H^+ exchange ameliorates cardiac dysfunction during stunning.[111]

Hata et al,[112] applying the time varying elastance concept and the oxygen consumption-pressure volume area relation in the excised cross-circulated canine-heart model, found that rapid correction of acidosis after global myocardial ischemia caused a transient overshoot of contractility followed by a post-acidotic stunning, which was not caused by a decreased contractile efficiency. Restoration of contractile function by calcium resulted in a Vo_2 intercept of the linear Vo_2-pressure volume area relation that exceeded control levels, while total oxygen cost was higher in post-acidotic than normal hearts. Inhibition of the Na^+-H^+ exchange system by selective inhibition with dimithylamiloride almost completely prevented the contractile and energetic abnormalities of the post-acidotic stunned myocardium in this model, implying that

rapid recovery of pH resulted in stunning through activation of the Na^+-H^+ exchange system. The important conclusion of this study must be that rapid recovery of pH after ischemia and the subsequent Ca^{2+} overload may underlie myocardial stunning, in a similar manner as post-acidotic stunned myocardium.

Future studies in *in vivo* models of pure regional stunning must examine whether inhibition of the Na^+-H^+ exchange system indeed contributes to attenuation of regional stunning and whether administration must occur prior to the onset of ischemia or reperfusion or can still have salutary effects when administrated after stunning has been established.

Ubiquinone and HMG-CoA reductase inhibitors

Ubiquinone (Coenzyme Q_{10}, CoQ_{10}) is under normal conditions present in most cells in which it functions as an essential cofactor, being an electron-carrier in oxydative phosphorylation. In high doses CoQ_{10} promotes stabilization of cell membranes and has been shown to possess antioxydative effects by acting as a free radical scavenger.[113] In order to investigate whether the antioxydative effect had a beneficial effect on myocardial stunning, Atar et al[114] fed CoQ_{10} to pigs for 20 days before the left anterior descending coronary artery was occluded for 8 minutes and reperfused for 2 hours, while the animals were under anesthesia. Compared to a control group, the stunning time (defined as the time interval in which systolic wall thickening returned to baseline) was reduced in the CoQ_{10} treated animals from 33 minutes to 14 minutes. The protective mechanism remained unknown but the authors speculated that the favorable action was caused by protection from free radical mediated reperfusion injury.

HMG-CoA (3-hydroxy-3-methylglutaryl coenzyme A) reductase is a key enzyme in the biosynthesis of cholesterol and HMG-CoA reductase inhibitors are therefore useful in the treatment of hypercholesterolemia. Pravastatin and simvastatin are competitive inhibitors of HMG-CoA reductase and prevent formation of mevalonic acid,[115] which is a precursor in the biosynthesis of both cholesterol and ubiquinone. A decreased synthesis of ubiquinone impairs the energy generating system, and may lead to further deterioration of cardiac function under pathophysiological conditions.[116] This could be especially true for simvastatin which inhibits synthesis of sterol equally in hepatic and non-hepatic cells, whereas pravastatin enters only hepatocytes. Ichihara et al[117] fed pravastatin and simvastatin (2 mg/kg po) for 3 weeks to dogs which, while under anesthesia, were subjected to a 15-minute coronary artery occlusion of the left anterior descending. After 2 hours of reperfusion systolic segment shortening of the post-ischemic myocardium was absent in the simvastatin group while it had recovered to 50% of baseline in the control and pravastatin groups. Levels of ATP, but not of any other metabolite, were about 20% lower in the simvastatin group compared to the control and pravastatin groups, suggesting that ischemia may have been more severe in the simvastatin group. However, without measurement of the effects of treatment modalities on collateral blood flow the significance of these findings remains unclear.

Propionyl-L-carnitine

Post-ischemic myocardium is characterized by glycogen depletion and an impairment of free fatty acid oxidation due to depletion of carnitine and citric acid cycle intermediates and fatty acylCoA-induced inhibition of adenine nucleotide translocase. In addition, glucose oxidation may be decreased due to fatty acylCoA induced inhibition of pyruvate dehydrogenase. In view of the importance of preservation of glucose oxidation for Ca^{++} homeostasis during early reperfusion, the carnitine deficiency and fatty acyl-CoA accumulation have been suggested to be causally related to the contractile dysfunction during reperfusion.[118] Administration of carnitine or L-propionyl carnitine might improve the capacity of the myocardium to use β-oxidation of fatty acids for energy production and for extraction of the harmful excess of fatty acid intermediates,[119,120] thereby enhancing glucose oxidation.[121]

Two periods of 10-minute total coronary artery occlusion each followed by 30 minutes of reperfusion resulted in 20% loss of myocardial tissue levels of carnitine in open-chest swine.[120] Three days of oral pretreatment with L-propionylcarnitine (100 mg/kg per day) increased carnitine tissue levels by 30% compared to non-treated animals both at baseline and at the end of the second 30-minute reperfusion period. This increase in carnitine tissue levels was associated with a borderline statistically significant improvement of post-ischemic regional myocardial segment shortening. In view of the concomitant reduction in mean aortic blood pressure it would appear that L-propionylcarnitine, despite maintaining adequate postischemia tissue levels of carnitine, failed to exert a beneficial effect on myocardial stunning.

Factors exacerbating myocardial stunning

If anti-ischemic actions of pharmacological agents and timely treatment with oxygen-derived free radical scavengers attenuates stunning, it may be assumed that conditions or agents that enhance ischemia or free radical production aggravate myocardial stunning. In man some of these conditions may be the consequence of life style or medication taken to treat clinical conditions. For instance, the HMG CoA reductase inhibitor simvastatin has been shown to exacerbate stunning in anesthetized dogs.[117]

Inhibition of endothelial derived relaxing factor

Hasebe et al investigated the effect of inhibition of nitric oxide (NO) on the severity of stunning in awake dogs. For this purpose the NO synthesis inhibitor N^6-nitro-L-arginine (L-NA, 30 μg/kg/min) was administered into the coronary artery starting 11 minutes before the onset of the 10-minute coronary artery occlusion while a maintenance dose (6 μg/kg/min) was started at the onset of reperfusion.[123] Compared to control (saline infusions) wall thickening was significantly less at 30 minutes reperfusion in both the subendocardium and subepicardium. The results could not be ascribed to differences in hemodynamics or myocardial perfusion in the presence of absence of L-NA during reperfusion. There was a slight increase in oxygen demand

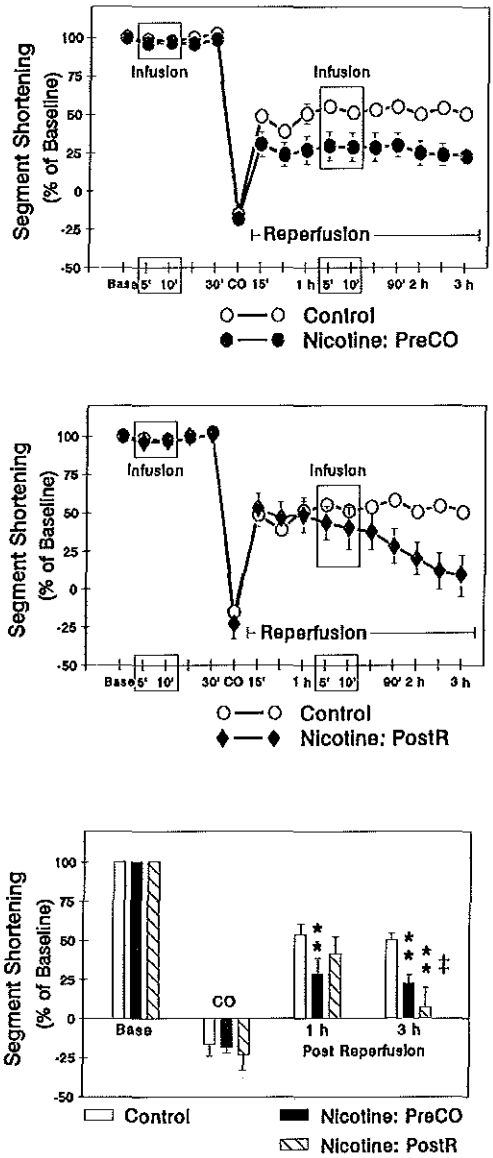

Fig 6. The effect of nicotine on segment shortening in the ischemic/reperfused left anterior descending coronary artery (LAD) bed, expressed as a percent of baseline preocclusions values. The top panel shows the data obtained during nicotine/saline infusions, before and during LAD occlusions, and after reperfusion for the control and nicotine-preocclusion groups. The middle panel shows the data obtained during nicotine/saline infusions, before and during LAD occlusion, and after reperfusion for the control and nicotine-postreperfusion groups. The bottom panel shows the statistical comparison of the three groups during coronary occlusions and at 1 and 3 hours after reperfusion. Base indicates baseline; CO, during coronary occlusion; PreCO, before occlusion; and PostR, after reperfusion. Data enclosed in the boxes were obtained during the nicotine/saline infusions. ‡p<.01 vs 1 hour after reperfusion; **p<.01 vs control. With permission from Circulation (124).

in the L-NA-treated animals during coronary artery occlusion in the face of similar coronary blood flows. It is highly unlikely that considered the amount of loss of function the enhanced stunning could be caused by more intense ischemia.[12] Consequently NO synthesis inhibition exacerbated stunning which could not be related to more severe ischemia during occlusion. The mechanism by which NO synthesis inhibition aggravated stunning remained unknown but the authors postulated that other actions of NO (such as on oxygen-derived free radical formation, platelet aggravation or neutrophil activation) could be involved.

Smoking

Przyklenk[124] investigated whether nicotine, infused in a dose mimicking the levels reached by humans after smoking a single cigarette, had a detrimental effect on contractile function of normal and stunned myocardium. Infusion of nicotine (80 μg/kg over a 10-minute period) at 30 minutes before a 15-minute coronary artery occlusion in anesthetized dogs did not affect regional segment shortening before and during ischemia (Fig. 6). In these animals, recovery of segment shortening of post-ischemic myocardium was 29% and 22% at 1 and 3 hours of reperfusion, respectively, while in the control animals segment shortening of the stunned myocardium recovered to 54% and 50%, respectively (both $p<0.05$ vs the nicotine-treated animals). When given at 1 hour of reperfusion, nicotine caused an almost complete loss of the partially recovered function. The deterioration in function was not the consequence of unfavorable changes in systemic hemodynamics or coronary blood flow, but additional experiments suggested that it was most likely mediated by oxygen derived free radicals acting on reversibly injured myocardium as infusion of the free radical scavenging agent N-2-mercaptopropionylglycine (50 mg/kg/hr) starting at 45 minutes of reperfusion prevented the deterioration in segment shortening by the nicotine infusion at 1 hour of reperfusion. From her data Przyklenk suggests that future studies should also investigate whether these observations can be confirmed during either active or passive smoking.

Magnesium deficiency

Herzog et al[71] reported a delayed recovery of function in magnesium deficient pigs subjected to a brief coronary artery occlusion compared to a group of control animals. The authors ascribed their finding to impaired free radical defense systems as levels of glutathione were 43% lower in the hypomagnesemia than in the control group.

Conclusion

Recovery of reversible post-ischemic myocardium can be enhanced by a variety of pharmacological interventions, that either recruit function after myocardial stunning has been established or which prevent the development of myocardial stunning by either an anti-ischemic action or inhibition of calcium-overload or formation of oxygen derived free radicals, in

particular during the early reperfusion phase. It is noteworthy, however, that only in the studies using beta-adrenergic receptor agonists and calcium sensitizing agents regional function could be restored to normal. All other preventive interventions only partially enhanced recovery. This may have been a consequence of the doses used, but it is likely that these interventions targeted only one aspect of stunning (severity of ischemia, calcium-overload or oxygen-derived free radicals).

We have restricted ourselves to describing the results of studies with a limited number of pharmacological agents, most of which are currently used in clinical practice. The list can easily be expanded with agents such as N-acetylcysteine,[126] vitamin E[127,128] or its hydrophilic analogue MDL 74,405,[129] which have all been shown to improve post-ischemic function similar to other antioxydants such as superoxyde dismutase + catalase,[130-132] mercaptopropionyl glycine[133] and desferrioxamine.[134]

At the present time a wide variety of pharmacological interventions are thus available for prevention attenuation or treatment of myocardial stunning, but the choice and hence the efficacy of specific treatment depends on the timing of administration.

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Chapter 3

End-Systolic Pressure Length Relations of Stunned Right and Left Ventricles After Inotropic Stimulation

Running title: Right and left ventricular stunning

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End-Systolic Pressure Length Relations of Stunned Right and Left Ventricles After Inotropic Stimulation

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Objectives and Results. Regional end-systolic pressure-segment length relationships (ESPSLR) were used to compare the degree of right and left ventricular stunning, induced by a 10 minute occlusion of the left anterior descending coronary artery (LADCA) and the response to subsequent atrial pacing (50 beats per minuteabove intrinsic heart rate) without and with dobutamine (2 µg/kg per min in 9 anesthetized open-chest pigs. From the ESPSLR, the slope E_{es} (at 100 mmHg for the left and 25 mmHg for the right ventricle) and the total area of the pressure-length relationship, the PLA, were determined. From the latter, the distribution into external work (EW) and potential energy (PE) as well as the efficiency of energy transfer (EET = EW/PLA) were calculated. In both the stunned left and right ventricular myocardium E_{es} and EW were reduced, according to the same linear regression equations ($\Delta E_{es} = 0.7\ E_{es,baseline} - 11.4$, $r^2 = 0.86$ and $\Delta EW = 0.4\ EW_{baseline} + 2.3$, $r^2 = 0.67$). EET of the stunned left and right ventricular segments decreased as PLA remained unchanged, due to an increase in PE. EET decreased from 0.84 ± 0.02 to 0.71 ± 0.03 ($P<.05$) in the stunned right ventricular segment and from 0.71 ± 0.02 to 0.44 ± 0.03 ($P<.05$) in the stunned left ventricular segment. Atrial pacing did not affect EET with respect to stunning levels, while the additional infusion of dobutamine restored E_{es}, EW and PE and consequently EET to baseline values.

Conclusions. In conclusion, the right ventricle is susceptible to stunning. During atrial pacing the EET was lower than expected from the E_{es}, which could, in agreement with the time varying elastance concept, be explained by an increase in afterload (a consequence of the decrease in SV). Dobutamine not only increased E_{es}, EW and EET, but also restored the relationship between E_{es} and EET in both ventricular stunned segments. (***Am J Physiol.*** **1993;265 (Heart Circ Physiol 34): H2099-H2109.)**

Keywords • myocardial stunning • regional contractility (E_{es}) • external work • efficiency of energy transfer • right ventricle • left ventricle • dobutamine • atrial pacing • pig

The consequences of brief periods of ischemia for parameters of regional myocardial function, such as systolic segment length shortening and systolic wall thickening, have been studied far less extensively for the right than for the left ventricle.[2,3,5,6,8,9,14] Since both systolic segment length shortening and systolic wall thickening are load dependent, their application in a comparative study involving both the right and left ventricles may be subjected to serious discussion.[1,9] In analogy to the time varying elastance concept,[33] Aversano er al[1] have studied the end-systolic left ventricular pressure-segment length relationship and shown that the slope of the line, connecting the end-systolic pressure-segment length points (E_{es}) produced by varying the loading conditions of the heart, provides a load independent index of regional myocardial contractility. Furthermore, the area enclosed by the ventricular pressure-segment length loop represents an index for the external work performed by the examined myocardial segment.[5,26,35] Recently, we[20] have extended the analogy of the former groups[1,5,25,35] and used the entire area enclosed by the end-diastolic and end-systolic pressure segment length relationships, the pressure length area (PLA), as an index for total mechanical work, which is the sum of the external work and potential energy.[33] With this approach, it was shown that during left ventricular stunning contractility (E_{es}) and external work decreased, but also that total mechanical work remained unchanged. The latter was caused by an increase in potential energy, which negated the decrease in external work and implied a decrease in the efficiency of energy transfer.[20] In the same study, we also showed that stimulation of stunned left ventricular myocardium with dobutamine increased E_{es} and external work, while PLA remained unchanged, resulting in a full recovery of the efficiency of energy transfer, thereby preventing an excessive increase in oxygen consumption.[20]

It has been shown that the time varying elastance concept both globally and regionally[6,15,22,30] is applicable to both ventricles and we therefore extended our analysis to the right ventricle in order to compare the degree of right and left ventricular stunning and the recruitability of E_{es}, external work and efficiency of energy transfer during chronotropic and inotropic stimulation. Right and left ventricular stunning was induced by a 10 min occlusion of the left anterior descending coronary artery, which is short enough to prevent the development of subendocardial necrosis of the left ventricle.[28]

Methods

General

All experiments were performed in accordance with the Guiding Principles in the Care and Use of Animals as approved by the Council of the American Physiological Society and under the regulations of the Committee on Animal Experimentation of the Erasmus University Rotterdam.

After an overnight fast, cross-bred Landrace x Yorkshire pigs (HVC, Hedel, the

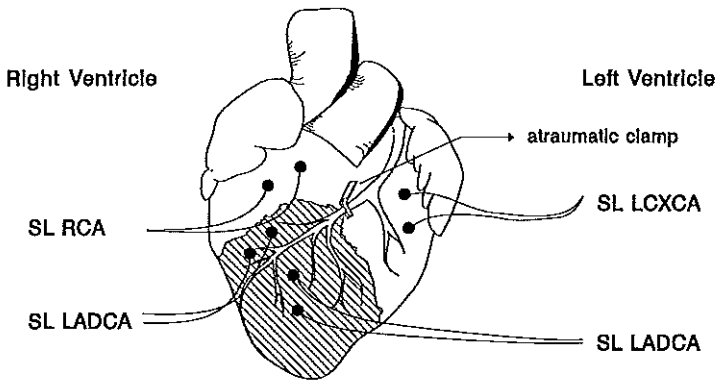

Fig 1. Illustration indicating the location of the ultrasound crystals in the left and right ventricular myocardium perfused by the left anterior descending coronary artery (LADCA, shaded area) and in the control regions, perfused by the left circumflex coronary artery (LCXCA) and right coronary artery (RCA). SL = segment length

Netherlands) of either sex (23-30 kg) were sedated with 20 mg/kg ketamine i.m. (AUV, Cuijk, The Netherlands), anesthetized with 5 mg/kg metomidate i.v. (Janssen Pharmaceutica, Beerse, Belgium), intubated and connected to a ventilator for intermittent positive pressure ventilation with a mixture of O_2 and N_2 (1:2, v/v). Respiratory rate and tidal volume were set and adjusted, when necessary, to keep arterial blood gases within the normal range.[31] Catheters (7F) were placed in the superior caval vein for (i) continuous infusion of 10-15 mg/kg/h sodium pentobarbitone (Sanofi, Paris, France), (ii) the administration of 4 mg of the muscle relaxant pancuronium bromide (Organon Teknika B.V., Boxtel, The Netherlands) prior to thoracotomy and for the infusion of dobutamine. Catheters were also positioned in the descending aorta for withdrawal of blood samples and measurement of central aortic blood pressure. A 7F Sensodyn micromanometer-tipped catheter (B. Braun Medical B.V., Uden, The Netherlands), inserted via the left carotid artery, was used to measure left ventricular pressure, while another microtipped catheter was advanced into the right ventricle via the right subclavian vein to monitor right ventricular pressure. After a midline sternotomy, the left mammary vessels were ligated and the second left rib was removed for ease of further instrumentation, and the heart suspended in a pericardial cradle. The adventitia surrounding the aorta was dissected free and an electromagnetic flow probe (Skalar, Delft, The Netherlands) placed around the vessel for measurement of ascending aortic blood flow. On the proximal third of the left anterior descending coronary artery (LADCA), a small segment was dissected free of its adventitia for subsequent positioning of an atraumatic arterial clamp. Pacing leads were attached to the left atrial appendage and connected to a pacing stimulator. Rectal temperature was monitored throughout the experiment and maintained between 37° C and 38° C using external heating pads.

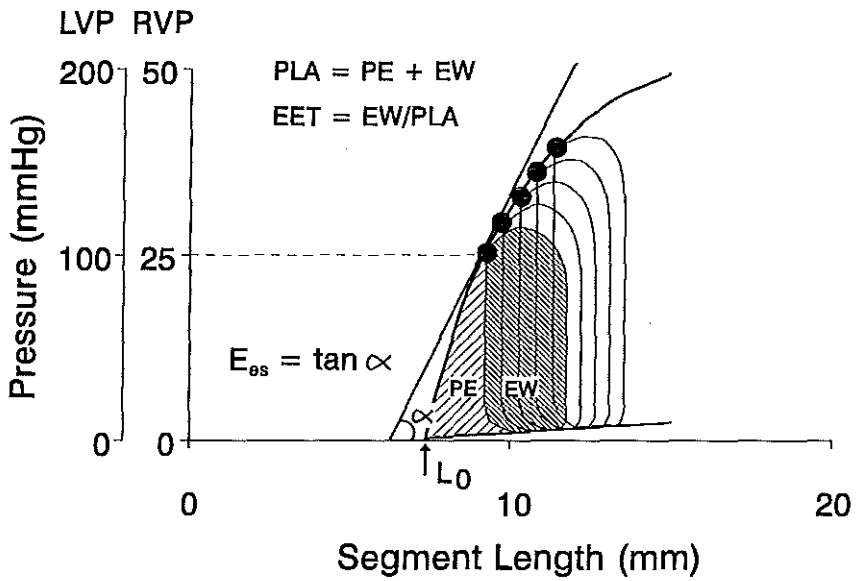

Fig 2. Scheme illustrating the calculation of indices for total mechanical work (PLA), potential energy (PE), external work (EW), efficiency of energy transfer (EET) and E_{es} from the left and right ventricular pressure segment length relationships. In this figure E_{es} for the left and right ventricle were determined at 100 mmHg and 25 mmHg, respectively. LVP = left ventricular pressure, RVP = right ventricular pressure, Lo = segment length at zero pressure.

Regional myocardial segment length shortening was measured by sonomicrometry (Triton Technology Inc., San Diego, CA, USA) using four pairs of ultrasonic crystals (Sonotek Corporation, Del Mar, CA, USA, Fig 1, diameter 2 mm). Two pairs were placed in the subendocardial regions of the left ventricle and the midmyocardium of the right ventricle supplied by the LADCA. The LADCA perfused area was identified by a short (< 5 sec) occlusion, causing a sharp demarcation between the oxygenated myocardium and the bulging blue myocardium deprived of oxygenated hemoglobin. The other two pairs were placed in regions of each ventricle remote from the distribution area of the LADCA (Fig 1).

Experimental protocol

After completing the surgical procedures, a 30-45 min stabilization period was allowed before baseline recordings were made of pressures in the descending aorta (AP), left and right ventricles (LVP and RVP, respectively) and their respective first derivatives (LVdP/dt and RVdP/dt), ascending aortic blood flow (cardiac output, CO) and segment length changes in the four regions. With the respirator switched off, the aorta was then gradually clamped to create a series of 10-20 beats with variable afterloads (duration 5-10 s) for the construction of the left ventricular end-systolic pressure-segment length relationships. The right ventricular end-systolic pressure-segment length relationships (ESPSLR's) were subsequently obtained by gradually

clamping the pulmonary artery over a similar period of 5-10 s, also with the respirator switched off. The ventricular pressures and segment length signals were digitized (sample rate 250 Hz) with an 8 bit AD-converter (Tiepie Engineering, Leeuwarden, The Netherlands) and stored on disk for further off-line analysis.

After all baseline data were recorded, the LADCA was occluded for 10 min. After recording of the systemic hemodynamic parameters and segment length changes, the coronary artery was reperfused for 30 min. At that time, the heart rate was raised by 50 beats per minuteand 5 min later an intravenous infusion of dobutamine (2 μg/kg per min was started for 10 min, while atrial pacing was continued. In earlier studies we have shown that 2 μg/kg per min dobutamine increases the pig's own heart rate by less than 50 beats per minute.[11] Collection of all data was repeated after 30 min of reperfusion, after 5 min of atrial pacing and at the conclusion of the dobutamine infusion.

Data analysis and statistics

Left and right ventricular systolic segment length shortening were calculated as the difference between lengths at end-diastole and end-systole and expressed as a percentage of end-diastolic length. Baseline end-diastolic segment length measurements were normalized to 10 mm. End-diastole was determined at the peak positive dP/dt while end-systole was determined at peak-negative dP/dt, which corresponds closely to the zero crossing point of the flowmeter tracing and thus the closure of the aortic and pulmonary valve of the left and right ventricle, respectively.[13]

The end systolic pressure-segment length relationships (ESPSLR's) of both ventricles were determined by fitting the end systolic pressure-segment length data points (Fig 2), which were calculated by an iterative algorithm, to a second order polynoma, similar to the method described extensively by Mirsky [24] and Van der Velden er al.[34] Briefly, since the length at zero pressure (L_0) is unknown, L_o was taken equal to zero and the end systolic pressure-segment length point for each individual beat was calculated on basis of the maximal elastance for that beat. Subsequently, linear regression was applied to the calculated end-systolic pressure-segment length point and a first estimate of L_0 was obtained. Using this new L_0-estimate and the maximal elastance for each beat, end-systolic pressure segment-length points were again calculated, applying linear regression, which resulted in a second estimate of L_0. This sequence of steps was continued until the change in L_0 was less than 0.25 mm. This usually took 3-4 iterations. With this procedure end-systole not necessarily coincides with end-systole based on the definition of peak positive and peak negative dP/dt. Since a second order polynoma described the ESPSLR more accurately than the linear regression, the slope of the ESPSLR became dependent on segment length. We, therefore, characterized the ESPSLR of the left ventricle by the slope (E_{es}) at 100 mmHg and the length at the zero pressure intercept (L_0; see also reference 20). For the right ventricle, we used the slope at 25 mmHg (Fig 2). In addition we determined the average of the area of 10 consecutive pressure-segment length loops as the index of the external work (EW,

TABLE 1. Hemodynamics at baseline, during 10 min occlusion and 30 min reperfusion of the left anterior descending coronary artery (LADCA), and subsequent chronotropic and inotropic stimulation in 9 anesthetized swine

	Baseline	Occlusion	Reperfusion	Atrial pacing[a]	Atrial pacing + Dobutamine[b]
MAP, mm Hg	95±3	84±2*	85±3*	87±3*	87±3*
HR, bpm	109±5	107±6°	99±6*	148±6*'	149±6*'
CO, L/min	2.48±0.16	2.03±0.16*	2.15±0.20*	1.98±0.22*	2.41±0.21'°
LVSP, mm Hg	112±3	103±3*	104±3*	100±4*	111±3'°
$LVdP/dt_{max}$, mm Hg/s	2320±220	1830±120*	1790±150*	1690±130*	3210±260*'°
LVEDP, mm Hg	11±1	14±1*	12±1	12±1	9±1
SVR, mm Hg/(L/min)	40±3	43±4	44±7	42±5	39±5
RVSP, mm Hg	36±2	31±1*	32±2	36±2	39±2'°
$RVdP/dt_{max}$, mm Hg/s	730±130	580±140*	590±140*	590±180*	1100±410*'°
RVEDP, mm Hg	7±1	8±1	8±1*	7±1	6±1'°

[a] Heart rate was increased by 50 beats per minuteover the heart rate measured after 30 min of reperfusion; [b] Dobutamine was infused at 2 µg/kg/min for 10 min. MAP = mean arterial blood pressure; HR = heart rate; CO = cardiac output; LVSP = left ventricular systolic pressure; $LVdP/dt_{max}$ = maximal rate of rise of left ventricular pressure; LVEDP = left ventricular end-diastolic pressure; RVSP = right ventricular systolic pressure; $RVdP/dt_{max}$ = maximal rate of rise of right ventricular pressure; RVEDP = right ventricular end-diastolic pressure. All data have been presented as mean±SEM.

* $P<.05$ vs Baseline

' $P<.05$ vs Reperfusion

° $P<.05$ vs Atrial pacing.

Fig 2). Potential energy (PE, Fig 2) was determined by integrating the second order polynoma from L_o to the end-systolic segment length (L_{es}) as described earlier.[20] The sum of potential energy and external work equals the total mechanical work performed by the myocardium pressure length area (PLA, Fig 2). The efficiency of energy transfer EET was defined as EW/PLA.[33]

All data have been presented as mean and standard error of the mean (SEM). Statistical analysis was performed for each variable by repeated measures of ANOVA. When significance was reached ($P<.05$), paired t-tests were applied with a Bonferroni correction for multiple measurements. The statistical difference between the regression equations were evaluated by analysis of covariance (ANCOVA), using a standard package (Statgraphics, Rockville, Maryland, USA).

RESULTS

Systemic hemodynamics

Occlusion of the LADCA caused a 11 ± 2% decrease ($P<.05$, Table 1) in mean arterial blood pressure, because of a fall in cardiac output (14 ± 4%, $P<.05$). Both $LVdP/dt_{max}$ and $RVdP/dt_{max}$ decreased by approximately 20% ($P<.05$). LVEDP increased by 3 ± 1 mmHg ($P<.05$) over its baseline value of 11 ± 1 mmHg, while RVEDP did not change. During reperfusion LVEDP decreased by 2 ± 1 mmHg, while none of the other parameters was significantly affected. Atrial pacing did not lead to significant changes apart from the increase in heart rate. During the

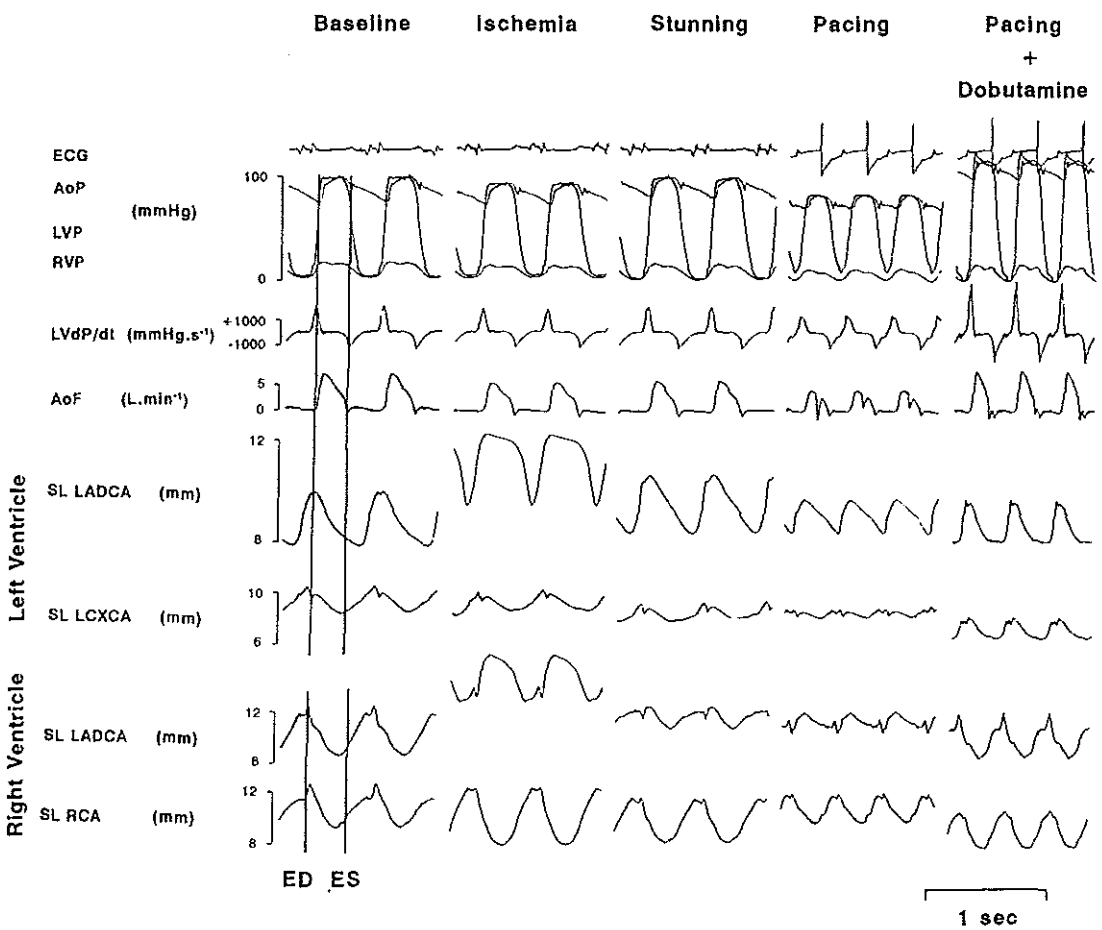

Fig 3. Original tracing showing hemodynamics and regional segment length changes for the control and stunned left and right ventricular myocardium. ECG = electrocardiogram; AoP, LVP and RVP are aortic, left ventricular and right ventricular pressure, respectively; LVdP/dt = first derivative of left ventricular pressure; AoF = aortic flow; SL = segment length; LADCA = left artery descending coronary artery; LCXCA = circumflex artery; RCA = right coronary artery; ES and ED are end-systole and end-diastole, respectively; End-diastole was determined at peak positive. LVdP/dt and RVdP/dt (not shown) for the left and right ventricle, respectively. End-systole was determined at peak negative peak dP/dt.

additional infusion of dobutamine, there were increases in both $LVdP/dt_{max}$ (by 108 ± 17%, $P<.05$) and $RVdP/dt_{max}$ (by 94 ± 20%, $P<.05$) and in left (to its baseline value) and right (to 10%over its baseline value) ventricular systolic pressures. Cardiac output also returned to baseline values (Table 1).

Regional segment length shortening

Occlusion of the LADCA resulted in bulging of the ischemic segment of the left ventricle as systolic segment length shortening (%SS) decreased to -4 ± 1%, and to an almost complete

TABLE 2. Systolic segment length shortening at baseline, during 10 min occlusion and 30 min reperfusion of the left anterior descending coronary artery (LADCA), and subsequent chronotropic and inotropic stimulation in 9 anesthetized swine

	Baseline	Occlusion	Reperfusion	Atrial pacing[a]	Atrial pacing + Dobutamine[b]
Left ventricle					
non-stunned area					
EDL, mm	10.0	10.5±0.1*	9.6±0.4	9.1±0.4*†	9.5±0.2*
ESL, mm	8.7±0.1	9.3±0.2*	8.4±0.4	8.3±0.4	8.4±0.3
SS, %	14±1	12±1	13±1	9±1*†	12±1°
stunned-area					
EDL (mm)	10.0	11.2±0.3*	10.4±0.2*	9.9±0.2†	9.8±0.1†
ESL (mm)	8.2±0.1	11.8±0.5*	9.5±0.2*	9.2±0.2*	7.8±0.4†°
SS (%)	18±1	-4±1*	10±1*	8±1*†	17±2†°
Right ventricle					
non-stunned area					
EDL (mm)	10.0	9.5±0.3	9.6±0.2	9.7±0.2	9.3±0.2*
ESL (mm)	8.3±0.1	7.9±0.4	8.3±0.2	8.4±0.2	7.9±0.2
SS (%)	17±1	17±3	14±1*	14±2*	15±2
stunned-area					
EDL (mm)	10.0	10.4±0.4	9.5±0.5	9.7±0.4	9.3±0.4
ESL (mm)	7.3±0.4	10.1±0.5*	8.1±0.3	8.3±0.4	7.0±0.4†°
SS (%)	27±4	3±2*	14±3*	14±3*	24±4†°

[a] Heart rate was increased by 50 beats per minuteover the heart rate measured after 30 min of reperfusion; [b] Dobutamine was infused at 2 µg/kg/min for 10 min. EDL = end-diastolic segment length (normalized to 10 mm at baseline); ESL = end-systolic segment length; SS = segment length shortening; All data have been presented as mean±SEM.

* $P<.05$ vs Baseline

† $P<.05$ vs Reperfusion

° $P<.05$ vs Atrial pacing.

loss of function of that of the right ventricle as %SS decreased to 3 ± 2% (Fig 3 and Table 2). Segment length shortening of the post-ischemic myocardium of both ventricles recovered only partially during reperfusion (to 61 ± 9% and 54 ± 7% of baseline for the left and right ventricles, respectively). Increasing heart rate by atrial pacing decreased segment length shortening by approximately 30% versus baseline in both stunned and non-stunned left ventricular myocardium, while segment length shortening of the stunned and of the non-stunned right ventricular segments were not affected. With the addition of dobutamine, segment length shortening of the stunned segments and the non-stunned segment of the left myocardium returned to baseline, while that of the non-stunned myocardium of the right ventricle remained unchanged (Fig 3 and Table 2).

Regional contractility

An example of the changes in E_{es} of the left and right ventricular segments during the course of the experimental protocol is shown in Fig 4. As shown, regional end-systolic pressure-segment length relationships decreased during stunning for both the right and left ventricular intervention areas, while they remained approximately constant for the control regions. E_{es} of the non-stunned myocardium of both ventricles was unchanged after 30 minutes of reperfusion following the 10 minute occlusion period (Table 3). Atrial pacing had only a minimal effect on E_{es}, but the

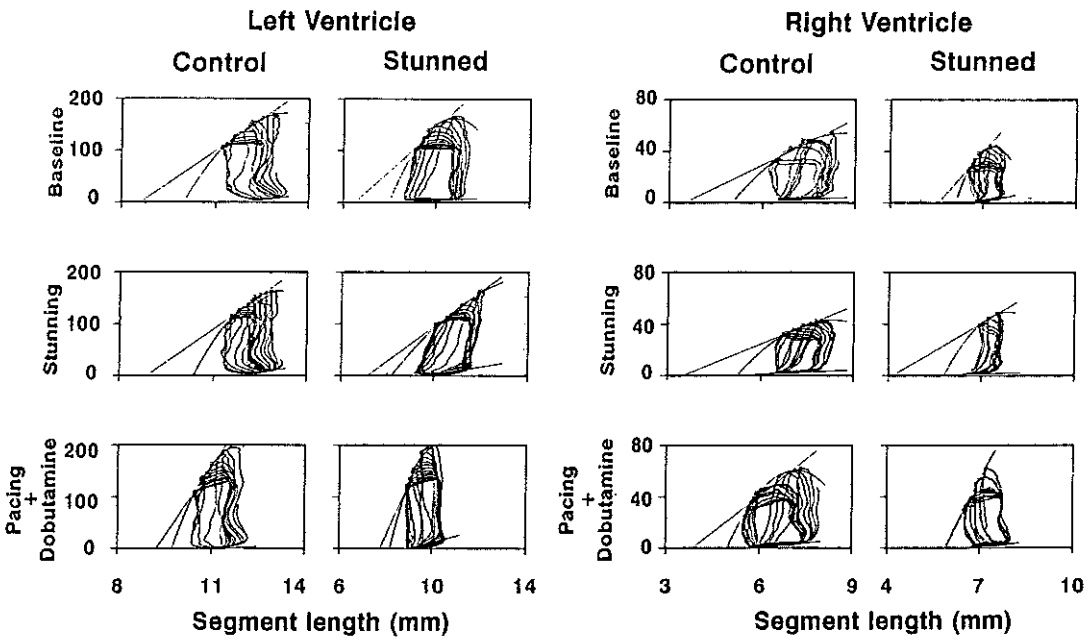

Fig 4. Examples showing end-systolic pressure-segment length relationships as well as pressure-segment loops obtained from afterload changes for both right and left ventricular segments.

TABLE 3. Regional Mechanics and Energetics at Baseline, After 10-min Occlusion and 30-min of Reperfusion of the LADCA and Subsequent Chronotropic and Inotropic Stimulation of Nine Anesthetized Swine

	Baseline	Reperfusion	Atrial pacing	Atrial pacing + dobutamine
		Left ventricle		
Non-stunned area				
E_{es}, mm Hg/mm	88±6	76±8	79±9	147±19*°
L_o, mm	7±0.6	7.3±0.8	7.1±0.8	8.4±0.7
EET	0.71±0.02	0.60±0.02	0.54±0.07*	0.77±0.05*°
Stunned area				
E_{es}, mm Hg/mm	77±10	30±5*	53±12	131±35*°
L_o, mm	7.8±0.5	8.1±0.5	7.7±0.5	7.9±0.6
EET	0.70±0.02	0.44±0.03*	0.40±0.04*	0.68±0.05*°
		Right ventricle		
Non-stunned area				
E_{es}, mm Hg/mm	30±7	43±10	43±10	64±13*°
L_o, mm	6.4±0.6	6.7±0.6	7.4±0.4	7.2±0.4
EET	0.82±0.03	0.76±0.02	0.67±0.03*	0.81±0.03°
Stunned area				
E_{es}, mm Hg/mm	33±6	23±4*	26±6	64±9*°
L_o, mm	4.9±0.6	5.6±0.9	5.8±0.9	5.2±0.9
EET	0.84±0.03	0.70±0.04*	0.61±0.07*	0.81±0.04*°

Values are means ± SE. Heart rate was increased by 50 beats per minute over the heart rate measured after 30 min of reperfusion. Dobutamine was infused at 2 μg/kg per min or 10 min. E_{es}, slope of end-systolic pressure segment-length relationship; L_o, optimal length; EET, efficiency of energy transfer.

* $P<.05$ vs Baseline

* $P<.05$ vs Reperfusion

° $P<.05$ vs Atrial pacing

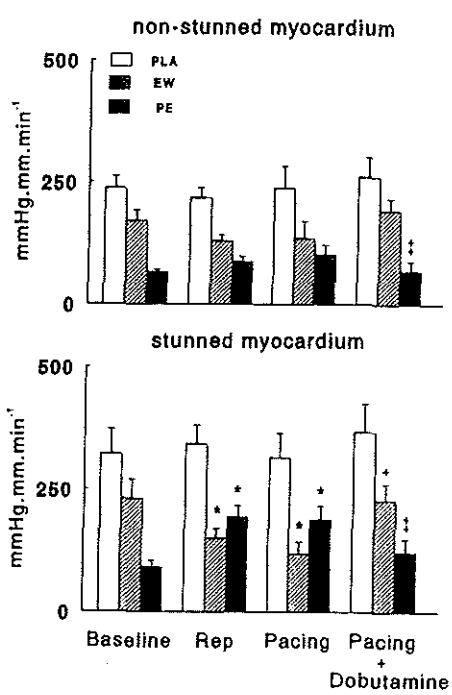

Fig 5. Total mechanical work (PLA), external work (EW) and potential energy (PE) before (Baseline) and after 30 min reperfusion (Rep) following a 10 min occlusion of the left anterior descending coronary artery and subsequent atrial pacing (Pacing) and the additional infusion of dobutamine (Pacing + Dobutamine) of non-stunned and stunned right ventricular myocardium. Data have been presented as mean±SEM (n = 9). * $P<.05$ vs Baseline; ⁺ $P<.05$ vs Reperfusion; ° $P<.05$ vs Atrial Pacing.

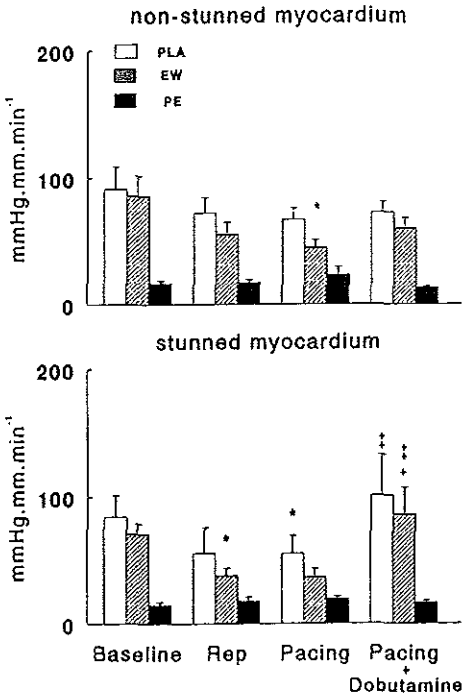

Fig 6. Total mechanical work (PLA), external work (EW) and potential energy (PE) before (Baseline) and after 30 min reperfusion (Rep) following a 10 min occlusion of the left anterior descending coronary artery and subsequent atrial pacing (Pacing) and the additional infusion of dobutamine (Pacing + Dobutamine) of non-stunned and stunned left ventricular myocardium. Data have been presented as mean±SEM (n = 9). * $P<.05$ vs Baseline; ⁺ $P<.05$ vs Reperfusion; ° $P<.05$ vs Atrial Pacing.

additional infusion of dobutamine caused a doubling of the E_{es} of both the left (184 ± 29%, $P<.05$) and right ventricle (214 ± 31%, $P<.05$) compared to baseline. After 30 min of reperfusion, E_{es} of the stunned myocardium had decreased in both the left (to 42 ± 6% of baseline, $P<.05$) and right ventricle (to 80 ± 8% of baseline, $P<.05$; Table 3). E_{es} of the two stunned myocardial segments was also not affected by atrial pacing, but increased both by a similar amount as in the non-stunned myocardium during the additional infusion of dobutamine. The intercepts at zero left ventricular pressure (L_o) for the non-stunned and stunned myocardium were unchanged for both ventricles after 30 min of reperfusion and were also not modified by atrial pacing and the additional infusion of dobutamine (Table 3).

Regional pressure-segment length area's

The total mechanical work (PLA) of the left ventricular myocardium inside and outside the

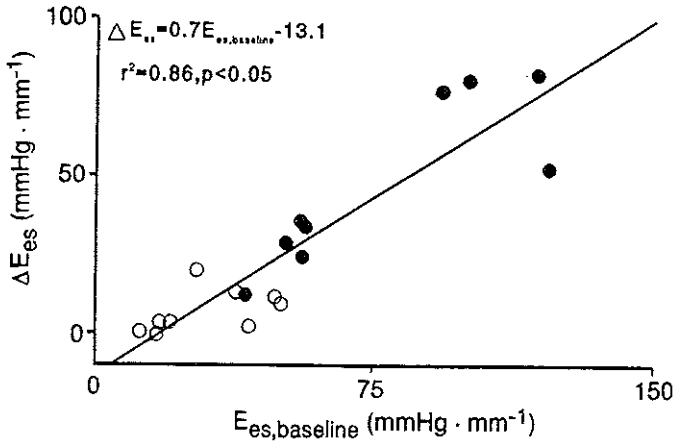

Fig 7. Relationship between the decrease in E_{es} (ΔE_{es}) after 30 min reperfusion following a 10 min occlusion of the left anterior descending coronary artery and E_{es} at baseline ($E_{es,baseline}$) for stunned left (●)and right (o) ventricular myocardium.

distribution area of the LADCA was unchanged at the end of the reperfusion period and not affected by atrial pacing and the additional dobutamine infusion (Fig 5). There were, however some striking differences in the pattern of the external work (EW) and potential energy (PE), the two components which determine PLA. While there were no marked changes in these two components in the non-stunned myocardium during the different experimental conditions, there were considerable changes in the stunned left ventricular myocardium. After 30 min of reperfusion, PE had doubled, while EW had decreased to 60% of baseline. Atrial pacing did not affect EW and PE, but both indices returned to baseline values during the additional infusion of dobutamine (Fig 5). From these data it follows that the efficiency of energy transfer (EET) of the non-stunned myocardium during baseline (0.71 ± 0.02, Table 3) did not change during the experiment, except after atrial pacing ($P<.05$ vs baseline). It decreased to 0.44 ± 0.03 ($P<.05$) after 30 min of reperfusion in the stunned myocardium. Atrial pacing did not further lower EET in the stunned region (0.40 ± 0.04), but the additional infusion of dobutamine restored EET to

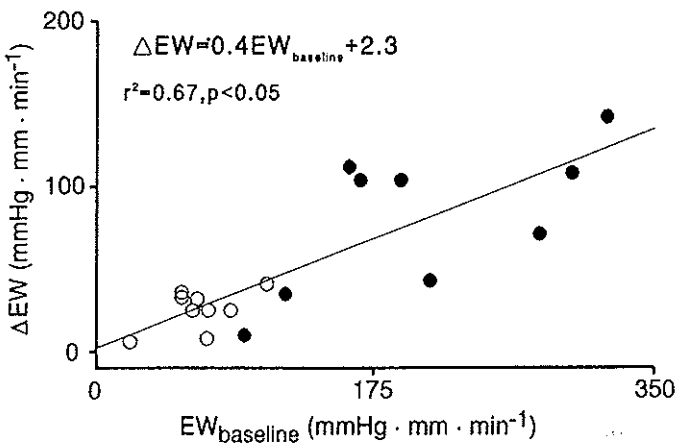

Fig 8. Relationship between the decrease in external work after 30 min reperfusion following a 10 min occlusion of the left anterior descending coronary artery (ΔEW) and the external work at baseline ($EW_{baseline}$) for stunned left (●) and right (o) ventricular myocardium.

baseline values (0.68 ± 0.05).

PLA of the non-stunned and stunned right ventricular myocardium also did not change during the experiment, except after atrial pacing ($P<.05$ vs baseline) (Fig 6). Since in the stunned region EW decreased and PLA did not change after reperfusion, EET decreased from 0.84 ± 0.03 to 0.70 ± 0.04 ($P<.05$). In the non-stunned myocardium, EET remained unchanged (0.82 ± 0.03 at baseline and 0.76 ± 0.02 after reperfusion (NS)). Atrial pacing had no further affect on the EET of the stunned region, but lowered the EET in the control region from 0.76 ± 0.02 to 0.67 ± 0.03 ($P<.05$ vs baseline) due to a decrease in EW. Dobutamine restored EW, PE (Fig 6) and EET (Table 3) to baseline values in the stunned as well as in the non-stunned segments.

Relationships between E_{es}, EW and EET

In Fig 7 we have related the decreases in E_{es} of the individual animals during stunning to their respective baseline values and observed that for both ventricles the decreases were linearly related ($r^2 = 0.86$) to the values determined at baseline. We could also demonstrate such a linear relationship for EW ($r^2 = 0.67$, Fig 8).

In Fig 9 the relation between E_{es} and EET (EW/PLA) has been depicted for the myocardial segments inside the distribution area of the LADCA for both ventricles. It proved that the data points obtained under baseline conditions and after stunning could be described by hyperbolic relationships: $EET = 1/(1 + 31/E_{es})$ ($r^2 = 0.61$) and $EET = 1/(1 + 6.6/E_{es})$ ($r^2 = 0.44$) for the left and right ventricle, respectively. The figure also shows that EET was considerably lower than expected from these relationships during atrial pacing, but the relationship was restored during the additional infusion of dobutamine.

DISCUSSION

It has recently been appreciated that right ventricular dysfunction might contribute to the hypotension and thereby, indirectly the degree of left ventricular dysfunction, during and after myocardial infarction.[8,29,32] This study shows, that even after brief periods of ischemia, induced by occlusion of the LADCA, the right ventricle also becomes stunned and thereby might, indirectly, contribute to the left ventricular dysfunction observed during left ventricular stunning.

Segment length shortening and E_{es}

In assessing the severity of right and left ventricular stunning, we used both systolic segment length shortening (%SS) and the slope of the end-systolic pressure segment-length relationship (E_{es}), the reason being that %SS may be mainly determined by (regional) end-diastolic and end-systolic volume changes and does therefore not necessarily reflect myocardial contractility. Some striking differences were indeed observed in the pattern of changes in %SS and E_{es}. Firstly, at baseline %SS of the right ventricular myocardium was slightly larger than that of the left

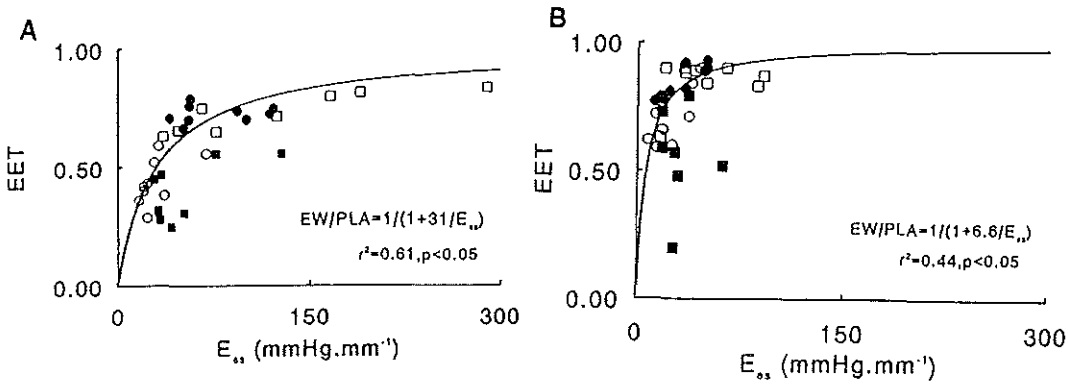

Fig 9. Hyperbolic relationship between efficiency of energy transfer EET (EW/PLA) and E_{es} for the left (A) and the right ventricle (B). Estimation of the curve was performed on data obtained at baseline (●) and after stunning (○). Notice that the EET points obtained during atrial pacing are below the EET-E_{es} relationship (■), while those obtained during the additional infusion of dobutamine are close to the EET-E_{es} relationship (□).

ventricular myocardium and showed, in agreement with earlier studies[6,7,13,14,26] fairly large regional differences (Table 2). On the other hand, E_{cs} (and also the maximum rate of rise in ventricular pressure) was higher for the left than for the right ventricle, which is also in agreement with earlier studies.[21,30,33] Secondly, both %SS and E_{es} were reduced significantly after 30 min of reperfusion following the coronary artery occlusion, but both parameters responded differently when the heart was stressed by atrial pacing. Systolic segment length shortening decreased, in both the stunned and non-stunned left ventricle, but was not affected in the right ventricular segments. E_{es}, on the other hand, tended to increase in the stunned left and right ventricular segments and remained unchanged in the non-stunned segments, which is compatible with the small effect of atrial pacing on E_{cs}. An interesting observation is that end-diastolic length of both (stunned and non-stunned) left ventricular segments decreased during pacing, while it did not change for the right ventricular segments. This decrease in end-diastolic segment length of the stunned left ventricle strongly suggests that in this segment the decrease in %SS was not due to a loss of contractile function but was caused by the decrease in stroke volume. The finding that E_{cs} of the stunned segments tended to increase supports this hypothesis. Finally, %SS was restored during infusion of dobutamine, which is in agreement with earlier investigations using inotropic stimulation.[2,3,11,12,20] E_{es}, on the other hand, increased to almost twice the baseline values. Hence, it appears to be preferable to use indexes such as E_{es}, when one wants to compare the effects of inotropic and chronotropic stimulation on contractility of stunned myocardium, which is working against different afterloads.

E_{es} in stunned left and right ventricular segments

Right ventricular stunning was accompanied by a decrease of contractility, although the absolute change was less than for the left ventricle. This might be due to a lower oxygen demand of the right ventricle before the ischemic period, resulting in a smaller oxygen debt during the

occlusion period. Indeed, PLA and E_{es}, two indexes of oxygen demand, were much lower for the right than for the left ventricle (Table 3), in accordance with the lower oxygen consumption per gram of right ventricular myocardium.[21] In addition, the observation that baseline contractility is a main factor determining the decrease of contractility during stunning for both the right and left ventricular myocardium, might be explained by the same working hypothesis since a higher contractility leads to a higher external work[30,33] and both factors are known to be related to a higher oxygen demand.[33] A similar observation has been reported by Kass er al,[16,17] who showed that in canine and human hearts, the regional ischemia-induced decrease in global E_{es} depended on its pre-ischemia values. These authors interpreted their results on basis of a two compartment model, describing the ischemic region with a smaller slope of the (passive) pressure-volume relationship (i.e. higher compliance) as the remote area.[16,17] The model calculations and the measurements indicated that the greater the active stiffness of the myocardial wall initially, the larger the effect of replacing a region of the myocardium by more compliant tissue.[16,17] Therefore, these authors interpreted their findings, solely on a mechanical basis. However, a recent study showed that purine efflux induced by a short period of ischemia is related to pre-ischemic myocardial function, indicating that a higher contractile function before the ischemic period leads to a higher ATP-catabolism during similar ischemic periods.[10]

In view of the non-linearity of the ESPSLR curves of both ventricles it might be argued that an index based on a linear relationship such as the preload-recruitable stroke work relationship would be preferable.[15,27] The advantage of the regional ESPSLR curves is, however, that, in addition to an estimation of E_{es} they also allow the calculation of indexes for external work, potential energy, total mechanical energy output and efficiency of energy transfer of the examined segments.[1,4,18,20,33] A further advantage of the time varying elastance concept is that it has established a relationship between E_{es} and efficiency of energy transfer.[4,18]

Efficiency of energy transfer in stunned left and right ventricular segments

Using the time varying elastance concept, Burkhoff er al [4] have shown that EET and E_{es} are related by $EET = 1/(1 + 0.5\ E_a/E_{es})$ in which E_a equals the ratio of mean arterial blood pressure and stroke volume. Myocardial stunning decreased mean arterial blood pressure and stroke volume to the same extent, which implies that 1) the EET of the non-stunned left ventricular segment remained unchanged as E_{es} did not change, while 2) the EET of the stunned left ventricular segment decreased due to a decrease in E_{es}. Atrial pacing decreased stroke volume but had no effect on mean arterial blood pressure, which resulted in a decrease in EET despite a virtually unchanged E_{es} (Fig 9). During the dobutamine infusion, the ratio of mean arterial blood pressure and stroke volume increased but did not return completely to baseline values. Nevertheless, the EET values obtained after dobutamine coincided with the relationship determined during baseline and stunning, suggesting a less pronounced roll of E_a during high contractile states, which is in agreement with data from Nozawa er al [27] and Morris er al [26]

The response of EET and the relationship between EET and E_{es} in the right ventricular

segments due to the 10 min occlusion, followed a similar pattern as the left ventricle, since the end-systolic right ventricular pressure decreased in a similar amount as stroke volume and probably E_a for the right ventricle also remained constant after coronary occlusion and subsequent reperfusion. The observed decrease of EET, therefore, mainly resulted from the decrease of E_{es}
of the right ventricular stunned region. Again the response of atrial pacing appeared unbeneficial, while the EET values after dobutamine coincided with the EET-E_{es} relationship.

In addition, by comparing the EET-E_{es} relationship for both ventricles, it can be shown that despite lower E_{es} values for the right ventricle as for the left ventricle during baseline conditions, EET values are almost similar because of the lower afterload or E_a for the right ventricle (Fig 9). Due to the steepness of the resulting hyperbolic relationship between EET and E_{es} for the right ventricle, EET is more sensitive to absolute changes in E_{es}. However, the small absolute decrease of E_{es} for the right ventricular stunned segment as compared to the left ventricular stunned segment, explains why EET was only modestly reduced (by 15%), while EET was reduced by 38% in the left ventricle.

Limitations of the study

Regional myocardial contractile function is often characterized by systolic segment length shortening or systolic wall thickening. Analogous to the time varying elastance concept (end-systolic pressure-volume relationships), Aversano er al have used the end-systolic pressure-segment length relationship to obtain an index of regional myocardial contractility,[1] which is more load-independent than segment length shortening. The time varying elastance concept also enables the calculation of the total mechanical work performed by the myocardium. The area inside the pressure-volume loop (mmHg.mm^3) represents external work, while the difference between total mechanical work (the area enclosed by the end-systolic and end-diastolic pressure-volume relationships and the systolic pressure-volume trajectory) and external work represents potential energy.[30,33] Several investigators have shown that, in analogy to the approach taken by Aversano er al, for both ventricles the area enclosed by the pressure-segment length loop, in spite of its difference in dimensions (mmHg.mm), is also a reliable index of external work and relates to regional oxygen consumption.[5,19,23,26,35] We have extended this analogy by considering the area (PLA) enclosed by the end-systolic and end-diastolic pressure segment length relationships and the systolic-pressure segment length trajectory (PLA).[20] The consequence of this assumption is that the dimensionless variable EET can be calculated from the ratio of EW and PLA. The close agreement between the present values of EET and the values obtained from pressure-volume relationships, obtained during baseline conditions and during infusion of dobutamine[27] are in accordance with this argument.

In constructing the ESPSLR, we only used gradual increases in afterload, which leaves the determination of potential energy open to criticism. We have, however, shown that ESPSLR's (and consequently also E_{es}, PE and PLA) constructed from changes in afterload alone are almost

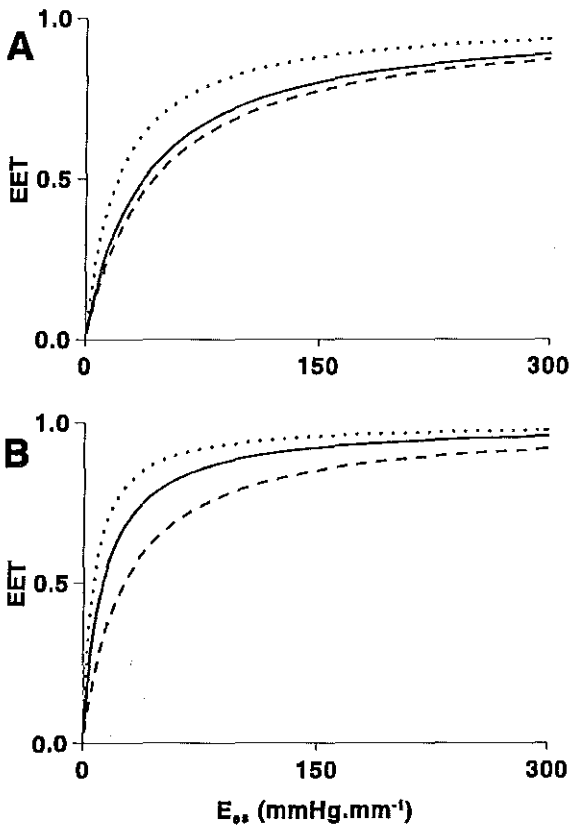

Fig 10. Hyperbolic relationships between EET and E_{es} for the stunned segments of the left (A) ventricle, calculated at 125 mmHg (dashed line), 75 mmHg (dotted line) and the working point (solid line) and for the right ventricle (B), calculated at 50 mmHg (dashed line), 25 mmHg (dotted line) and the working point (solid line). The working points for the left and right ventricle were ~ 100 mmHg and 35 mmHg, respectively.

identical to the ESPSLR's constructed by varying both pre- and afterload.[20] Similar to earlier investigators,[4,30,33,34] we found that the ESPSLR were described more accurately with a non-linear than with a linear relationship. This finding makes the slope of the ESPSLR (E_{es}) segment-length dependent. To characterize contractility we used the E_{es} at 100 mmHg for the left ventricle and the E_{es} at 25 mmHg for the right ventricle. To examine the possibility that our conclusions were determined by the choice of these pressure values, we also determined E_{es} and EET at end-systolic pressures of 125 and 75 mmHg for the left ventricle and at 50 mmHg for the right ventricle. E_{es} calculated at different end-systolic pressures changed similarly for both the right and left ventricular stunned segments during reperfusion, atrial pacing and the additional infusion of dobutamine, indicating that the relative response of the E_{es} values is similar in the range of end-systolic pressures normally encountered during our experiments.

A distinct difference was found between the hyperbolic relationships of EET and E_{es} for both stunned segments, when analyzed for the different end-systolic pressures (Fig 10). We therefore also analyzed the relationships between EET and E_{es} at the end-systolic pressures found during

each steady state before the changes in afterload were applied ("working point"). Although these working points were different between and during experiments, the resulting hyperbolic relationship was close to the 100 mmHg curve for the left ventricle (compare Fig 9 with Fig 10). However, the choice of 25 mmHg used for the analysis of the right ventricular data, resulted in quantitatively different conclusions at the working point for the right ventricle (Fig 10). Since it is theoretically better to estimate these relationships at a constant end-systolic pressure[18] and 25 mmHg was in the working range found in our studies, this approach was followed in the present study.

In conclusion, this study confirms that after a brief occlusion of the LADCA, not only the left ventricle, but also the right ventricle became regionally stunned and therefore might contribute to left ventricular dysfunction. The decreases in E_{es} and EW depended on their baseline values and were considerably less for the right than for the left ventricle, but for both ventricles, the decreases in E_{es} and EW could be described by the same linear relationship. Because for both stunned ventricular area's EW decreased and the PLA was less affected, the efficiency of energy (EET = EW/PLA) was decreased. During baseline conditions and stunning E_{es} and EET of both ventricular stunned segments were related by a hyperbolic relationship.

During atrial pacing the relationships were uncoupled as EET was lower than expected from the E_{es}, which could, in agreement with the time varying elastance concept, be explained by an increase in E_a (a consequence of the decrease in SV). Dobutamine not only increased E_{es}, EW and EET, but also restored the relationship between E_{es} and EET in both ventricular stunned segments.

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Chapter 4

Myofibrillar Ca^{2+} Sensitization Predominantly Enhances Function and Mechanical Efficiency of Stunned Myocardium

Running title: Ca^{2+} sensitization and myocardial stunning

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Myofibrillar Ca^{2+} Sensitization Predominantly Enhances Function and Mechanical Efficiency of Stunned Myocardium

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Background. Myocardial stunning is not only characterized by a decreased regional postischemic function, but also by a relatively high oxygen consumption (ie, decreased mechanical efficiency). Several lines of evidence suggest that the underlying mechanism may involve a decreased sensitivity of the myofibrils to calcium, but *in vivo* evidence is lacking. We therefore evaluated this hypothesis *in vivo* using EMD 60263, a calcium-sensitizing agent, which is devoid of any phosphodiesterase-inhibiting properties.

Methods and Results. We first established the effect of two consecutive doses of EMD 60263 (0.75 and 1.5 mg/kg; i.v.; n = 7), administered at 15 min intervals, on segment length shortening (SLS), external work index EW (the area inside the left ventricular pressure-segment length loop), myocardial oxygen consumption (MVO_2) and mechanical efficiency (EW/MVO_2) in anesthetized pigs with normal myocardium. After the highest dose of EMD 60263, SLS in the distribution area of left anterior descending coronary artery (LADCA) had increased from 13 ± 1% at baseline to 17 ± 1% ($P<.05$). EW, myocardial oxygen consumption (MVO_2) and mechanical efficiency (EW/MVO_2 per beat) were, however, not significantly affected (123 ± 10%, 98 ± 9% and 85 ± 13% of baseline, respectively). In 14 other anesthetized pigs myocardial stunning was induced by two sequences of 10 min LADCA occlusion and 30 min of myocardial reperfusion. After induction of stunning the two doses of EMD 60263 (n = 7) or saline (3 mL and 6 mL; n = 7) were infused. In the distribution area of the LADCA the stunning protocol caused decreases in SLS from 16 ± 1% to 8 ± 1% ($P<.05$) and in EW to 49 ± 5% of baseline ($P<.05$), while MVO_2 was only minimally affected ($P>.05$). Consequently, mechanical efficiency had decreased to 59 ± 8% of baseline ($P<.05$). Infusion of saline did not affect any of these regional myocardial variables, but after administration of EMD 60263 SLS recovered dose-dependently to 15 ± 2% after the highest dose of the drug. EW and mechanical efficiency also recovered dose-dependently to 89 ± 4% ($P<.05$ vs stunning) and to 88 ± 7% (NS vs baseline) of baseline, respectively. In the not-stunned segment SLS increased from 15 ± 2% (at baseline) to 18 ± 2% (after the highest dose), while EW per beat was not changed significantly. An adrenergic mode of action of EMD 60263 was excluded by blocking the alpha- and beta-adrenoceptors with phentolamine and propranolol, respectively, 15 min before administration of EMD 60263 (ie, 15 min into the second reperfusion period) in 5 additional experiments. In these experiments the EMD 60263-induced increases in SLS and EW were not attenuated. Because EMD 60263 decreased heart rate from 106 ± 4 to 76 ± 3 beats per minute ($P<.05$) in the animals with stunned myocardium we performed 5 experiments with the specific negative chronotropic compound zatebradine (UL-FS 49; 0.1 - 0.5 mg/kg) to rule out bradycardia as a contributing factor to the effects of EMD 60263. These doses of zatebradine lowered heart rate from 116 ± 5 to 55 ± 1 beats per minute ($P<.05$), but had no effect on SLS of stunned and not-stunned myocardium.

Conclusions. Calcium sensitization affects function and mechanical efficiency of the stunned myocardium more profoundly than of not-stunned myocardium, lending support to the hypothesis that Ca^{2+} desensitization of the myofibrils is involved in myocardial stunning. (***Circulation.*** **1994;90:959-969)**

Key Words • EMD 60263 • systemic hemodynamics • regional myocardial function • myocardial oxygen consumption

The mechanism underlying the prolonged contractile dysfunction after a short period of myocardial ischemia ("myocardial stunning") is still unknown. Proposed hypotheses include a reduced ability to synthesize high energy phosphates, impairment of regional perfusion, impairment of the sympathetic neural responsiveness, generation of free radicals, activation of leukocytes, reduction in the activity of creatine kinase and disturbances in the calcium homeostasis, but most of these mechanisms have been refuted (for review see References 1 and 2). The current view holds that generation of free radicals and disturbances in the calcium handling of the myocardial cell, mechanisms that are not mutually exclusive, are the two most likely mechanisms that cause this reversible postischemic dysfunction.[1]

Transient calcium overload as found during the early reperfusion phase may lead to disturbances in the calcium homeostasis and/or decreased sensitivity of the myofilaments to calcium.[3,4] Several groups of investigators have shown that the capacity of cardiac sarcoplasmic reticulum to transport Ca^{2+} decreases time-dependently during ischemia[5-7], which suggests that a reduced function of the Ca^{2+} pump might play a role in the mechanism leading to stunning. In a recent study, however, we have shown that in stunned myocardium of intact open-chest pigs the phosphorylation state of phospholamban was unchanged, and the Ca^{2+} uptake by the sarcoplasmatic reticulum was even slightly increased[8], while regional myocardial contractile function was still severely depressed.[9] These data suggest that a change in the active Ca transport of the sarcoplasmic reticulum may not be the principal cause of the contractile dysfunction of stunned myocardium. Marban and co-workers found no differences in intracellular Ca^{2+} transients in isolated ferret hearts before and after stunning and therefore concluded that the crucial lesion in stunning occurs at a later stage of the excitation-contraction process ie, the responsiveness of the myofilaments to calcium.[4,10] Up till now, *in vivo* evidence of this hypothesis has been difficult to obtain because agents which possess Ca^{2+} sensitizing properties, such as AR-L 57[11,12], sulmazole [13,14] and pimobendan [15,16] increase myocardial contractility predominantly via inhibition of phosphodiesterase and thus by increasing the Ca^{2+} transient.

In the present study we report on the cardiovascular effects of the thiadiazinone derivative EMD 60263 (Figure 1) in pigs with stunned myocardium. In *in vitro* experiments this compound has been shown to be a potent Ca^{2+} sensitizer devoid of any phosphodiesterase-inhibiting properties (I. Lues and M. Klockow, personal communication November 1993). The same investigators also demonstrated that in voltage-clamped myocytes the presence of EMD 60263 affected the delayed rectifier current I_{Kr} in a way which is characteristic for class III antiarrhythmic action. Potassium channel blockade might potentially increase myocardial contractility by increasing the action potential duration and therefore Ca^{2+} influx. This mechanism does not contribute to a positive inotropic effect of EMD 60263, however, as its enantiomer EMD 60264, which only differs from EMD 60263 because it lacks Ca^{2+} sensitizing properties, exerts a negative inotropic action (U. Ravens, personal communications, November 1993).

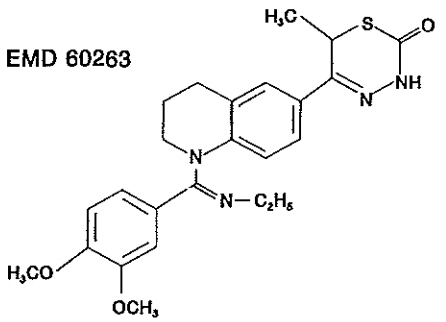

Fig 1. Diagram shows the chemical structure of EMD 60263.

In the present study myocardial stunning was induced by two cycles of 10 min coronary artery occlusion, separated by 30 min of reperfusion. This protocol causes a prolonged depression of regional myocardial function without myocardial necrosis[17,18], while Ca^{2+} uptake by the sarcoplasmic reticulum and phosphorylation of phospholamban is not depressed.[8] The effect of EMD 60263 on regional myocardial function was assessed by studying both the systolic segment length shortening (SLS) and the external work (EW) performed by the stunned and not-stunned myocardial segments. External work was estimated from the area enclosed by the left ventricular pressure-segment length loop, which has been validated as a reliable index by several groups of investigators.[19,20] Because a major characteristic of stunned myocardium is also a relatively high oxygen consumption for the amount of work performed[21,22], a phenomenon which may also point towards a decrease in the calcium sensitivity[23], we also evaluated the effect of EMD 60263 on mechanical efficiency, which is the amount of external work performed by the myocardium per unit consumed O_2.

Methods

Animal care

All experiments were performed in accordance with the guiding principles in the care and use of animals as approved by the Council of the American Physiological Society and under the regulations of the animal care committee of the Erasmus University Rotterdam, Rotterdam, the Netherlands.

Surgical Preparation

After an overnight fast cross-bred Landrace x Yorkshire pigs of either sex (23-30 kg, n = 31) were sedated with ketamine i.m.(20-30 mg/kg, Apharmo, Huizen, The Netherlands) and

anesthetized with sodium pentobarbital i.v. (20 mg/kg, Sanofi, Paris, France). The animals were 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: pH between 7.35 and 7.45; pCO_2 between 35 mm Hg and 45 mm Hg; pO_2 between 120 mm Hg and 180 mm Hg. Catheters were inserted into the superior caval vein for continuous administration of sodium pentobarbital (5 mg/kg/h) and haemaccel (Behringwerke A.G., Marburg, FRG) for replacing blood withdrawn during sampling. Fluid-filled catheters were positioned in the descending aorta for withdrawal of blood samples and to monitor the central aortic blood pressure. Through the left carotid artery a micromanometertipped catheter (B. Braun Medical B.V., Uden, The Netherlands) was inserted into the left ventricle for measuring left ventricular blood pressure and, by electrical differentiation, its first derivative (LVdP/dt. After administration of pancuronium bromide (4 mg, 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 placed around the ascending aorta for measurement of aortic blood flow (cardiac output). A proximal segment of the left anterior descending coronary artery (LADCA) was then dissected free for placement of an atraumatic clamp for occluding the coronary artery (n = 24) or a Doppler flow probe (n = 7), while the accompanying vein was cannulated for collection of local coronary venous blood.

Regional myocardial segment length shortening was measured by sonomicrometry (Triton Technology Inc., San Diego, CA, USA). One pair of the ultrasonic crystals was implanted inside the distribution area of the LADCA and another pair inside the distribution area of the left circumflex coronary artery (LCXCA). The crystals of each pair were positioned in the midmyocardial layer approximately 10-15 mm apart.

In order to determine regional blood flows the left atrial appendage was cannulated for injection of a batch of $1\text{-}2x10^6$ carbonized plastic microspheres (15 ± 1 μm (SD) in diameter) labelled with either ^{46}Sc, ^{95}Nb, ^{103}Ru, ^{113}Sn or ^{141}Ce (NEN Company; Dreieich, FRG). Starting 15 s before the injection of microspheres, blood was withdrawn from a femoral artery at a rate of 10 mL/min until 60-65 s after completion of the injection of the microspheres. At the end of each experiment the area perfused by the LADCA was identified by ligation of the coronary artery at the site of occlusion and injection of patent blue violet (Sigma, St. Louis, MO, USA) via the left atrial catheter. The heart and a number of other organs were excised and handled as described previously to determine regional blood flows.[24]

Experimental protocols

After the preparation had remained stable for at least 30 min following completion of the instrumentation, baseline values were obtained for systemic hemodynamic variables, regional myocardial function and arterial and coronary venous blood gases. Samples were collected for the measurement of hemoglobin concentration, oxygen saturation, pH, pCO_2 and pO_2.

We first evaluated the effect two doses of EMD 60263 (0.75 mg/kg and 1.5 mg/kg, dissolved in 3 mL and 6 mL saline, respectively) on global systemic hemodynamics, regional systolic segment length shortening, external work, myocardial oxygen consumption and mechanical efficiency in seven animals with a normal intact coronary circulation. Each dose was infused over a 3 min period and infusions were separated by a 15 min interval. In these animals LADCA blood flow was measured with the Doppler flow probe.

In 14 animals a batch of radioactive microspheres was injected for the measurements of regional myocardial blood flow. The LADCA was then occluded for 10 min and subsequently the myocardium reperfused for 30 min. This sequence of 10 min occlusion and 30 min of reperfusion was then repeated. At the end of the second reperfusion period in 7 animals 0.75 mg/kg EMD 60263, dissolved in 3 mL saline, was infused over a period of 3 min. Fifteen min later, this was followed by a second dose of 1.5 mg/kg EMD 60263 dissolved in 6 mL saline and again infused over a period of 3 min. In 7 other animals, the same volumes of saline were infused at similar time intervals. Systemic hemodynamics variables, segment length changes and regional myocardial blood flows were determined at baseline, at the end of the second 30 min reperfusion period (stunning) and 15 min after infusion of each dose of EMD 60263 or saline.

In five additional animals, the alpha- and beta-adrenoceptors were blocked 15 min before administration of the first dose of EMD 60263 (ie, after 15 min reperfusion following the second 10 min occlusion). Alpha- and beta-adrenoceptor blockade was achieved with 1 mg/kg phentolamine and 0.5 mg/kg propranolol followed by an infusion of 0.5 mg/kg/h, respectively.[25] During the course of the experiments it was found that EMD 60263 did not only affect regional myocardial function, but also exerted a pronounced bradycardic effect. In order to assess whether the bradycardia contributed to the changes in regional myocardial function, we performed another five experiments in which stunning was induced as described above and 0.1 mg/kg of the specific negative chronotropic agent zatebradine (UL-FS 49)[26,27] was administered intravenously at the end of the second 30 min reperfusion period. At 15 min intervals this was followed by doses of 0.2 mg/kg and 0.5 mg/kg. In these experiments no radioactive microspheres were injected.

Data analysis and presentation

Systolic segment length shortening (SLS) was calculated from the tracings as 100% x (EDL - ESL)/EDL, in which EDL and ESL are the segment length at end-diastole and end-systole, respectively. These time points were defined as the opening and closure of the aortic valves, respectively. Left ventricular pressure and myocardial segment length were digitized (sample rate 250 Hz) on a personal computer using an 8 bit AD converter. The area inside the left ventricular pressure-segment length loop was calculated and multiplied by 10 mm/$EDL_{Baseline}$ to arrive at a normalized index for external work (EW).

Oxygen consumption of the myocardium perfused by the LADCA (MVO_2) was calculated as the product of the local transmural myocardial blood flow (using the radioactive microsphere

data in the animals with stunning and the Doppler flow measurements in the animals without stunning) and the difference in the oxygen contents of the arterial and local coronary venous blood.[28] Mechanical efficiency was defined as the ratio of external work and myocardial oxygen consumption (EW/MVO_2 per beat). Because the index EW reflects external work, but has not its dimensions, we have expressed the changes in EW/MVO_2 per beat as percentage change of baseline.

The 14 animals were arbitrarily assigned to treatment with saline or EMD 60263 at the end of the second 30 min period ("stunning") and because no differences existed between the groups, the values of those groups obtained at baseline as well as those obtained at the end of the second 30 min reperfusion period ("stunning") were pooled. Statistical significance of the changes induced by the stunning protocol were evaluated by the Student's paired t-test. The effects of EMD 60263 and saline during stunning were assessed by two-ways analysis of variance with repeated measures and Bonferroni adjustment (BMDP Statistical Software Inc., Los Angeles, CA, USA). Statistical significance was accepted for $P<.05$ (two-tailed). All data have been presented as arithmetic mean ± SEM.

Drugs

EMD 60263 (supplied by Prof. Dr. P. Schelling, E. Merck, Darmstadt, Germany) was dissolved in saline to obtain infusion rates of 1 and 2 mL/min for the doses of 0.25 mg/kg/min and 0.5 mg/kg/min, respectively. Zatebradine (UL-FS 49) was a gift from Dr. J. Dämmgen (Dr. Karl Thomae GmbH, Biberach an der Riss, Germany) and also dissolved in saline. Propranolol hydrochloride (ICI-Pharma, Rotterdam, The Netherlands) and phentolamine-methanosulfonide (Ciba-Geigy, Basel, Switzerland) were also dissolved in saline.

Results

Effect of EMD 60263 in anesthetized pigs

Systemic hemodynamics

Infusion of EMD 60263 caused a slight decrease in mean arterial blood pressure (from 85 ± 1 mm Hg to 78 ± 3 mm Hg, $P<.05$), owing to a fall in diastolic arterial blood pressure (13 ± 3%, $P<.05$) as systolic arterial blood pressure remained unchanged (Table 1). The decrease in diastolic arterial blood pressure appeared to be secondary to a prolongation of the duration of diastole as heart rate decreased dose-dependently by as much as 37 ± 3% from its baseline value of 118 ± 5 bpm ($P<.05$). Cardiac output decreased as the increase in stroke volume (34 ± 7%, $P<.05$) was not sufficient to compensate for the decrease in heart rate. LVdP/dtmax, left ventricular end-diastolic pressure and systemic vascular resistance (calculated as the ratio of mean arterial blood pressure and cardiac output) were not affected.

TABLE 1. Effect of the Ca^{2+} sensitizer EMD 60263 on systemic hemodynamics of seven anesthetized open-chest pigs

	Baseline	EMD 60263 (μg/kg)	
		0.75	1.5
HR, bpm	118±5	94±5*	74±5*
SAP, mm Hg	103±1	102±1	101±3
MAP, mm Hg	85±1	81±1	78±3*
DAP, mm Hg	76±1	72±2*	67±3*
CO, L/min	2.2±0.1	2.0±0.2*	1.8±0.1*
SV, mL	19±1	21±2	25±3*
LVdP/dtmax, mm Hg/s	2300±160	2410±180	2420±130
LVEDP, mm Hg	7.1±1.1	7.1±1.0	7.3±1.1
SVR, mm Hg/(L/min)	39±2	42±4	44±3

HR indicates heart rate; SAP, MAP, and DAP, systolic, mean, and diastolic arterial pressure, respectively; CO, cardiac output; SV, stroke volume; LvdP/dt, maximal rare of rise in left ventricular pressure; LVEDP, left ventricular end-diastolic pressure; and SVR, systemic vascular resistance. The two doses of EMD 60263 were administered over 3 minutes at 15-minute intervals; data were obtained 15 minutes after administration of each dose. Vaues are mean±SEM.

*P<.05 vs baseline.

Regional myocardial performance

EMD 60263 caused slight and similar increases (P<.05) in the systolic segment length shortening in the distribution areas of both the LADCA (from 13 ± 1% to 17 ± 1%) and the LCXCA (from 13 ± 1% to 17 ± 2%) (Table 2). In the same table is also shown that external work (EW per beat) also increased to the same extent in both areas (23 ± 13% and 28 ± 11% in the distribution areas of the LADCA and LCXCA, respectively). Taking into account the EMD 60263-induced decrease in heart rate it can be calculated that EW per min did not change.

EMD 60263 had no effect on oxygen extraction in the distribution area of the LADCA and, because LADCA blood flow did also not change, also no effect on myocardial oxygen consumption (MVO_2). Mechanical efficiency (EW/MVO_2 per beat) was not affected after the lowest dose, but tended to decrease after the highest dose of EMD 60263.

Effect of EMD 60263 in anesthetized pigs with stunned myocardium

Systemic hemodynamics

The two cycles of 10 min coronary artery occlusion and 30 min of reperfusion caused a slight decrease in mean arterial blood pressure (from 91 ± 2 mm Hg to 86 ± 2 mm Hg, P<.05),

TABLE 2. Effect of the Ca^{2+} Sensitizer EMD 60263 on Regional Myocardial Function and Myocardial Oxygen Consumption in Seven Anesthetized Open-Chest Pigs

	Baseline	EMD 60263 (μg/kg)	
		0.75	1.5
LADCA			
SLS, %	13±1	14±1	17±1*
EW/beat, mm Hg. mm	129±9	147±8	158±17
$cvO_{2\text{-}sat}$, %	30±2	30±2	30±3
CBF, mL/min	27±4	25±4	25±4
MVO_2, μmol/min	79±11	72±10	76±12
EW/MVO_2, % baseline	100	99±5	85±13
LCXCA			
SLS, %	13±1	15±1*	17±2*
EW/beat, mm Hg.mm	114±7	130±14	137±13*

LADCA indicates left anterior descending coronary artery; SLS, segment length shortening; EW, external work; $cvO_{2\text{-}sat}$, coronary venous oxygen saturation; CBF, coronary blood flow; MVO_2, myocardial oxygen consumption; and LCXCA, left circumflex coronary artery. The two doses of EMD 60263 were administered over 3 minutes at 15-minute intervals; data were obtained 15 minutes after administration of each dose. Values are mean±SEM.

* $P<.05$ vs baseline

which was primarily caused by the fall in cardiac output from 2.64 ± 0.15 L/min to 2.30 ± 0.06 L/min ($P<.05$) as systemic vascular resistance was only slightly increased (Table 3). Because heart rate was not affected, stroke volume fell in parallel with cardiac output. LVdP/dtmax was decreased by 19 ± 3%, while left ventricular end-diastolic pressure was unchanged (Table 3).

Infusion of saline after 30 min of reperfusion following the second 10 min occlusion had no effect on any of the systemic hemodynamic variables. On the other hand, administration of EMD 60263 resulted in dose-dependent decreases in mean arterial blood pressure, owing to a decrease in diastolic arterial blood pressure (by as much as 14 ± 5%, $P<.05$), while systolic arterial blood pressure was unaffected (Table 3). The decrease in diastolic arterial blood pressure appeared to be again secondary to a prolongation of the duration of diastole, as heart rate decreased dose-dependently by as much as 28 ± 3% ($P<.05$) after administration of the higher dose of EMD 60263. Stroke volume increased (25 ± 7%, $P<.05$), which was not enough to compensate for the decrease in heart rate, as cardiac output still fell by 11 ± 4% after the higher dose. Systemic vascular resistance, LVdP/dtmax and left ventricular end-diastolic pressure were also not affected by EMD 60263 in this series of experiments.

TABLE 3. Effect of the Ca^{2+} Sensitizer EMD 60263 on Systemic Hemodynamics of Anesthetized Open-Chest Pigs After Myocardial Stunning

			Change From Stunning			
			Saline (n=7)		EMD 60263 (n=7)	
	Baseline (n=14)	Stunning (n=14)	1.0 mL/min	2.0 mL/min	0.75 mg/kg	1.5 mg/kg
HR, bpm	108.1±1.6	105.6±3.6	-0.4±2.5	-1.9±3.5	-14.1±1.6†	-30.3±3.2†
SAP, mm Hg	113.9±2.1	106.9±2.6*	-0.9±2.9	4.0±2.6	-3.4±3.2	-2.1±4.9
MAP, mm Hg	91.1±1.7	86.4±2.4*	-0.6±3.2	2.4±2.8	-5.6±3.4	-8.4±4.3
DAP, mm Hg	80.4±1.6	77.8±2.5	-1.6±2.6	0.4±2.5	-6.0±3.5	-11.3±4.0†
CO, L/min	2.64±0.15	2.30±0.06*	0.04±0.1	0.06±0.08	-0.09±0.05	-0.23±0.08
SV, mL	24.5±1.3	22.0±0.8*	0.2±0.5	1.0±1.1	2.2±0.5†	5.3±1.3†
LVdP/dtmax, mm Hg/s	2240±105	1810±105*	43±74	39±67	37±28	97±98
LVEDP, mm Hg	9.4±0.7	10.3±0.9	-0.6±0.4	0.6±0.7	-0.14±1.62	1.0±1.38
SVR, mm Hg/(L/min)	36.0±2.3	38.0±1.5	0.0±1.0	1.0±1.0	1.0±2.0	2.0±3.0

Definitions are as in Table 1. Pigs undewent two periods of 10 minutes occlusion of the left anterior descending coronary artery (LADCA) each followed by 30 minutes of reperfusion. Stunning values were obtained after 30 minutes of reperfusion following the second LADCA occlusion. Saline and EMD 60263 were administered over 3 minutes at 15-minute intervals; data were obtained 15 minutes after administration of each dose. Values are mean±SEM.

* $P<.05$ vs Baseline (only for values obtained during stunning)

† EMD 60263-induced change from stunning is significantly different ($P<.05$) from the saline-induced change from stunning at comparable time point.

Regional myocardial segment length shortening

During each of the two occlusions there was a complete loss of systolic segment length shortening in the distribution area of the LADCA, while there was only a partial recovery during the subsequent reperfusion periods (from 16 ± 1% at baseline to 8 ± 1% of the end of the second reperfusion period). Figure 2 shows that in the animals, treated with a saline infusion, segment length shortening remained depressed. However, administration of EMD 60263 caused a dose-dependent recovery of segment length shortening to baseline levels.

Systolic segment length shortening in the not-stunned (LCXCA-perfused) myocardium (15 ± 2% at baseline) did not change during the induction of stunning in the adjacent myocardium. Infusion of saline had no effect on SLS of the not-stunned myocardium, but increased to 18 ± 2% after the highest dose of EMD 60263 (Figure 2). This increment is very similar to that observed in the animals which did not undergo the stunning protocol (Table 2). Compared to the increase in the stunned myocardium, the effect in the not-stunned segment was much less. The latter was most clearly demonstrated by SLS_{LADCA}/SLS_{LCXCA}, which had decreased from 1.09 ± 0.16 to 0.61 ± 0.16 after induction of stunning. While this ratio did not change during infusion of saline, there was an increase to 0.75 ± 0.15 and 0.86 ± 0.12 after 0.75 mg/kg and 1.5 mg/kg of EMD 60263, respectively (both $P<.05$ versus stunning).

External work

The stunning protocol caused a decrease ($P<.05$) in the external work per beat of the LADCA-perfused myocardium to 49 ± 5% of baseline (Figures 3 and 4), and because heart rate

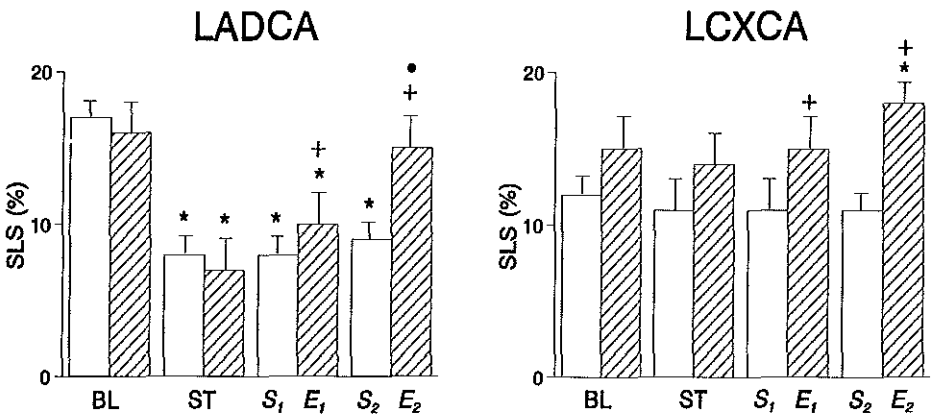

Fig 2. Bar graphs show systolic segment length shortening (SLS) at baseline (BL), during stunning (ST), and after infusion of 2 vol saline (3 [S_1] and 6 [S_2] mL) or two doses of EMD 60263 (0.75 [E_1] and 1.5 [E_2] mg/kg). Values are mean±SEM from seven animals in each group. *$P<.05$ vs baseline; †$P<.05$ vs stunning; •, EMD 60263-induced changes from stunning significantly different from saline-induced changes from stunning. LADCA indicates left anterior descending coronary artery; LCXCA, left circumflex coronary artery.

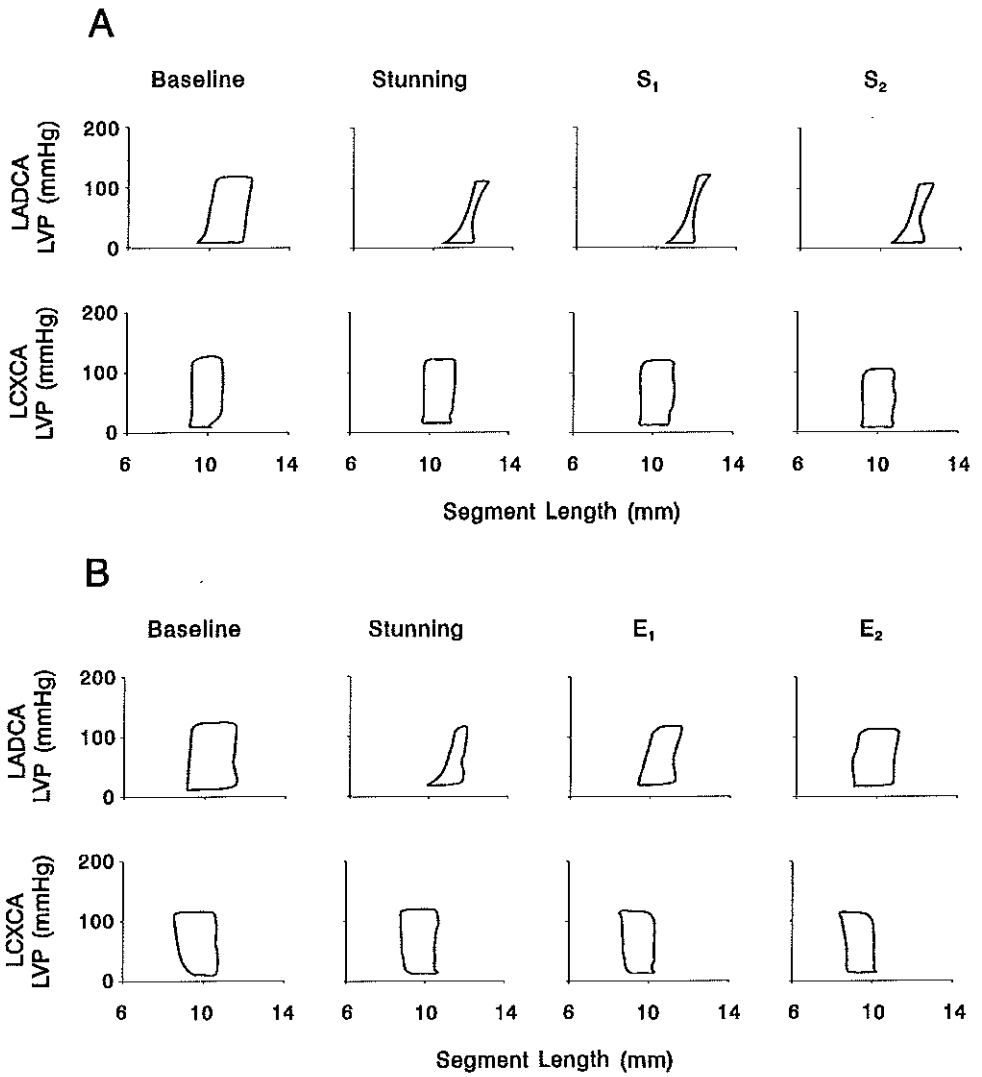

Fig 3. Representative example of left ventricular pressure (LVP)-segment length tracing after saline (A, n=7) or EMD 60263 (B, n=7) in pigs with stunned myocardium. Stunning was induced in the myocardium supplied by the left anterior descending coronary artery (LADCA). Area supplied by the left circumflex coronary artery (LCXCA) served as not-stunned area. Two doses of EMD 60263 were administered over 3 minutes at 15-minute intervals: E_1=0.75 mg/kg, E_2=1.5 mg/kg. In the saline-treated animals 3mL (S_1) and 6 mL $S(_2)$ saline was administered.

did not change, there was a similar decrease (to 48 ± 5% of baseline, $P<.05$) in external work per min (data not shown). Infusion of saline had no effect on EW per beat (Figures 3 and 4) and EW per min of the stunned myocardium, however, after administration of EMD 60263, EW per beat increased dose-dependently to 89 ± 4% of baseline after the highest dose ($P<.05$ vs stunning; Figure 4), while EW per min increased to only 63 ± 5% of baseline because of the lower heart rate ($P<.05$ vs stunning; not shown).

The stunning protocol caused minor decreases in both EW per beat (to 92 ± 7% of baseline) and EW per min (to 92 ± 9% of baseline) of the not-stunned segment, because of the decrease in left ventricular systolic pressure (Figure 4). There were no further changes during the infusion of saline, but EW per beat had increased to 105 ± 5% of baseline after infusion of the highest dose of EMD 60263 (Figure 4), while EW per min had further decreased to 75 ± 7% of baseline (ie, 17 ± 6% lower than the value observed during stunning, $P<.05$).

Because of the intravenous route of administration, EMD 60263 caused increases in external work performed by both the stunned and not-stunned segments, we also calculated the ratio of the EW performed by the LADCA and the EW performed by the LCXCA (EW_{LADCA}/EW_{LCXCA}), in order to assess whether differences in regional performance were attenuated after administration of EMD 60263. After the stunning protocol this ratio was decreased from 1.24 ± 0.13 to 0.70 ± 0.12. Infusion of saline did not affect this ratio, but after the first and second dose of EMD 60263 this ratio had increased to 0.94 ± 0.24 and 1.02 ± 0.21, respectively (both $P<.05$ vs stunning).

Regional myocardial blood flow and vascular resistance

Thirty minutes after the second occlusion, transmural blood flow in the distribution area of the LADCA had decreased from 161 ± 7 mL/min/100g to 132 ± 10 mL/min/100 g ($P<.05$), a decrease that was equally distributed over the transmural layers as the subendocardial/subepicardial blood flow ratio (baseline value 1.16 ± 0.06) did not change. Neither infusion of saline, nor that of EMD 60263 resulted in any significant changes. In the myocardium supplied by the LCXCA, transmural perfusion (baseline value 179 ± 9 mL/min/100 g) and its distribution (baseline value subendocardial/subepicardial blood flow ratio 1.10 ± 0.04) were unchanged after the occlusion-reperfusion sequences. Infusion of saline or EMD 60263 also did not affect perfusion of the not-stunned myocardium.

Vascular resistance of the LADCA was increased from 0.58 ± 0.03 mm Hg/(mL/min/100g) to 0.69 ± 0.04 mm Hg/(mL/min/100 g) ($P<.05$) after the stunning protocol, but did not change any further during the subsequent infusion of saline or EMD 60263. The vascular resistance of the LCXCA was not affected by either the occlusion-reperfusion sequence or the subsequent infusion of saline or EMD 60263.

Regional myocardial oxygen consumption

Oxygen saturation in the vein accompanying the LADCA (baseline 24 ± 2%) was

unchanged (25 ± 2%) following the two sequences of 10 min occlusion and 30 min reperfusion. From these data and the regional myocardial blood flow data it follows that oxygen consumption of the stunned myocardium had decreased from 452 ± 21 μmol/min/100g to 378 ± 30 μmol/min/100g ($P<.05$), before the infusion of saline or EMD 60263 was started. There was a similar decrease in MVO_2 per beat, because heart rate was not affected. After administration of EMD 60263, coronary venous oxygen saturation was 29 ± 4%, which was not significantly different from the value before administration. MVO_2 was 380 ± 35 μmol/min/100g and 338 ± 34 μmol/min/100g after 0.75 mg/kg and 1.5 mg/kg of EMD 60263, respectively (both values $P<.05$ vs the MVO_2 before the stunning value). MVO_2 per beat was not significantly affected despite the decrease in heart rate. Because coronary venous oxygen saturation, myocardial blood flow and heart rate did not change, MVO_2 per min and MVO_2 per beat of the stunned myocardium remained unchanged during infusion of saline.

TABLE 4. Effect of the Ca^{2+} Sensitizer EMD 60263 on Systemic and Regional Hemodynamics of Anesthetized Open-Chest Pigs After Myocardial Stunning and Adrenergic Receptor Blockade

			EMD 60263	
Parameter	Baseline	Stunning + α- and ß-Adrenergic Receptor Blockade	0.75	1.5
HR, bpm	104±5	72±2*	58±1†	50±3†
SAP, mm Hg	107±1	86±6*	93±5	98±4†
MAP, mm Hg	85±2	68±5*	72±4	69±3
DAP, mm Hg	74±2	59±4*	63±4	55±3
CO, L/min	2.4±0.1	1.6±0.3*	1.7±0.2	1.9±0.3
SV, mL	23±1	23±4	29±4†	39±8†
LvdP/dtmax, mm Hg/s	2260±220	1050±160*	1070±240	1150±210
LVEDP, mm Hg	9±1	15±2*	18±1	14±1
SVR, mm Hg/(L/min)	36±1	47±9*	47±8	39±5
SLS_{LADCA}, %	19±3	9±1*	14±3†	20±2†
SLS_{LCXCA}, %	19±2	16±2	18±2†	23±2†
$EW/beat_{LADCA}$, mm Hg.mm	207±35	86±25*	136±32†	188±22†
$EW/beat_{LCXCA}$, mm Hg.mm	184±19	132±27	168±26†	211±27†

Definitions are as in Table 1; SLS, segment length shortening; LADCA, left anterior descending coronary artery; LCXCA, left circumflex coronary artery; and EW, external work. Pigs undewent two periods of 10 minutes occlusion of the LADCA each followed by 30 minutes of reperfusion. Stunning values were obtained after 30 minutes of reperfusion following the second LADCA occlusion. Saline and EMD 60263 were administered over 3 minutes at 15-minute intervals; data were obtained 15 minutes after administration of each dose. Values are mean±SEM

* $P<.05$ vs baseline (only for values obtained during stunning).

† $P<.05$ vs stunning + α- and ß-adrenergic receptor blockade.

Myocardial efficiency of regional myocardium

In view of the large decrease in the external work index EW (to 49 ± 5% of baseline) compared to the decrease in oxygen consumption per beat (to 86 ± 6% of baseline) it follows that after stunning mechanical efficiency of the LADCA-perfused myocardium (EW/MVO_2 per beat) was decreased to 58 ± 6% of baseline ($P<.05$). Infusion of saline had no effect on mechanical efficiency as both external work and myocardial oxygen consumption were unaltered (to 60 ± 10% and 60 ± 11% of baseline after the first and second infusion, respectively; both $P<.05$ vs baseline). Because EMD 60263 increased external work without an adverse effect on MVO_2 per beat, the EW/MVO_2 per beat ratio had increased to 72 ± 11% and 88 ± 7% (NS vs baseline) after 0.75 mg/kg and 1.5 mg/kg of EMD 60263, respectively. It is noteworthy that after the higher dose of EMD 60263 mechanical efficiency of the stunned myocardium was not different from the corresponding measurement in the animals without stunning.

In this series of experiments oxygen extraction of the not-stunned myocardium (distribution of the LCXCA) was not measured and oxygen consumption of that segment could therefore not be determined. Because in the first series of experiments (pigs without stunning) EMD 60263 had no effect on myocardial oxygen extraction of control myocardium, we assumed that in the animals with stunned myocardium EMD 60263 had also no effect on oxygen extraction of the not-stunned myocardium. If true the ratio of EW and transmural blood flow per beat (CBF) also reflects mechanical efficiency. Figure 5 clearly shows that EMD 60263 had no significant effect on the EW/CBF of the not-stunned myocardium.

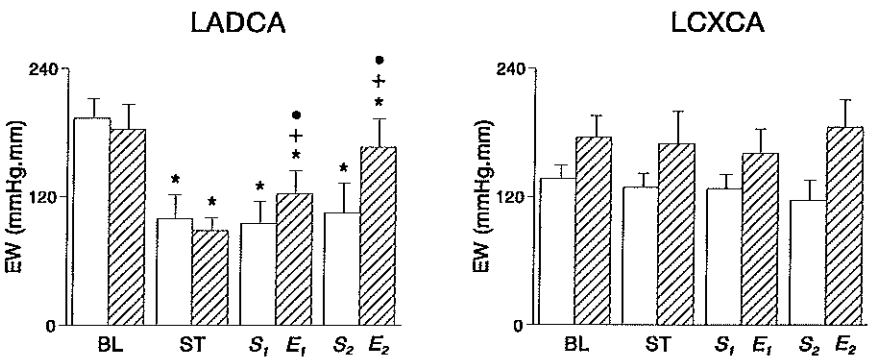

Fig 4. Bar graphs show effect of saline (open columns, n=7) or EMD 60263 (hatched columns, n=7) on external work (EW) in pigs with stunned myocardium. Stunning was induced in the myocardium supplied by the left anterior descending coronary artery (LADCA). The area supplied by the left circumflex coronary artery (LCXCA) served as not-stunned area. Two doses of EMD 60263 were administered over 3 minutes at 15-minute intervals: E_1=0.75 mg/kg, E_2=1.5 mg/kg. In the saline-treated animals 3 mL (S_1) and 6 mL (S_2) saline was administered. BL indicates baseline; ST, stunning. *$P<.05$ vs baseline; +$P<.05$ vs stunning; ●, EMD 60263-induced changes from stunning significantly different from saline-induced changes from stunning.

TABLE 5. Effect of the Specific Negative Chronotropic Compound Zatebradine (UL-FS 49) on Global and Regional Myocardial Performance in Five Anesthetized Pigs With Stunned Myocardium

			Zatebradine		
	Baseline	Stunning	0.1 mg/kg	0.2 mg/kg	0.5 mg/kg
HR, bpm	113±7	116±5	89±7†	68±3†	55±1†
SAP, mm Hg	112±1	103±4*	102±4	105±5	109±5
MAP, mm Hg	90±2	83±3*	78±4	75±2†	70±3†
DAP, mm Hg	79±3	78±5	66±4	62±3†	53±3†
CO, L/min	2.7±0.2	2.6±0.2	2.4±0.1	2.1±0.1†	1.8±0.1†
SV, mL	23±2	23±2	27±2†	31±2†	33±3†
$LVdP/dt_{max}$, mm Hg/s	2610±290	2180±190*	2360±300	2100±240	1960±160
LVEDP, mm Hg	8±1	7±1	9±2	10±1	13±1†
SVR, mm Hg/(L/min)	33±3	33±3	33±3	37±2	41±4
SLS_{LADCA}, %	17±2	9±1*	10±2	11±1	11±2
SLS_{LCXCA}, %	15±1	14±1	15±1	15±2	16±2

Definitions are as in Table 1; SLS, segment length shortening; LADCA, left anterior descending coronary artery; LCXCA, left circumflex coronary artery; and EW, external work. Stunning values were obtained after two cycles of 10 minutes of LADCA occlusion and 30 minutes of reperfusion. Zatebradine was administered over 3 minutes at 15-minute intervals; data were obtained 15 minutes after administration of each dose. Values are mean±SEM.

* $P<.05$ vs baseline (only for values obtained during stunning)

† Zatebradine-induced change from stunning is significantly different ($P<.05$) from the saline-induced change from stunning.

Regional blood flows and vascular resistances

Blood flow and vascular resistance in a number of major organs (brain, kidneys, adrenals and skeletal muscle) were not significantly affected by the induction of myocardial stunning. The subsequent infusion of saline also did not lead to any significant changes. After EMD 60263, however, blood flow to the kidneys (by 12%, $P<.05$) and the muscular part of the diaphragm decreased. This was due to the decrease in mean arterial blood pressure, because vascular resistance of these organs was not affected (not shown).

Effect of EMD 60263 in anesthetized pigs with stunned myocardium after α- and ß-adrenoceptor blockade

Systemic hemodynamics

Blockade of the alpha- and beta-adrenoceptor, starting 15 min after the second 10 min LADCA occlusion period, resulted in a pronounced depression of arterial blood pressure, cardiac output, heart rate and LVdP/dtmax and an increase in left ventricular end-diastolic pressure, while stroke volume was only marginally affected (Table 4). In the presence of adrenoceptor blockade EMD 60263, however, caused similar changes in systemic hemodynamic variables as in animals without adrenoceptor blockade: dose-dependent decreases in mean and diastolic arterial blood pressure and heart rate, a dose-dependent increase in stroke volume, while systolic

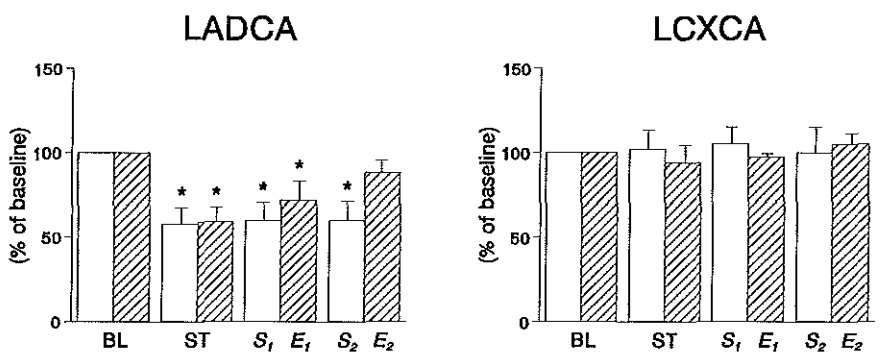

Fig 5. Bar graphs show effect of saline (open columns, n=7) or EMD 60263 (hatched columns, n=7) on mechanical efficiency (external work [EW]/myocardial oxygen consumption [MVO_2]) in pigs with stunned myocardium. Because the index EW reflects external work but does not have its dimensions, and in the not-stunned segment mechanical efficiency was approximated by EW/transmural blood flow per beat (EW/CBF perbeat), all values are presented as percentage of baseline (±SEM). Stunning was induced in the myocardium supplied by the left anterior descending coronary artery (LADCA). The area supplied by the left circumflex coronary artery (LCXCA) served as not-stunned area. Two doses of EMD 60263 were administered over 3 minutes at 15-minute intervals: E_1=0.75 mg/kg, E_2=1.5 mg/kg. In the saline-treated animals 3 mL (S_1) and 6 mL (S_2) saline was administered. BL indicates baseline; ST, stunning. *$P<.05$ vs baseline.

arterial blood pressure, cardiac output, LVdP/dtmax and systemic vascular resistance were not significantly altered (Table 4).

Regional myocardial performance

After adrenoceptor blockade SLS of the stunned myocardium had decreased from 19 ± 3% at baseline to 9 ± 1%, while SLS of the not-stunned myocardium was not affected (baseline 19 ± 2%). In the presence of adrenoceptor blockade EMD 60263 caused dose-dependent increase in SLS of the stunned myocardium to 20 ± 2% after the highest dose ($P<.05$), an increase which is very similar to the EMD 60263-induced increase, when the adrenoceptors were not blocked (Figure 2). In the not-stunned segment SLS increased dose-dependently up to 23 ± 2% after the last dose of EMD 60263. After adrenoceptor blockade external work of the stunned myocardium had decreased to 44 ± 11% of baseline values and, because of the fall in systolic arterial blood pressure, external work of the not-stunned segment had decreased to 73 ± 12% of baseline ($P<.05$, Table 4). Adrenoceptor blockade did not affect the EMD 60263-induced effects on external work, however, as the increases (Table 3) were very similar to the EMD 60263-induced increases observed in the absence of adrenoceptor blockade (Figure 3).

Effect of the specific bradycardic agent zatebradine (UL-FS 49) on the function of stunned myocardium

In Table 5 it is shown that zatebradine in doses of 0.1 mg/kg to 0.5 mg/kg lowered heart rate dose-dependently from 116 ± 5 bpm to 55 ± 1 bpm. Similar to EMD 60263 there were dose-dependent decreases in mean and diastolic arterial blood pressure, while systolic arterial blood pressure was unchanged. Systolic segment length shortening had decreased from 17 ± 2% at baseline to 9 ± 1% at the end of the second reperfusion period. Lowering heart rate with zatebradine had no significant effect on systolic segment length shortening of the stunned and not-stunned myocardium (Table 5).

Discussion

Myocardial stunning is a transient event which is characterized by a depressed function and by a relatively high oxygen consumption for the amount of work performed by the stunned myocardium. As a matter of fact several authors have shown that oxygen consumption of stunned myocardium is equal to or even exceeds that of normal myocardium[21,22,29], although the latter is not a common finding.[9,30] Still there is a consensus that stunned myocardium uses oxygen less efficiently than normal myocardium.[23] The mechanisms underlying the oxygen wastage (ie, decreased mechanical efficiency) of stunned myocardium are unknown, but it is unlikely that it is caused by mitochondrial uncoupling[31], an increase in basal metabolism[32] or

changes in the Ca^{2+}-ATPase activity of the sarcoplasmic reticulum.[10,33] We have reported earlier that in the same model as used in the present study, the calcium sequestering properties of the sarcoplasmic reticulum were unchanged during stunning.[8] It is therefore most likely that an inefficient utilization of ATP by the contractile apparatus is a major factor contributing to the oxygen wastage. A decrease in the sensitivity of the contractile regulatory proteins to calcium and the consequent changes in the myosin ATPase activity could explain an increase in the ratio of external work over oxygen consumption and the depressed contractile function of stunned myocardium. The aim of the present study was therefore to elucidate whether in *in vivo* experiments the calcium-sensitizing actions of EMD 60263 could restore function as well as mechanical efficiency of stunned myocardium. Traditionally the not-stunned myocardium adjacent to the stunned myocardium serves as a control segment. One can not exclude, however, that the response of the not-stunned segment is affected by the alterations in the adjacent stunned myocardium. For instance, in porcine myocardium it has been shown that phosphorylation of phospholamban is affected when the adjacent myocardium is exposed to a prolonged period of ischemia.[34] We therefore performed a series of experiments in which the effect of the EMD 60263 was studied in pigs which had not undergone the stunning protocol. The results in Tables 1 and 3 show that the responses of the systemic hemodynamic variables were very similar in both models. The results presented in Table 2 show that in the distribution area of the LADCA, EMD 60263 slightly increased SLS and EW per beat, but had no effect on LADCA blood flow and oxygen extraction in that segment of the myocardium. Consequently, oxygen consumption was unchanged and mechanical efficiency, did not change (85 ± 13% of baseline after the higher dose of EMD 60263).

Recruitment of inotropic reserve in stunned myocardium has been be accomplished by stimulation with adrenoceptor agonists such as dopamine and epinephrine[35-37], calcium[38] and by post-extrasystolic potentiation.[39] In addition to inotropic stimulation it is also possible to enhance function of stunned myocardium by increasing myocardial blood flow with vasodilators such as dipyridamole, papaverine and nitroglycerine.[29] In the present study, we showed that EMD 60263 increased systolic segment length shortening of both the stunned and not-stunned porcine myocardium. The data presented in Figure 2 also demonstrated that the effect on the stunned myocardium was much more pronounced than on the not-stunned myocardium, and that differences in performance between the two areas were almost completely abolished. Comparison of the data presented in Table 2 and Figure 2 also teaches that the effects of EMD 60263 on wall function and external work in the animals with and without myocardial stunning were very similar to those observed in the not-stunned myocardium (distribution area of LCXCA). This action of EMD 60263 was not attenuated when experiments were repeated after alpha- and beta-adrenoceptor blockade, thereby excluding adrenergic stimulation and phosphodiesterase inhibition[40-42] as the cause of the positive inotropic action of EMD 60263. Furthermore, coronary blood flow was not increased by EMD 60263, which eliminated coronary vasodilation as a potential factor to the inotropic effects of EMD 60263. A complicating factor

in the present study was the lowering of the heart rate by EMD 60263 as several groups of investigators have shown that bradycardia improves contractile function of ischemic myocardium.[26,43] The effect of bradycardia on function of postischemic myocardium is, however, not well documented. We therefore studied the effect of bradycardia on postischemic function by administration of the specific negative chronotropic agent zatebradine and found that we could not restore function, when we lowered the heart rate over an even wider range than with EMD 60263. Hence, we may conclude that the EMD 60263-induced bradycardia does not play an important role in the improvement in function. Finally, because *in vitro* experiments have shown a negative inotropic effect of the enantiomer EMD 60264, it is most likely that EMD 60263 exerted its effect by increasing the sensitivity of the contractile proteins to calcium. At first glance, our results appear to be in contrast with those reported by Heusch *et al.*[11], who reported that the increases in systolic wall thickening were very similar for not-stunned and stunned myocardium after administration of AR-L 57. It must be kept in mind, however, that this drug increases myocardial contractility predominantly by phosphodiesterase inhibition.[12] The fact that in the present study EMD 60263 exhibited no vasodilator properties in the systemic vascular bed or a number of regional vascular beds, a usual finding for calcium sensitizers with phosphodiesterase inhibitor activity is also in agreement with the *in vitro* studies, which showed that EMD 60263 is almost completely devoid of any phosphodiesterase inhibitor activity.

Parallel to the decreases after stunning and increases after EMD 60263 in systolic segment length shortening, we found decreases and increases in the external work index EW. This is not surprising, because the former is derived from the relation between the changes in segment length and left ventricular pressure. When we related EW to the MVO_2 per beat, we confirmed the decrease in mechanical efficiency of stunned myocardium[23]. A striking finding in the present study is, however, that this decrease in mechanical efficiency for stunned segment could be reversed with EMD 60263. This action of EMD 60263 appears to be specific for stunned myocardium as EMD 60263 had no effect on mechanical efficiency of the same myocardial segment when it was administered in the pigs without stunning (Table 2). These data provide strong support for the hypothesis that calcium desensitization plays a role in the relative oxygen wastage of stunned myocardium. Gross *et al.*[44] have also shown a beneficial effect of the thiadiazinone derivative EMD 57033 on bioenergetics of skinned ventricular trabeculae. On the other hand, no beneficial effects on bioenergetics were observed in isolated heart studies using EMD 53998[45] and pimobendan.[46] The last studies[45,46] do not necessarily contradict the present results as we found only an effect of EMD 60263 on mechanical efficiency of stunned (Figure 5) and not on that of the normal myocardium (Table 2).

In none of the two series of experiments with EMD 60263 the global contractility index, LVdP/dtmax, increased, although both SLS and stroke volume increased. A number of factors could explain this observation as LVdP/dtmax, in addition to myocardial contractility, also depends on heart rate and loading conditions of the heart. We have shown that in anesthetized pigs with normal hearts LVdP/dtmax increases markedly when heart rate is raised from 60 bpm

to 100 bpm[47]. In this respect lowering heart rate should induce a decrease in LVdP/dtmax. It could thus be argued that the positive inotropic effects of EMD 60263 counteracted a possible negative effect of the bradycardia on LVdP/dtmax. Nevertheless administration of zatebradine, which is devoid of inotropic actions[48], did neither improve regional function of the stunned myocardium (Table 5) and did not led to a decrease in LVdP/dtmax. It is quite feasible that an intracoronary administration using much lower doses of EMD 60263 would have provided an improvement in regional function without pronounced global hemodynamic effects.

The absence of an increase in LVdP/dtmax can also be explained by intracellular changes in Ca^{2+} handling induced by the Ca^{2+} sensitizing properties of EMD 60263. Lee and Allen have shown that increasing Ca^{2+} sensitivity, and thereby increasing the troponin binding constant for Ca^{2+}, causes a slower release of Ca^{2+} from troponin and hence a prolonged time course of tension.[49] This implies that Ca^{2+} remains bound longer to troponin and since the sarcoplasmic reticulum function in our experimental model is not impaired[8], a decreased Ca^{2+} transient might result.[49] These effects can counteract each other, leading to an unchanged LVdP/dtmax.

From the finding that the effects of the Ca^{2+} sensitizer, EMD 60263, are more pronounced in stunned myocardium than in the not-stunned myocardium, we conclude that Ca^{2+} desensitization may play a role in the events leading to postischemic myocardial dysfunction and oxygen wastage.

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Chapter 5

On the Changes in Diastolic Function of Post-ischemic Myocardium Produced by Increases of the Myofibrillar Sensitivity to Calcium in Anesthetized Pigs

Running title: Calcium sensitization and diastolic function.

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Objectives. This study was carried out to investigate whether recovery of regional systolic function of stunned myocardium by increasing sensitivity of the myofibrils to Ca^{2+} was accompanied by impairment of diastolic function.

Background. The clinical usefulness of Ca^{2+} sensitizing agents has been questioned because the beneficial effect on systolic function might be offset by an adverse effect on diastolic function. The latter is feasible because in stunned myocardium and during other pathological conditions an increase in diastolic Ca^{2+} levels can occur, which in combination with an increased sensitivity of the myofibrils to Ca^{2+} could impair left ventricular relaxation and left ventricular filling.

Methods. In anesthetized pigs myocardial stunning was produced by 2 sequences of a 10 min coronary artery occlusion and 30 min of reperfusion. The animals were then allocated to one of four groups and received either 2 volumes (3 and 6 ml) of saline at 15 min intervals (control, n=7), two doses (1.5 and 3.0 mg/kg) of the calcium sensitising agent EMD 60263 (n=8) or its enantiomer EMD 60264 (n=6) which lacks calcium sensitising properties, or two infusions (1 and 3 µg/kg per min) of dobutamine (n=7). Regional myocardial perfusion was measured with radioactive microspheres and segment length changes were examined with two pairs of ultrasound crystals implanted in the stunned and remote normal not stunned myocardium.

Results. The stunning protocol reduced systolic segment shortening (SS) of the post ischemic myocardium from 17±1 % at baseline to 9±1 %, while SS of the normal myocardium remained unaffected (14±1 % at baseline). Saline or EMD 60264 did not improve the depressed systolic function of stunned myocardium. EMD 60263 dose-dependently increased SS to 24±2 % in the stunned region and to 22±1 % in the normal region. EMD 60263 not only abolished the postsystolic segment shortening induced by the stunning protocol, but also delayed the onset diastolic of segment lengthening (SL, 210 ± 40 ms for both regions compared to 0 ms for saline). These changes in regional wall motion were accompanied by a decrease in heart rate from 100±4 beats/min during stunning to 54±5 beats/min and an increase in stroke volume from 27±1 ml to 45±3 ml. To exclude bradycardia as a cause for these changes hearts were paced to restore heart rate to stunning level. Atrial pacing at stunning level (HR_{stun}) decreased SS in both regions to baseline levels. Furthermore, HR_{stun} decreased the delay in onset of SL in the stunned region, but did not effect that of the normal region. Increasing heart rate to 30 beats/min above stunning level further deteriorated SS and increased the delay in onset of SL. The impairment of early SL was reflected in a decrease in stroke volume by 39±7 % ($P<.05$ versus stunning). In contrast dobutamine at similar heart rates increased SS in both regions, but did not delay the onset of SL or impair stroke volume.

Conclusion. Increasing Ca^{2+} sensitivity of the myofibrils in stunned myocardium in excess of that needed to restore systolic segment shortening is accompanied by similar increases in systolic shortening in normal myocardium. At this high dose the onset of relaxation is delayed in both stunned and remote normal myocardium, while the rate of relaxation was not affected. The data suggest that, at least in this model, of EMD 60263 impairs diastolic function only at doses that are in excess of doses needed to restore systolic function.

Key Words • Ca^{2+} sensitizers • systemic hemodynamics • diastolic myocardial function • myocardial oxygen consumption

A decreased sensitivity of the myofibrils to calcium has been reported to underlie the depressed contractile function of stunned myocardium. Consequently, we previously investigated the effects of the calcium sensitizer EMD 60263 (a thiadiazinone derivate) on contractile function in anesthetized pigs and observed that EMD 60263 preferentially increased systolic segment shortening and mechanical efficiency of stunned myocardium versus that of remote normal (not stunned) myocardium.[1] We also observed in that study that the effects of EMD 60263 were unmitigated in the presence non-selective α- and β-adrenoceptor blockade, indicating that the increased regional contractile function could not be attributed to increased Ca^{2+} sensitivity via α-adrenergic stimulation or increased cAMP production via phosphodiesterase inhibition (a property of the calcium sensitizer pimobendan).

Despite the beneficial effects of an increased Ca^{2+} sensitivity on systolic contractile function the therapeutic potential of Ca^{2+} sensitizing agents has been questioned, because it might concomitantly produce adverse effects on diastolic function. For instance, increased diastolic intracellular Ca^{2+} levels, which have been observed both in diseased human hearts[2] and stunned myocardium,[3,4] could in combination with an increased Ca^{2+} sensitivity of the myofibrils impair left ventricular relaxation and filling resulting in a reduction in stroke volume.

In a porcine model of myocardial stunning[1,5,6] we investigated whether regional diastolic function of stunned and remote normal myocardium is impaired by increases in responsiveness of the myofibrils to Ca^{2+}. Although in a previous study a dose of 1.5 mg/kg of EMD 60263 was able to restore systolic contractile function in stunned myocardium, we did not observe an increase in end-diastolic pressure, a decrease in end-diastolic segment length or a decrease in stroke volume, that would indicate a disturbance in relaxation.[1] These findings suggest that the dose was either too low to impair diastolic function or that the concomitant bradycardia may have concealed disturbances in diastolic function. In the present study we therefore not only administered EMD 60263 in a dose that was in excess of that required to normalize systolic function of the stunned myocardium, but also restored heart rate back to pre-drug levels. Finally, we compared the effects of EMD 60263 on diastolic function to that of equipotent doses (with respect to systolic function) of the β-adrenergic receptor agonist dobutamine both at spontaneous and matched heart rates.

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 Council of the American Physiological Society and with the prior approval of the Animal Care Committee of the Erasmus University Rotterdam, Rotterdam, The Netherlands.

Experimental groups

Four groups of animals were studied (Fig. 1). After myocardial stunning was produced the animals received either two doses of saline (control group), two doses of EMD 60263 or its enantiomer EMD 60264, or two infusions of dobutamine. The effects of EMD 60264 were studied because it lacks the Ca^{2+} sensitising properties of EMD 60263 but shares its inhibitory action on the delayed rectifier K^+ current (I_{Kr}), which contributes to the negative chronotropic effects of EMD 60263.[1] The actions of EMD 60263 were compared to dobutamine as this drug enhances contractility by elevating cyclic AMP levels. The latter not only leads to an increase in Ca^{2+} transients, but also decreases the sensitivity of myofibrils to Ca^{2+} which acts to enhance diastolic relaxation.

Surgical Preparation

Overnight fasted, cross-bred Landrace x Yorkshire pigs of either sex (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). Then, the animals 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 controlling respiratory rate and tidal volume. Fluid-filled catheters were positioned in the superior caval vein for administration of haemaccel (Behringwerke A.G., Marburg, FRG) for replacing blood withdrawn during sampling and in the descending aorta for monitoring the aortic blood pressure. A micromanometer-tipped catheter (B. Braun Medical B.V., Uden, The Netherlands) was inserted into a carotid artery and positioned in the left ventricle (LV) for recording of LV blood pressure and its first derivative (LVdP/dt).

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. Pacing leads were attached to the right atrial appendage and connected to a pacemaker. An electromagnetic flow probe (Skalar, Delft, The Netherlands) was placed around the ascending aorta for measurement of ascending aortic blood flow. A proximal segment of the left anterior descending coronary artery (LADCA) was dissected out for later placement of an atraumatic clamp, while the vein accompanying the LADCA was cannulated for collection of coronary venous blood draining the LADCA perfused myocardial region.

Regional myocardial segment shortening was measured by sonomicrometry (Triton Technology Inc., San Diego, CA, USA) with one pair of ultrasonic crystals inserted within the distribution territory of the LADCA and another pair inside the distribution territory of the left circumflex coronary artery (LCXCA). Crystals were positioned in the midmyocardial layer approximately 10 mm apart.

Regional myocardial blood flows were determined in all groups but the EMD 60264 group by injecting 1-2x10^6 radioactive microspheres [15 ± 1 μm (SD) in diameter] labeled with either ^{46}Sc, ^{95}Nb, ^{103}Ru, ^{113}Sn or ^{141}Ce (NEN Company; Dreieich, FRG) into the left atrial appendage

using the arterial reference sampling techniques for calibration. At the end of each experiment the remote normal myocardium was identified by left atrial injection of patent blue violet (Sigma, St. Louis, MO, USA) after ligation of the LADCA at the site of occlusion. After the animals were killed with an overdose of pentobarbital the heart was excised and the LV handled as described earlier in order to obtain regional myocardial blood flow data.[8]

Experimental Protocols

After hemodynamic variables had been stable for at least 30 min following completion of the instrumentation, baseline values of systemic hemodynamics and regional myocardial function were recorded, arterial and coronary venous blood samples collected for determination of hemoglobin, oxygen saturation and blood gas values, and a batch of radioactive microspheres was injected into the left atrium for determination of regional myocardial blood flows. The myocardium in the territory of the LADCA was then stunned by two sequences of a 10 min proximal LADCA occlusion and 30 min of reperfusion. At the end of the second 30 min reperfusion period animals were allocated to the four different groups (Fig. 1). Seven animals served as control and received two infusions of saline (3 and 6 ml/min over 3 min) at 15 min intervals, while two groups of animals received two infusions (0.5 and 1.0 mg/kg over 3 min, total doses of 1.5 and 3.0 mg/kg, respectively) of either EMD 60263 (n=8) or enantiomer EMD 60264 (n=6). Because of the bradycardia produced by EMD 60263 the measurements after the highest dose were repeated after heart rate was restored to levels recorded after stunning was produced (HR_{st}) and after increasing heart rate to 30 beats/min above the heart rate measured at stunning (HR_{st+30}). This last set of data facilitated comparison with the data obtained in group four, in which animals received two consecutive dobutamine infusions at rates of 1 and 3µg/kg

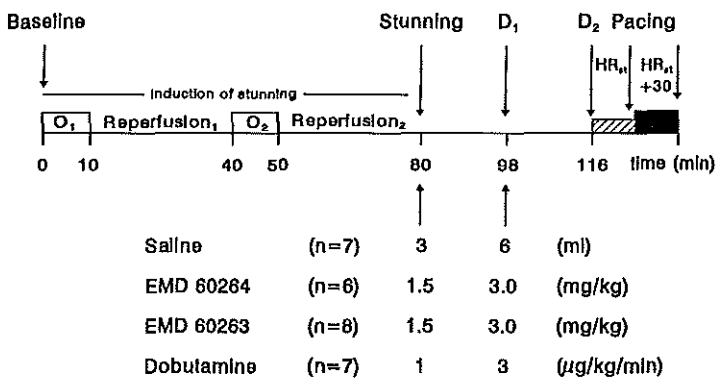

Fig 4. Schematic presentation of the experimental protocols. Infusion of the low dose was started after the measurements at stunning were obtained (t=80 min). After the effects of the low dose were measured at D_1 (t=90 min) the second dose was administered. Following measurement of the effects of the second dose at D_2,(t=116 min) heart rate was increased by atrial pacing in the animals which received saline (control), EMD 60263 or EMD 60264 in order to compare the results to those obtained at stunning and with dobutamine at comparable heart rate.

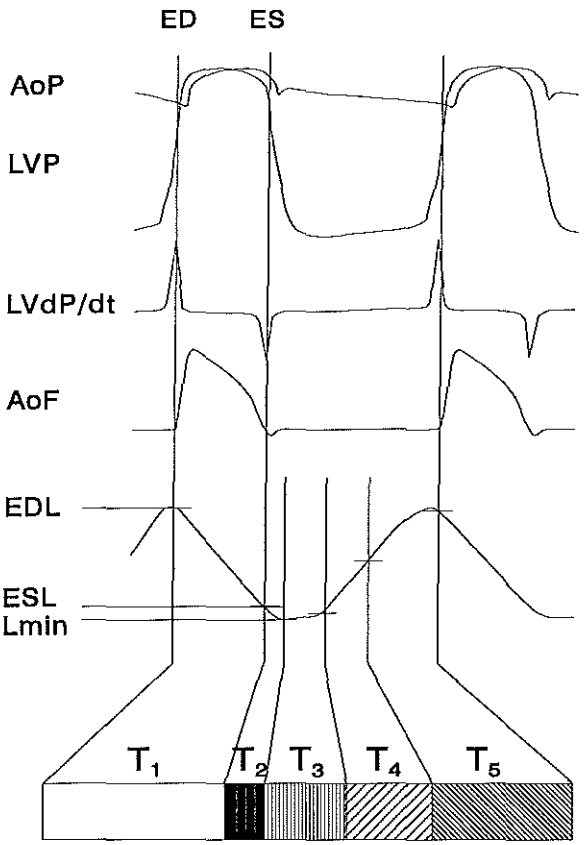

Fig 4. Definition of the time intervals measured from the segment length tracings. T_1 and T_2, duration of ejection and post ejection shortening, respectively; T_3, the time interval between maximum shortening and the onset of segment lengthening; T_4, the time interval in which 50% of segment legnthening is achieved. T_5, the time interval from 50% of relaxation. ED (end-diastole) and ES (end-systole) were defined at the opening and closure of the aortic valve, respectively. AoP, aortic pressure; LVP, left ventricular pressure; LVdP/dt, left ventricular dP/dt; AoF aortic blood flow; EDL, end-diastolic length; ESL, end-systolic length.

per min, with each infusion lasting 15 min. The increase in heart rate by the high dose of dobutamine was approximately 30 beats/min.

Drugs

EMD 60263 and EMD 60264 (courtesy of Prof. P. Schelling, E Merck Darmstadt) and dobutamine (Dobutrex, Eli Lilly Nederland B.V. Nieuwegein, The Netherlands) were dissolved in physiological saline. Fresh solutions were prepared on the day of each experiment.

Data analysis and presentation

Figure 2 is a presentation of systemic hemodynamic and segment length recordings. Systolic

segment shortening (SS) was computed as 100% x (EDL-ESL)/EDL, in which EDL and ESL are the segment length at the time of opening and closing of the aortic valves, respectively. The systolic ejection period was defined as T_1, so that the velocity of segment shortening was computed as the mean velocity during the systolic ejection phase (EDL-ESL/T_1). Post-systolic segment shortening (PSS) was calculated as 100% x (ESL-L_{min}/EDL), in which L_{min} is the minimum segment length after closure of the aortic valves. To describe in greater detail the segment dynamics during diastole we divided diastole into four periods and measured segment length changes during these four phases of diastole defined as T_2-T_5. T_2 describes the period between the end of systole and the occurrence of L_{min}, while T_3 was defined as the interval between the occurrence of L_{min} and onset of rapid segment lengthening. The latter is defined as the instant at which the rate of segment lengthening increased sharply (Fig. 2). T_4 was defined as the time between the onset of rapid segment lengthening and 50% of total diastolic segment lengthening, while the remainder of diastole was defined as T_5. Mean velocity of relaxation was computed for the period needed to achieve 50% of segment lengthening (ΔSL during T_3+T_4 divided by the duration of T_3+T_4). The area inside the LV pressure-segment length was determined as described earlier and used as an index of external work (EW).[9,10]

Myocardial oxygen consumption (MVO_2) of the perfusion territory of the LADCA was computed as the product of regional mean transmural myocardial blood flow and the difference in the oxygen contents of the arterial and coronary venous blood.[11] Mechanical efficiency, defined as the ratio of the regional external work and regional myocardial oxygen consumption (EW/MVO_2), could only be determined for the distribution territory of the LADCA as oxygen consumption of the distribution area of the LCXCA was not measured. All mechanical efficiency data have been normalized to their respective baseline values.

All data are presented as mean ± SEM. Intragroup comparisons were made with Duncan's new-multiple range-test once analysis of variance (randomized block design) had revealed that the samples represented different populations. Intergroup comparisons (comparing responses to treatment in time) were made using two way ANOVA followed by unpaired t-test. Statistical significance was accepted for $P<.05$ (two-tailed).

Results

Systemic hemodynamics (Table 1)

The occlusion - reperfusion protocol produced a slight drop in mean arterial blood pressure (6 ± 3%, $P<.05$), secondary to a decrease in cardiac output (15 ± 4%, $P<.05$) and stroke volume (11 ± 6%). This decrease in cardiac output and stroke volume was primarily caused by the decrease in global LV contractility, reflected by the 21 ± 5% decrease in $LVdP/dt_{max}$ ($P<.05$), as heart rate, LV systolic and LV end-diastolic pressure did not change.

Subsequent infusion of saline did not lead to further changes in any of the systemic

Table 1. Hemodynamic Changes produced by Intravenous Infusions of Saline (Control, n=7), EMD 60264 (n=6), EMD 60263 (n=8) and Dobutamine (n=7) in Anesthetized Pigs with Stunned Myocardium.

		Baseline	Stunning	D_1	D_2	Pacing	Pacing +30
CO (liters/min)	Control	2.8 ± 0.2	2.4 ± 0.1*	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*	1.9 ± 0.1*	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*#	2.5 ± 0.2	2.2 ± 0.3*
	Dobutamine	3.0 ± 0.4	2.5 ± 0.2	2.7 ± 0.1	3.1 ± 0.2*#		
HR (beats/min)	Control	107 ± 2	104 ± 7	105 ± 6	102 ± 6	106 ± 4	138 ± 6*
	EMD 60264	108 ± 4	109 ± 4	91 ± 3*	80 ± 5*	108 ± 4	
	EMD 60263	109 ± 5	100 ± 4	73 ± 5*#	54 ± 5*#	100 ± 4	130 ± 5*
	Dobutamine	111 ± 2	109 ± 4	115 ± 4	133 ± 4*#		
LVSP (mm Hg)	Control	111 ± 3	104 ± 4	104 ± 3	108 ± 3	109 ± 5	104 ± 3
	EMD 60264	111 ± 4	102 ± 3	93 ± 4	79 ± 3*	74 ± 5*	
	EMD 60263	113 ± 2	108 ± 3	106 ± 3	109 ± 4	103 ± 8*	86 ± 6*#
	Dobutamine	109 ± 2	106 ± 2	110 ± 2	111 ± 2		
LVEDP (mm Hg)	Control	9 ± 1	9 ± 1	9 ± 1	10 ± 1	8 ± 1	7 ± 1*
	EMD 60264	7 ± 1	6 ± 1	7 ± 1	8 ± 1	7 ± 1	
	EMD 60263	10 ± 1	10 ± 1	11 ± 1*	14 ± 1*#	10 ± 1	13 ± 2
	Dobutamine	10 ± 2	10 ± 1	9 ± 1	8 ± 1*#		
$LVdP/dt_{max}$ (mm Hg/s)	Control	2110 ± 190	1660 ± 170*	1670 ± 160	1700 ± 150	1660 ± 140	1790 ± 160
	EMD 60264	2030 ± 240	1560 ± 120*	1470 ± 140*	1210 ± 100*+	1120 ± 110*+	
	EMD 60263	2130 ± 130	1640 ± 120*	1620 ± 130*	1720 ± 130*	1870 ± 120	1670 ± 160*
	Dobutamine	2610 ± 190	1940 ± 160*	2790 ± 130*#	4180 ± 190*#		
$LVdP/dt_{min}$ (mm Hg/s)	Control	-2120 ± 150	-1780 ± 210*	-1730 ± 150	-1930 ± 300	-2010 ± 300	-2230 ± 260
	EMD 60264	-2450 ± 350	-2060 ± 310*	-1240 ± 140+	-870 ± 70+	- 870 ± 90+	
	EMD 60263	-2110 ± 90	-1740 ± 80*	-1140 ± 120+	-890 ± 110+	-1220 ± 130+	-1320 ± 160
	Dobutamine	-1920 ± 130	-1560 ± 120*	-1820 ± 120*	-2400 ± 330		
DAP (mm Hg)	Control	79 ± 3	75 ± 4	74 ± 2	76 ± 2	79 ± 3	80 ± 3+
	EMD 60264	78 ± 4	74 ± 2	63 ± 3+	49 ± 2+	50 ± 3+	
	EMD 60263	82 ± 3	78 ± 3	67 ± 3+#	61 ± 3+#	75 ± 7	68 ± 5#
	Dobutamine	81 ± 1	80 ± 1	81 ± 2	79 ± 2		
MAP (mm Hg)	Control	89 ± 3	84 ± 4	83 ± 2	86 ± 2	89 ± 3	87 ± 3
	EMD 60264	92 ± 5	85 ± 3	75 ± 4+	61 ± 4+	61 ± 5+	
	EMD 60263	91 ± 2	87 ± 3	79 ± 3+*	77 ± 3+*†	84 ± 7	73 ± 6#
	Dobutamine	90 ± 1	88 ± 1	91 ± 2	90 ± 2		
SV (ml)	Control	27 ± 2	23 ± 1	23 ± 1	24 ± 1	22 ± 1	17 ± 1+
	EMD 60264	24 ± 1	19 ± 2	21 ± 1	24 ± 1+	17 ± 2	
	EMD 60263	27 ± 1	27 ± 1	34 ± 2+*	45 ± 3+*	25 ± 2	17 ± 2+*
	Dobutamine	27 ± 3	23 ± 2	24 ± 2	24 ± 2		
SVR ($dynes.s/cm^5$)	Control	2640 ± 240	2880 ± 160	2800 ± 160	2880 ± 240	3200 ± 400	3040 ± 320
	EMD 60264	2960 ± 240	3360 ± 160	3280 ± 240	2640 ± 240+	2880 ± 240	
	EMD 60263	2560 ± 160	2720 ± 240	2720 ± 240	2720 ± 240*	2880 ± 320	2960 ± 320
	Dobutamine	2560 ± 320	2960 ± 320	2720 ± 160	2320 ± 80+		

CO=cardiac output; HR=heart rate; LVSP and LVEDP=left systolic and end-diastolic pressure, respectively; $LVdP/dt_{max}$ and $LVdP/dt_{min}$ = maximal rate of rise and fall of left ventricular pressure, respectively; DAP=diastolic arterial blood pressure; MAP=mean arterial blood pressure; SV=stroke volume; SVR=systemic vascular resistance; D_1 and D_2 =3 ml and 6 ml of saline for control, 1.5 mg/kg and 3.0 mg/kg for EMD 60264 and EMD 60263 and 1 μg/kg per min and 3 μg/kg per min for dobutamine, respectively.Pacing is pacing at a heart rate similar to the heart rate during pacing. Pacing+30 is pacing at a heart rate of 30 beats/min above the heart rate during stunning (which is equal to the heart rate after D_2 in the dobutamine group). Values presented are mean value± SEM. *P <0.05 vs baseline; +P <0.05 vs stunning; # changes vs stunning are significantly different from changes vs stunning in control animals; + changes vs stunning are significantly different from changes vs stunning in the dobutamine group. † changes vs stunning are significantly different from changes vs stunning in the EMD 60264 group.

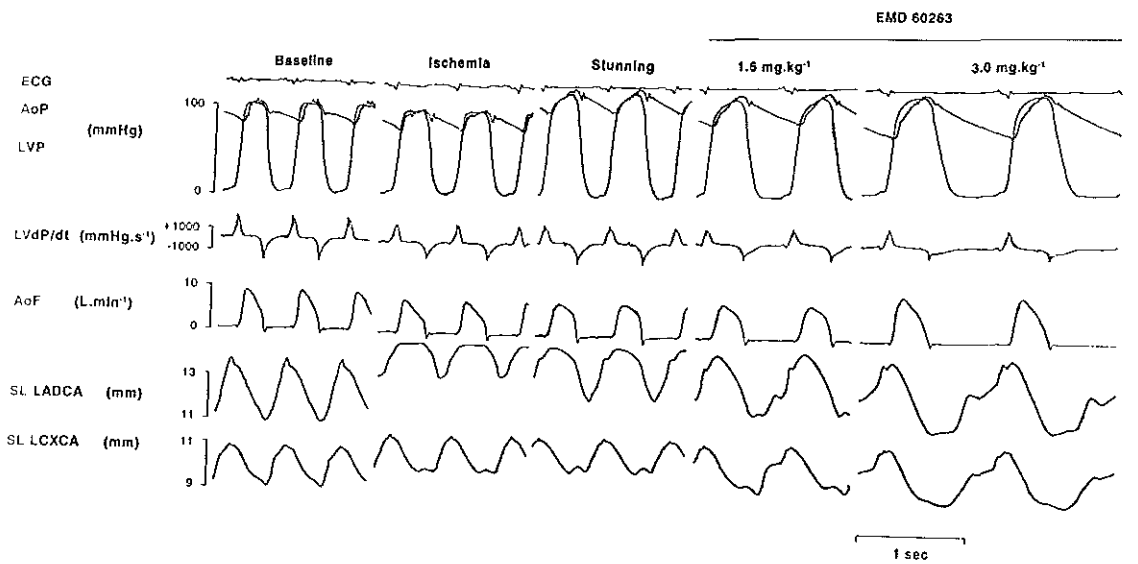

Fig 3. This recording of a representative experiment with EMD 60263 shows that the depressed systolic segment shortening recovered already during infusion of the low dose of EMD 60263 and that in both the stunned (LADCA) and not stunned regions (LCXCA) the onset of segment lengthening was delayed after administration of the high 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.

hemodynamic variables demonstrating excellent hemodynamic stability of the experimental preparation. Atrial pacing to increase heart rate by 30 beats/min (HR_{st+30}) caused a 2±1 mmHg decrease in LV end-diastolic pressure ($P<.05$ vs stunning) and a 27±3% decrease in stroke volume ($P<.05$ vs stunning).

EMD 60264 caused dose-dependent decreases in heart rate, aortic blood pressure, $LVdP/dt_{max}$ and cardiac output. Following administration of the high dose, the decrease in heart rate (26 ± 7%) exceeded that in cardiac output (10 ± 5%) so that stroke volume increased by 25±8% ($P<.05$). The high dose also decreased systemic vascular resistance (21±7%, $P<.05$), but had no effect on LV end-diastolic pressure. Atrial pacing to restore heart rate to HR_{st} had no effect on any of the global hemodynamic variables except for stroke volume which decreased to stunning levels. In these animals heart rate could not be increased to HR_{st+30} due to atrioventricular block.

EMD 60263 produced dose-dependent decreases in heart rate (up to 46 ± 3% of the value obtained after production of stunning), mean and diastolic arterial blood pressure (12 ± 4% and 22 ± 3%, respectively), and cardiac output (10 ± 4%), while stroke volume increased by 69±7% ($P<.05$). LV systolic blood pressure, systemic vascular resistance and $LVdP/dt_{max}$ remained unchanged, while the maximal rate of fall in LV pressure decreased ($LVdP/dt_{min}$) from -1740 ± 80 mmHg s^{-1} to -890 ± 110 mmHg s^{-1} ($P<.05$) and LV end-diastolic pressure increased from 10 ± 1 mmHg to 14 ± 1 mmHg ($P<.05$). Atrial pacing to increase heart rate to HR_{st} resulted in a

Table 2. Changes in Regional Post-systolic Shortening produced by Intravenous Infusions of Saline (Control), EMD 60264, EMD 60263 and Dobutamine in Anesthetized Pigs with Stunned Myocardium.

		Baseline	Stunning	D_1	D_2	Pacing	Pacing +30
LADCA							
EDL (mm)	Control	11.0 ± 0.7	12.0 ± 0.8*	11.8 ± 0.7+	11.8 ± 0.7	11.4 ± 0.7+	10.9 ± 0.7+
	EMD 60264	9.8 ± 0.9	10.7 ± 1.1*	10.8 ± 1.1	10.7 ± 1.1	10.4 ± 1.1+	
	EMD 60263	12.0 ± 1.2	13.0 ± 1.4*	12.8 ± 1.3	12.6 ± 1.3+	11.7 ± 1.2+	10.8 ± 1.1+
	Dobutamine	11.2 ± 0.6	12.9 ± 0.8*	11.9 ± 0.8+	11.2 ± 0.6+		
SS(%)	Control	17 ± 1	8 ± 1*	8 ± 1	9 ± 2	9 ± 2	6 ± 2
	EMD 60264	19 ± 1	8 ± 1	7 ± 1	6 ± 2	4 ± 3	
	EMD 60263	17 ± 1	9 ± 1*	15 ± 1*#	24 ± 2*#	17 ± 1+	13 ± 1
	Dobutamine	21 ± 2	10 ± 2	15 ± 2*#	17 ± 2*#		
PSS (%)	Control	3.1 ± 0.9	7.2 ± 0.8*	6.2 ± 0.8	5.6 ±1.1	5.1 ± 0.7+	4.9 ± 0.6+
	EMD 60264	1.5 ± 0.6	9.0 ± 1.1*	9.0 ± 1.2	7.3 ± 1.1	7.2 ± 1.4	
	EMD 60263	1.3 ± 0.4	7.0 ± 0.8*	3.2 ± 0.4*#†	0.5 ± 0.2*#†	0.8 ± 0.3*#†	0.3 ± 0.2*#
	Dobutamine	2.3 ± 1.0	8.6 ± 0.7*	4.4 ± 0.8*#†	2.0 ± 0.5*#†		
V_{ss}(mm/s)	Control	6.9 ± 0.5	3.3 ± 0.7*	3.6 ± 0.7	3.7 ± 0.8	3.6 ± 0.7	2.8 ± 0.9
	EMD 60264	7.0 ± 0.9	3.2 ± 0.6*	3.0 ± 0.8	2.3 ± 0.8	1.3 ± 1.4	
	EMD 60263	8.2 ± 1.5	4.3 ± 1.1*	6.0 ± 1.0+	7.8 ± 1.4+	7.0 ± 1.4+	5.4 ± 0.7
	Dobutamine	5.2 ± 0.2	4.4 ± 0.2*	5.2 ± 0.2+	6.2 ± 0.2+		
V_{SL}(mm/s)	Control	15.8 ± 2.8	18.5 ± 6.2	13.3 ± 3.0	15.6 ± 2.8	13.5 ± 2.8	22.4 ± 4.4+
	EMD 60264	9.8 ± 1.6	10.9 ± 1.3	7.7 ± 1.5+	5.3 ± 1.4+	13.3 ± 2.5	
	EMD 60263	13.4 ± 2.0	12.6 ± 2.5	9.9 ± 3.8	10.2 ± 2.7	15.3 ± 4.6	14.9 ± 4.5
	Dobutamine	14.6 ± 2.9	13.3 ± 2.1	13.0 ± 2.4	13.2 ± 2.5		
LCXCA							
EDL (mm)	Control	9.9 ± 0.8	10.1 ± 0.7	10.0 ± 0.7	10.1 ± 0.8	9.5 ± 0.6	9.2 ± 0.6+
	EMD 60264	10.5 ± 0.8	10.3 ± 0.8	10.4 ± 0.8	10.7 ± 0.8	10.4 ± 0.8	
	EMD 60263	11.4 ± 1.1	11.6 ± 1.2	11.6 ± 1.2	11.5 ± 1.2	10.2 ± 0.9+	9.4 ± 1.0+
	Dobutamine	12.7 ± 0.7	13.2 ± 0.8	12.8 ± 0.7	12.4 ± 0.6+		
SS (%)	Control	12 ± 1	11 ± 2	11 ± 2	11 ± 1	11 ± 2	9 ± 2
	EMD 60264	14 ± 2	13 ± 1	13 ± 1	13 ± 1	12 ± 2	
	EMD 60263	14 ± 1	14 ± 1	17 ± 1*#	22 ± 1*#	12 ± 1	8 ± 1+
	Dobutamine	16 ± 2	13 ± 2	15 ± 2	15 ± 2+		
PSS (%)	Control	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	
	EMD 60263	2.1 ± 0.5	1.5 ± 0.7	0.6 ± 0.4+	0.5 ± 0.3	0.8 ± 0.4	0.6 ± 0.2
	Dobutamine	1.1 ± 0.2	2.4 ± 0.4+	1.3 ± 0.4+	1.3 ± 0.2+		
V_{ss}(mm/s)	Control	4.4 ± 0.3	4.1 ± 0.8	3.9 ± 0.6	4.1 ± 0.7	3.8 ± 0.8	3.4 ± 0.8
	EMD 60264	5.9 ± 0.8	5.3 ± 0.7	5.0 ± 0.7	4.9 ± 0.7	4.7 ± 0.7+	
	EMD 60263	5.9 ± 0.9	5.5 ± 1.0	5.8 ± 0.7	6.2 ± 0.9	4.0 ± 0.4	2.9 ± 0.4
	Dobutamine	8.3 ± 1.3	6.6 ± 1.3	8.2 ± 1.4	9.6 ± 1.5+		
V_{SL}(mm/s)	Control	6.2 ± 1.0	7.3 ± 1.4	6.8 ± 1.1	8.0 ± 1.6	7.4 ± 1.2	9.7 ± 2.3
	EMD 60264	11.2 ± 2.7	7.1 ± 1.0	8.3 ± 1.9	6.7 ± 1.6	6.6 ± 1.5	
	EMD 60263	10.2 ± 1.2	9.6 ± 1.2	13.4 ± 3.2	7.3 ± 1.6	10.6 ± 1.7	13.9 ± 6.2
	Dobutamine	8.3 ± 1.5	10.4 ± 1.5	9.3 ± 1.4	13.7 ± 3.4		

LADCA=left anterior descending coronary artery; LCXCA=left circumflex coronary artery; EDL= end-diastolic segment length; SS=systolic segment shortening; PSS=post-systolic segment shortening; V_{ss}=mean velocity of systolic segment shortening; V_{SL}=mean velocity of segment shortening between the onset of rapid segment lengthening and 50% of total diastolic segment lenthening. D_1 and D_2 are 3 ml and 6 ml saline for the control group, 1.5 mg/kg and 3 mg/kg for the EMD 60263 and 1 μg/kg/min and 3 μg/kg/min for the dobutamine group, respectively. Pacing is pacing at heart rate similar to the heart rate during pacing; Pacing+30 is pacing at a heart rate of 30 beats/min above the heart rate during stunning (which is equal to the heart rate after D_2 in the dobutamine group).

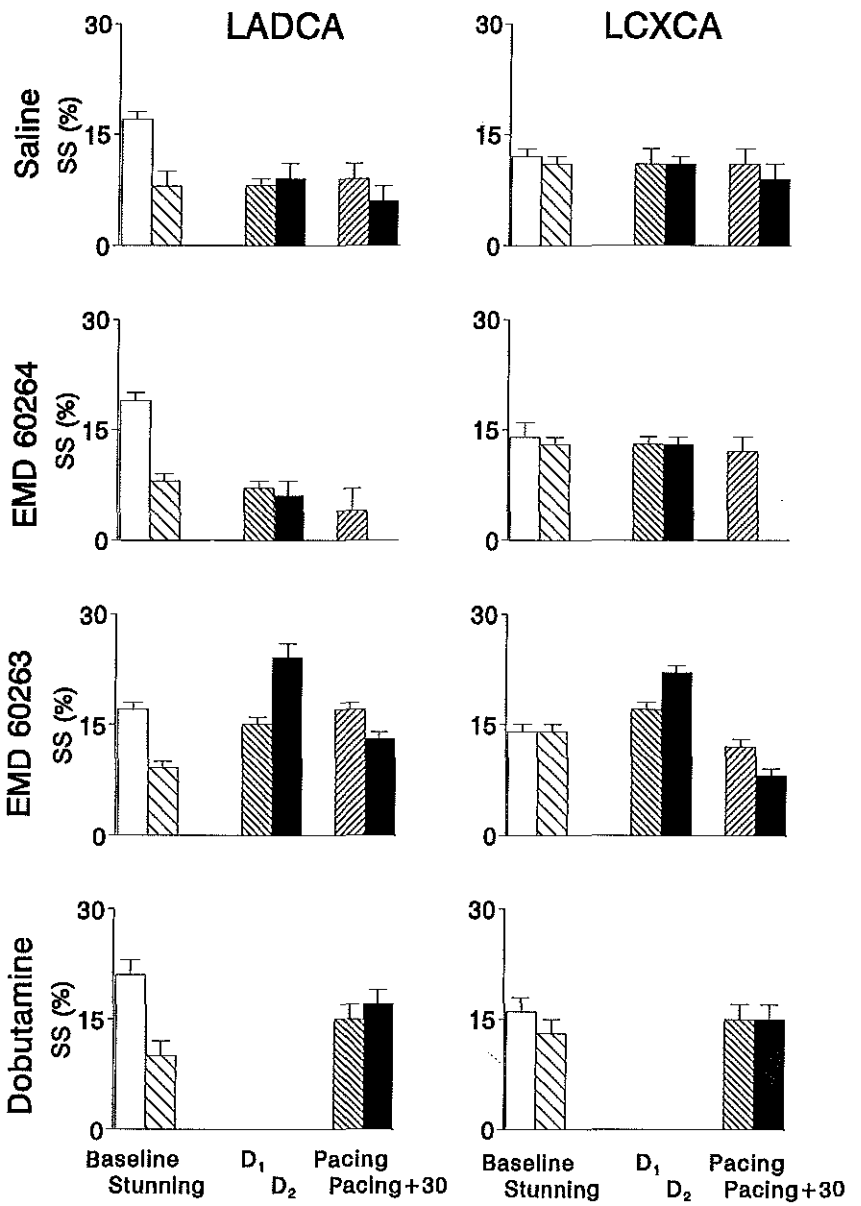

Fig 4. Effect of saline, EMD 60264, EMD 60263 and dobutamine on systolic segment shortening (SS) of stunned (perfused by the left anterior descending coronary artery, LADCA) and not stunned (perfused by the left circumflex coronary artery, LCXCA) myocardium. EMD 60264 (n=6) and EMD 60263 (n=8) were administered in doses of 1.5 mg/kg (D_1) and 3.0 mg/kg (D_2)while dobutamine (n=7) was indused at rates of 1 µg/kg per min (D_1) and 3 µg/kg per min (D_2). In the control animals (n=7) the saline infusions were 3 ml (D_1) and 6 ml (D_2). All data are mean ± SEM, * P<.05 versus baseline; $^+$ P<.05 versus stunning. P<.05 vs D_2

Table 3. Effects of EMD 60263 and dobutamine on regional myocardial perfusion and oxygen consumption of stunned porcine myocardium.

	Baseline	Stunning	D_1	D_2	D_2 + pacing	Wash-out
Control group (n=7)[1]						
LADCA perfusion territory						
transmural flow (ml/min/100g)	156 ± 6	121 ± 10*	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	
CVR (mmHg/(ml/min/100g))	0.58 ± 0.03	0.71 ± 0.03	0.79 ± 0.08	0.72 ± 0.04	0.74 ± 0.05	
cvO_2 saturation (%)	23 ± 3	25 ± 3	23 ± 4	24 ± 4	23 ± 3	
$\Delta(aO_2$ cont-cvO_2 cont) (μmol/ml)	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)	441 ± 26	353 ±26*	331 ± 29	362 ± 12	353 ± 17	
LCXCA perfusion territory						
transmural flow (ml/min/100g)	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	
CVR (mmHg/(ml/min/100g))	0.54 ± 0.03	0.54 ± 0.04	0.64 ± 0.10	0.59 ± 0.02	0.61 ± 0.05	
EMD 60263 (n=8)[2]						
LADCA perfusion territory						
transmural flow (ml/min/100g)	167 ± 5	122 ± 8*	119 ± 11	118 ± 10	110 ± 17	
endo/epi	1.07 ±0.09	1.16 ± 0.09	1.13 ± 0.09	1.17 ± 0.08	1.07 ± 0.07	
CVR (mmHg/(ml/min/100g))	0.55 ±0.02	0.73 ± 0.04*	0.70 ± 0.05	0.68 ± 0.06	0.75 ± 0.12	
cvO_2 saturation (%)	28 ± 2	29 ± 4	29 ± 4	29 ± 4	23 ± 3	
$\Delta(aO_2$ cont-cvO_2 cont) (μmol/ml)	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)	484 ± 27	328 ± 19*	334 ± 27	334 ± 12	357 ± 35	
LCXCA perfusion territory						
transmural flow (ml/min/100g)	180 ± 7	164 ± 10*	138 ± 13	129 ± 10$^+$	116 ± 17$^+$	
endo/epi	1.08 ± 0.06	0.99 ± 0.05*	0.99 ± 0.04	0.97 ± 0.04	0.79 ± 0.05$^+$	
CVR (mmHg/(ml/min/100g))	0.52 ± 0.03	0.54 ± 0.03	0.60 ± 0.04	0.62 ± 0.05	0.70 ± 0.09$^+$	
Dobutamine (n=7)						
LADCA perfusion territory						
transmural flow (ml/min/100g)	164 ± 16	130 ± 20*	172 ± 19	188 ± 12$^+$		130 ± 20
endo/epi	1.04 ± 0.06	1.06 ± 0.10	0.92 ± 0.05	0.86 ± 0.06		1.04 ± 0.08
CVR (mmHg/(ml/min/100g))	0.58 ± 0.05	0.78 ± 0.12	0.56 ± 0.05	0.49 ± 0.03$^+$		0.79 ± 0.13
cvO_2 saturation (%)	34 ± 3	34 ± 3	44 ± 3$^+$	40 ± 3$^+$		31 ± 2
$\Delta(aO_2$ cont-cvO_2 cont) (μmol/ml)	2.60 ± 0.21	2.58 ± 0.18	2.37 ± 0.17	2.36 ± 0.16		2.91 ± 0.13
MVO_2 (μmol/min/100g)	412 ± 27	323 ± 37	410 ± 33	440 ± 37$^+$		380 ± 68
LCXCA perfusion territory						
transmural flow (ml/min/100g)	169 ± 19	186 ± 31	221 ± 26	237 ± 18$^+$		170 ± 28
endo/epi	0.99 ± 0.05	0.82 ± 0.14	0.80 ± 0.10	0.78 ± 0.08		0.80 ± 0.08
CVR (mmHg/(ml/min/100g))	0.58 ± 0.07	0.56 ± 0.09	0.46 ± 0.07	0.39 ± 0.04$^+$		0.59 ± 0.08

LADCA=left anterior descending coronary artery; LCXCA=left circumflex coronary artery; D_1 and D_2 are 3 ml and 6 ml saline for the control group, 1.5 mg/kg and 3 mg/kg for the EMD 60263 and 1 μg/kg/min and 3 μg/kg/min for the dobutamine group, respectively. D_2 + pacing is pacing at a heart rate of 30 beats/min above the heart rate during stunning (which is equal to the heart rate after D_2 in the dobutamine group); CVR=coronary vascular resistance; cvO_2 saturation=coronary venouw O_2 saturation in the great cardiac vein draining the LADCA perfusion territory; $\Delta(aO_2$ cont-cvO_2 cont)=difference in the O_2 content of arterial and coronary venous blood; MVO_2=O_2 consumption; (n)=number of observations; All data are mean ± SEM; *$P<.05$ vs baseline; $^+P<.05$ vs stunning; [1]) n=6 for D_2 + pacing; [2]) n=7 for D_2 + pacing.

slight increase in LV systolic pressure ($P<.05$ vs high dose of EMD 60263 and stunning) and normalization of both LV end-diastolic pressure and stroke volume. $LVdP/dt_{min}$, recovered to -1120 ± 130 mmgHg.s^{-1} but remained well below the levels observed during stunning. Increasing heart rate to $HR_{stun+30}$ caused a deterioration of all global hemodynamic variables except for $LVdP/dt_{min}$ which was maintained.

Infusions of dobutamine produced dose-dependent increases in heart rate (23 ± 5%), $LVdP/dt_{max}$ (125 ± 22%), $LVdP/dt_{min}$ (60 ± 25%) and cardiac output (28 ± 10%), while arterial blood pressure remained unchanged. From these data it follows that a lowering of the systemic vascular resistance (18 ± 5%) prevented an increase in aortic blood pressure and that the increase in cardiac output was caused by the increase in heart rate. Stroke volume was maintained despite the increase in contractility which was possibly due to the decrease in left ventricular end-diastolic pressure from 10 ± 1 mmHg to 8 ± 1 mmHg ($P<.05$).

Segment length changes

Systolic segment shortening (Table 2, Figs. 3 and 4)

During both 10 min coronary artery occlusions, there was an increase in end-diastolic length (EDL), complete loss of systolic segment shortening (SS) and the appearance of prominent early postsystolic shortening in the distribution area of the LADCA (Fig.3). During the second reperfusion period SS recovered to approximately 50% of baseline, while end-diastolic segment length increased to 10% above baseline and post-systolic segment shortening persisted. EDL and SS in the distribution territory of the LCXCA were not affected by the stunning protocol.

Neither saline nor EMD 60264 had any effect on EDL or SS in the stunned and remote normal myocardium (Fig. 4). In the saline group increasing heart rate to 30 beats/min above stunning levels decreased SS of stunned myocardium ($P<.05$) and tended to decrease SS in the normal myocardium, which was due to a decrease in EDL with no change in ESL. In the animals that received EMD 60264, atrial pacing to increase heart rate to levels observed at stunning also decreased EDL or SS of the stunned myocardium, though the latter failed to reach statistical significance

After infusion of 1.5 mg/kg EMD 60263, SS in the distribution territory of the LADCA returned to baseline values, while in the remote normal segment SS increased only slightly. Following infusion of the high dose of EMD 60263 SS of the LADCA perfused territory increased to 24 ± 2%, which even exceeded the 17 ± 1% ($P<.05$) at baseline, while SS of the normal myocardium in the LCXCA territory increased from 14±1% at stunning to 22±1%. Raising heart rate to baseline values was accompanied by decreases in SS to baseline values in both stunned and normal myocardium, while a further increase to 30 beats/min above baseline caused SS to fall below baseline value, especially in the normal myocardium. The EMD 60263-induced increase in SS was accompanied by a slight decrease in end-diastolic length, which did not reach statistical significance. Atrial pacing further reduced end-diastolic segment length, but this was not different from the reduction observed in the control group.

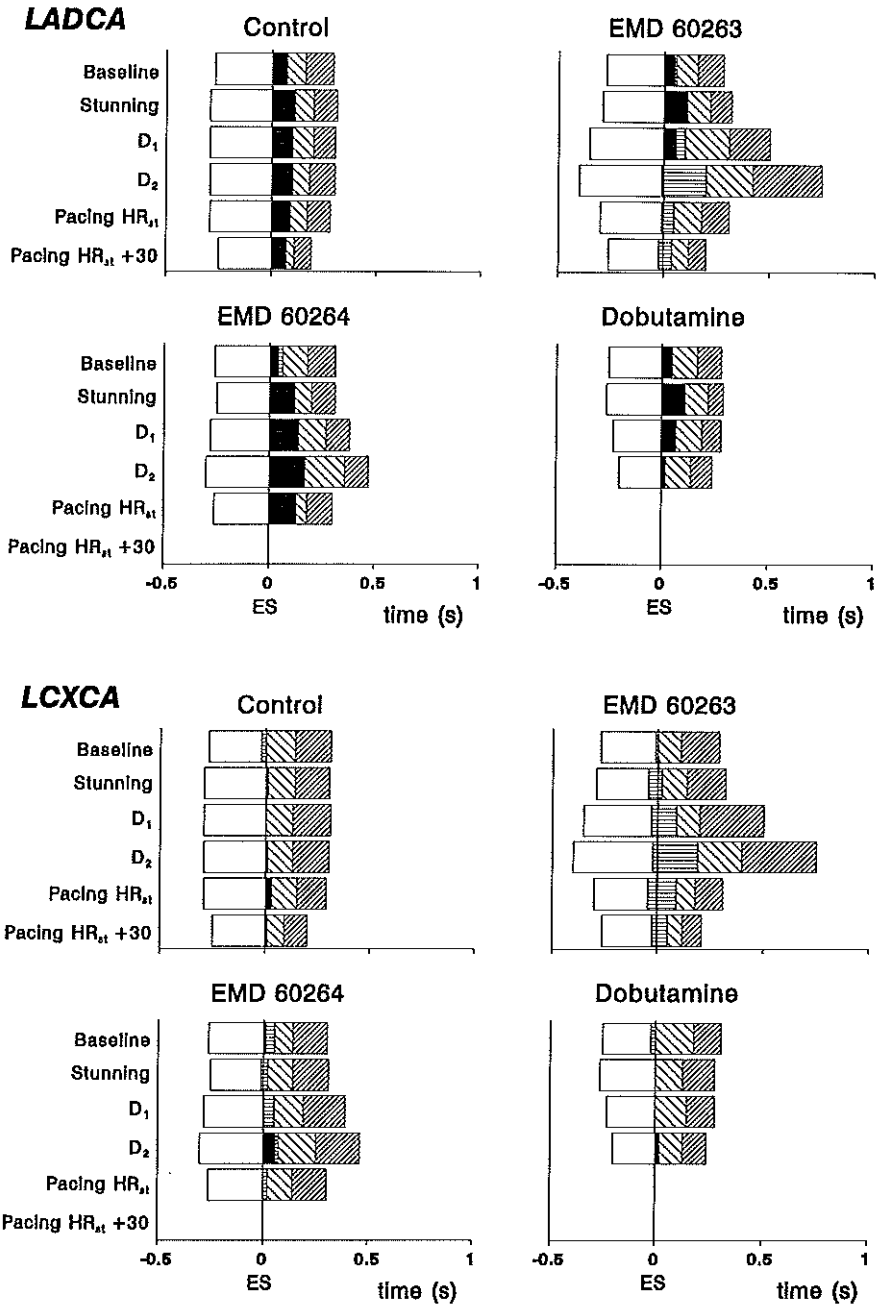

Fig 5. Effect of EMD 60263, EMD 60264 and dobutamine on the different time phases of the segment length changes of stunned (LADCA) and remote normal (LCXCA) myocardium. Notice that the high dose of EMD 60263 delayed the onset of segment lengthening (▤), and that the effect was attenuated when heart rate was increased by atrial pacing. For further details see legends of figures 2 and 4.

Infusion of dobutamine caused dose-dependent increases in the SS of the stunned myocardium, although baseline values were not reached ($P<.05$). In the remote normal region SS was not affected. End-diastolic segment length decreased in both the stunned and the remote normal myocardium.

The mean velocity of segment shortening (V_{SS}) was decreased during stunning in all the experimental groups (Table 2). Infusion of saline had no effect on V_{SS}, while infusion of EMD 60264 tended to decrease V_{SS} in both the stunned and normal myocardium. On the other hand, infusion of EMD 60263 restored V_{SS} to pre-stunning values in the stunned region (98 ± 5% of baseline value), but had no effect on the V_{SS} of the normal (LCXCA) region. Dobutamine also increased V_{SS} and reached levels above baseline level in both stunned and normal myocardium to 121 ± 4% ($P<.05$) and 120 ± 12% (ns) of baseline, respectively.

Post-systolic segment shortening and diastolic relaxation (Table 2, Fig. 5)

Post-systolic shortening in the stunned myocardium was not affected by either saline or EMD 60264, while it was completely abolished after infusion of EMD 60263 or dobutamine. Atrial pacing did not affect post-systolic segment shortening in any of the experimental groups.

In both the saline and the EMD 60264 treated animals the stunned and normal segments started to lengthen immediately after the segments reached their minimum length. EMD 60263 produced a delay in onset of segment shortening, so that T_3 was 210 ± 30 ms ($P<.05$) after administration of the high dose. After the onset of segment lengthening the time needed to reach 50% of diastolic segment lengthening for both myocardial segments was not different from baseline. However, when heart rate was raised to stunning levels the EMD 60263-induced delay in segment lengthening was attenuated in the stunned, but not in the normal myocardial region. A further increment in heart rate to 30 beats/min above stunning levels did not further decrease the delay in segment lengthening in either region. Infusion of dobutamine decreased the period of post-systolic shortening, but did not affect the time between minimum length and onset of segment lengthening.

Regional myocardial perfusion and oxygen consumption (Table 3).

Myocardial stunning decreased transmural blood flow by 23±3% (n=22), which was equally distributed across the left ventricular wall, while coronary vascular resistance increased by 30%. In the control animals there were no further changes after infusion of saline or when heart rate was increased to 30 beats/min above the heart rate observed during stunning. The difference in the arterial and coronary venous O_2 contents remained also unaffected by the stunning protocol. Consequently, oxygen consumption of the stunned myocardium decreased in parallel with transmural blood flow. In the remote normal myocardium mean transmural blood flow and its distribution as well as coronary vascular resistance were not significantly affected.
Administration of EMD 60263 had no effect on coronary blood flow and vascular resistance in the stunned myocardium. Because the difference in the arterial and coronary venous O_2 contents

Table 4. Effect of EMD 60263 and dobutamine on external and mechanical efficiency of stunned porcine myocardium.

	Baseline	Stunning	D_1	D_2	D_2 + pacing
Control group					
LADCA perfusion territory					
EW_{beat} (mmHg.mm)	190 ± 17	95 ± 20*	100 ± 18	108 ± 23	63 ± 18*
$MVO_{2\ beat}$ (µmol/100g)	4.1 ± 0.3	3.4 ± 0.2	3.2 ± 0.3	3.6 ± 0.2	2.6 ± 0.1
ME (%)	100	79 ± 5*	85 ± 6	81 ± 5	79 ± 6
LCXCA perfusion territory					
EW_{beat} (mmHg.mm)	136 ± 10	120 ± 14	115 ± 12	114 ± 14	84 ± 21*
EMD 60263					
LADCA perfusion territory					
EW_{beat} (mmHg.mm)	185 ± 21	84 ± 11*	159 ± 13*	243 ± 21*	89 ± 9
$MVO_{2\ beat}$ (µmol/100g)	4.5 ± 0.3	3.3 ± 0.2*	4.7 ± 0.4*	6.4 ± 0.5*	2.7 ± 0.3
ME (%)	100	84 ± 3*	95 ± 3*	97 ± 4*	89 ± 6
LCXCA perfusion territory					
EW_{beat} (mmHg.mm)	161 ± 16	145 ± 17*	161 ± 12	241 ± 15*	58 ± 8*
Dobutamine					
LADCA perfusion territory					
EW_{beat} (mmHg.mm)	220 ± 22	125 ± 14*	194 ± 17*	206 ± 23*	
$MVO_{2\ beat}$ (µmol/100g)	3.7 ± 0.2	2.9 ± 0.4	3.6 ± 0.3	3.3 ± 0.3	
ME (%)	100	65 ± 10*	90 ± 10	104 ± 16*	
LCXCA perfusion territory					
EW_{beat} (mmHg.mm)	175 ± 25	138 ± 28	160 ± 32	187 ± 26*	

LADCA=left anterior descending coronary artery; LCXCA=left circumflex coronary artery; D_1 and D_2 are 3 ml and 6 ml saline for the control group, 1.5 mg/kg and 3 mg/kg for the EMD 60263 and 1 µg/kg/min and 3 µg/kg/min for the dobutamine group, respectively. D_2 + pacing is pacing at a heart rate of 30 beats/min above the heart rate during stunning (which is equal to the heart rate after D_2 in the dobutamine group); EW=external work; MVO_2=oxygen consumption; ME=mechanical efficiency; all data are mean ± SEM. *P<.05 vs baseline; *P<.05 vs stunning; [1] n=6 for D_2 + pacing.

also remained unaffected, oxygen consumption in these animals paralleled the changes in transmural blood flow. Increasing heart rate to 30 beats/min above stunning levels had no effect on myocardial blood flow, its distribution or oxygen extraction. In the normal segment the endo-epi blood flow ratio decreased from 0.97±0.04 to 0.79±0.05 ($P<.05$) when the pacing rate was increased to 30 beats/min above the heart rate measured following stunning. This was caused by a 75% increase in the vascular resistance in the endocardial layer, while resistance in the epicardial layer was unaffected. Dobutamine caused a dose-dependent increase in transmural blood flow (up to 58 ± 15%) of the stunned myocardium and therefore a decrease in the coronary vascular resistance. Although oxygen extraction decreased (reflected by the increase in the O_2 saturation in the great cardiac vein) myocardial oxygen consumption increased significantly with the highest dose of dobutamine.

Mechanical efficiency (Table 4).

In the distribution territory of the LADCA, mechanical efficiency was decreased to approximately 80% of baseline after producing stunning, a condition which was not affected by the saline infusions in the control group. Administration of EMD 60263 caused dose-dependent increases in mechanical efficiency, which returned to baseline after administration of the higher dose. When the pacing rate was raised by 30 beats/min above baseline there was a trend towards a decrease in mechanical efficiency (p=NS). During administration of dobutamine, mechanical efficiency also recovered dose-dependently.

Discussion

The present study in open-chest pigs demonstrates that high doses of the calcium sensitizer EMD 60263 increased systolic segment shortening in the stunned myocardium more than in the remote normal myocardium. Another major finding was that during diastole EMD 60263 abolished post-systolic shortening in the stunned myocardium. However, the higher dose delayed the onset of relaxation in both stunned and normal segments, although without adversely affecting global left ventricular function. When the hearts were paced to compensate for the EMD 60263-induced bradycardia relaxation normalized. These findings indicate that the delay of relaxation produced by the increased Ca^{2+} sensitivity of the myofilaments, did not exert a detrimental effect on regional and global left ventricular function.

Solaro et al[12] have proposed that the calcium sensitising effects of thiadiazinone derivates is not due to an increased affinity of troponin C to calcium, but due to an increased activity of myofibrillar Mg^{2+}ATPase, which enhances the crossbridge interaction between actin and myosin.[12] This action of thiadiazinone derivates prolongs the duration of the attachment of crossbridges between actin and myosin, leading to an increase in both peak force and duration of the contraction and hence to an increase in the time-force integral of muscle contraction.

Ravens et al[7] recently described similar effects for EMD 60263 in isolated guinea-pig papillary muscle. Our *in vivo* results in this study are consistent with those findings. The increase in segment shortening and mean velocity of systolic segment shortening from stunning may result from the augmentation in peak force and the EMD 60263 induced increase of the duration of contraction could explain the delay in onset of segment lengthening.

The delay in segment lengthening could be interpreted as a disturbance in diastolic relaxation as the high dose also produced an increase in left ventricular end-diastolic pressure. However, the velocity of segment lengthening and the end-diastolic segment length were not decreased by the calcium sensitiser, while stroke volume simultaneously increased to above baseline value. Induction of bradycardia to similar heart rates in an identical model of myocardial stunning with the specific bradycardic agent zatebradine did not induce a delay in onset of segment lengthening. Because at the same time zatebradine also increased left ventricular end-diastolic pressure and stroke volume (though significantly less) without affecting end-diastolic segment length, it is likely that the delay in onset of segment lengthening may be caused by an increase in the duration of contraction.[1]

A disturbance in relaxation would be expected to be most detrimental when the duration of diastole is shortened i.e. at higher heart rates. Thus, to study the physiological significance of the observed delay in segment lengthening we paced the heart to produce heart rates that equaled values measured following stunning and during dobutamine infusion. Atrial pacing to restore heart rate to stunning levels resulted in decreased systolic shortening in both stunned and remote normal myocardium. The effects of atrial pacing in the presence of EMD 60263 on diastolic segment length changes, however, differed between these two regions. Whereas the delay in the onset of segment lengthening (T_3) decreased in the stunned region (ns vs baseline), T_3 remained unaffected in the remote normal myocardium. The different response to pacing between the stunned and remote normal myocardium were unexpected, particular in view of evidence that in normal myocardium pacing can induce an increase in intracellular calcium, both during systole and diastole[13-15] which would tend to enhance the EMD 60263 induced diastolic relaxation abnormalities. On the other hand, Kusuoka et al reported that pacing increases intracellular inorganic phosphate levels which would reduce the myofibrillar sensitivity to calcium.[16] This may explain why despite an increased diastolic intracellular calcium concentration pacing improved relaxation in the stunned myocardium (i.e. reduced the time delay to onset of segment lengthening). The observation that the relaxation delay persisted in the normal area during pacing could be interpreted to suggest that there was still a difference in myofibrillar calcium sensitivity between stunned and remote normal myocardium after administration of EMD 60263.

When we paced the hearts at 30 beats/min above stunning value to compare the effects of the calcium sensitiser with the effects of dobutamine at similar heart rates, stroke volume fell further in the EMD 60263-treated group. The increase in left ventricular end-diastolic pressure and decreases in end-diastolic segment length in both stunned and remote normal myocardium regions (which were slightly larger than in the control group) could be due to the slowing of

relaxation by EMD 60263. Also left ventricular work decreased, which was not accompanied by a fall in myocardial oxygen consumption so that mechanical efficiency decreased. In comparison, dobutamine improved global LV pump function and decreased left ventricular end-diastolic pressure. Dobutamine also decreased end-diastolic segment length, but as it decreased end-systolic segment length even further external work returned to baseline value. Similarly as myocardial oxygen consumption per beat did not alter with dobutamine mechanical efficiency also returned to baseline value. The beneficial effect of dobutamine despite the increase in heart rates is probably due to the lusitropic effect of β-adrenergic stimulation, which stimulates not only the uptake of calcium by the sarcoplasmic reticulum but also desensitises troponin to calcium. This is also supported by the observation that dobutamine did not produce a time delay in the onset of segment lengthening.

Conclusion

Increasing Ca^{2+} sensitivity of the myofibrils in stunned myocardium in excess of that needed to restore systolic segment shortening is accompanied by similar increases in systolic shortening in normal myocardium. At this high dose the onset of relaxation is delayed in both stunned and remote normal myocardium, while the rate of relaxation was not affected. The data suggest that, at least in this model, of EMD 60263 impairs diastolic function *only* at doses that are in excess of doses needed to restore systolic function.

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Chapter 6

The effect of a thiadiazinone derived Ca^{2+} sensitizer on the responsiveness of Mg^{2+}-ATPase to Ca^{2+} in myofibrils isolated from stunned and not-stunned porcine and human myocardium

Running title: Ca^{2+} sensitization of cardiac myofibrillar Mg^{2+}-ATPase

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The effect of the thiadiazinone derived Ca^{2+} sensitizer on the responsiveness of Mg^{2+}-ATPase to Ca^{2+} in myofibrils isolated from stunned and not-stunned porcine and human myocardium

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Previously we showed in an *in situ* porcine model that the thiadiazinone derivative [+]EMD 60263, a putative Ca^{2+} sensitizer with minimal phosphodiesterase III inhibitory properties, increased contractility more profoundly in stunned than in not-stunned myocardium. The aim of the present investigation was to study the mechanism of action by determining the *in vitro* effects of [+]EMD 60263 on the Ca^{2+} responsiveness of the Mg^{2+}-dependent ATPases of myofibrils and sarcoplasmic reticulum membrane vesicles isolated from normal ventricle of swine and hypertrophic septum of cardiomyopathic patients. Contamination of the myofibrils with sarcoplasmic reticulum membranes was excluded by testing the effect of the sarcoplasmic reticulum Ca^{2+} pumping ATPase inhibitor thapsigargin. The plasma concentrations at which [+]EMD 60263 exerted its inotropic effect in the *in situ* porcine model were found to be submicromolar. [+]EMD 60263 stimulated concentration dependently (1-10 µM) the submaximally activated Mg^{2+}-ATPase (at pCa 6.1) of pig heart myofibrils. [+]EMD 60263 (10 µM) shifted the pCa_{50} of porcine myofibrillar Ca^{2+} stimulated, Mg^{2+} dependent ATPase from 6.00 ± 0.05 to 6.67 ± 0.05, whereas the [-]enantiomer EMD 60264 had no significant effect. Although the effect was much less at 1 and 3 µM, [+]EMD 60263 (10 µM) also stimulated the maximal myofibrillar Mg^{2+}-ATPase activity. The Hill coefficient, reflecting the steepness of the fitted pCa/Mg^{2+}-ATPase curves at half-maximal activation were not affected by [+]EMD 60263 (10 µM). [+]EMD 60263 (10 µM) had no effect on sarcoplasmic reticulum Ca^{2+}-stimulated, Mg^{2+}-dependent ATPase from swine heart. The thiadiazinone derivative [+]EMD 57033 (10 µM) but not its [-]enantiomer EMD 57439 had similar, although less potent, effects on the pig heart myofibrillar Mg^{2+}-ATPase activity as compared to [+]EMD 60263. [+]EMD 60263 (3 µM) produced a significantly larger leftward shift of the pCa^{2+}/Mg^{2+}-ATPase activity curve of myofibrils isolated from the stunned compared to the adjacent not-stunned myocardium (ΔpCa_{50}'s caused by the presence of [+]EMD 60263 amounted respectively + 0.57 ± 0.04 and + 0.42 ± 0.05) in the *in situ* porcine model. The effects of [+]EMD 60263 on myofibrillar Mg^{2+}-ATPase of hypertrophic human heart were identical to those observed with porcine heart myofibrils. The results indicate that the positive inotropic action of [+]EMD 60263 observed in the *in situ* porcine model of stunned myocardium, may be primarily due to myofilament sensitization to Ca^{2+} and that this compound may have a similar action on diseased human myocardium. **(J Biochem Pharmacol 1996 in press)**

Abbreviations Sarcoplasmic reticulum, SR; Adenosine-5'-triphosphatase, ATPase; ethylene glycol bis(ß-aminoethylether) N,N,N',N'-tetraacetic acid, EGTA; dithiothreitol, DTT; 4-morpholino-propane sulfonic acid, MOPS; inorganic phosphate, P_i; phenylmethane-sulfonylfluoride, PMSF; phosphodiesterase III, PDE III; left anterior desecending coronary artery, LADCA; left circumflex coronary artery (LCXCA).

Key words • cardiac myofibrils • cardiac sarcoplasmic reticulum • human • pig • Ca^{2+} stimulated, Mg^{2+}-ATPase • thiadiazinone derivatives • stunning

Myocardial contractility can be modulated by two principal mechanisms, *i*) the amplitude of the cytosolic [Ca^{2+}] transient, *ii*) the responsiveness of the myofilaments to Ca^{2+} [1,2] or *iii*) a combination of both mechanisms. The myofilament Ca^{2+} sensitivity is in part related, to an enhancement of the Ca^{2+} binding to the myofilaments and this is reflected by an increased myofibrillar Mg^{2+}-ATPase activity [3,4]. Most positive inotropic drugs used clinically, such as digitalis, α- and ß-adrenergic agonists and phosphodiesterase (PDE) inhibitors act by increasing cytosolic and sarcoplasmic reticulum (SR) Ca^{2+} loading that leads to an increase in magnitude of the Ca^{2+} transient [5]. A number of PDE inhibitors, e.g. sulmazole, pimobendan and several thiadiazinone derivatives appear to produce their positive inotropic effect not only through increased cytosolic [Ca^{2+}] transient as they also exert a Ca^{2+} sensitizing action in both skinned and intact cardiac muscle preparations [1,6,7]. Either method of increasing myocardial contractile function may be damaging as the increased Ca^{2+} transient can potentially result into chronic overloading of the cell with Ca^{2+} [8] and the increased myofilament Ca^{2+} sensitization has the potential to prolong the time of relaxation and to impair diastolic filling of the heart [9].

Thiadiazinone derivatives vary widely in their potency to sensitize myofilaments to Ca^{2+} and to increase cellular cyclic AMP level via their PDE III inhibitory action [10]. However, *in vitro* studies with skinned fibers and soluble preparations of PDE III have indicated that the optical isomers of the racemic thiadiazinone EMD 53998 possess a remarkable separation of Ca^{2+} sensitizing and PDE III inhibitory activities [11] which can be clearly distincted. Thus, compared to the racemate EMD 53998, the [-]enantiomer EMD 57439 (Fig. 1) is a "pure" PDE III inhibitor with almost no Ca^{2+} sensitizing activity, while the [+]enantiomer EMD 57033 (Fig. 1) is a potent Ca^{2+} sensitizer with a weak PDE III inhibitory activity.

Recently, we reported on the *in vivo* cardiovascular effect of the thiadiazinone derivative [+]EMD 60263 (for its chemical structure see Fig. 1) in pigs with regionally stunned myocardium [12,13]. In these studies the effect of [+]EMD 60263 on regional myocardial function was assessed by measuring the systolic segment shortening, external work and mechanical efficiency of stunned and not-stunned myocardium [12]. It was shown that [+]EMD 60263 increased systolic segment shortening of both the stunned and not-stunned myocardium but the effect on the stunned was much more pronounced than on the not-stunned myocardium. Furthermore, [+]EMD 60263 restored mechanical efficiency of stunned myocardium to baseline levels, while that of not-stunned myocardium was unaffected. The action of [+]EMD 60263 was not attenuated when experiments were repeated after α- and ß-adrenergic receptor blockade, thereby excluding adrenergic stimulation or PDE inhibition as the cause of the positive inotropic action of [+]EMD 60263 [12]. The enantiomer [-] EMD 60264 (Fig. 1) has been investigated in the same model and found to have no effects on systolic segment shortening, external work and mechanical efficiency (unpublished results). In another study we demonstrated that in the

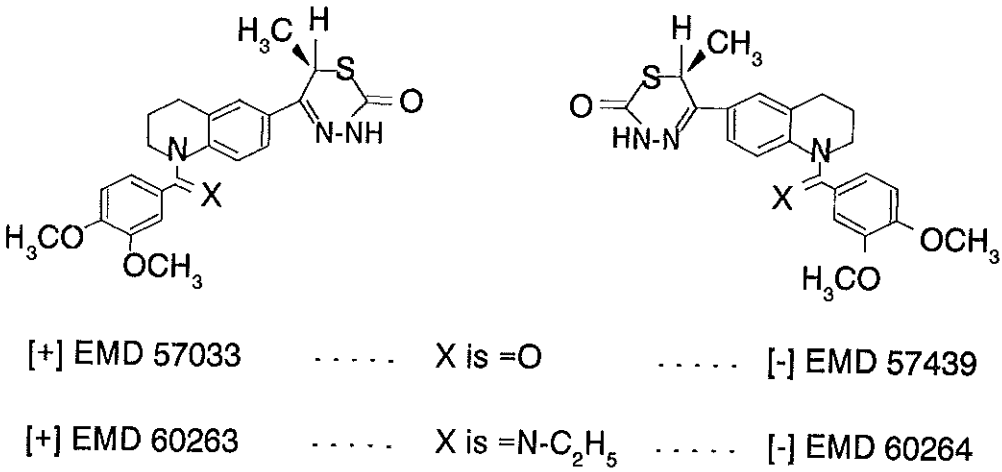

Fig 1 Chemical structures of the [+]enantiomers EMD 57033 and EMD 60263 (left) and [-]- enantiomers EMD 57439 and EMD 60264 (right) (see also refs. 11, 12 and 17).

same porcine model the rate of SR Ca^{2+} uptake was slightly increased in stunned compared to not-stunned myocardium [14]. The data supported the hypothesis that a decreased sensitivity of the myofilaments to Ca^{2+} and not a decreased SR Ca^{2+} pumping activity, is involved in the mechanism of stunning [15,16].

The aim of the present investigation was to complement the data on the thiadiazinone derivatives of our *in vivo* studies [12,13] with data obtained with subcellular preparations *in vitro*. We therefore studied the effects of [+]EMD 60263 and its [-]enantiomer EMD 60264 on the Ca^{2+} responsiveness of the Mg^{2+} -ATPases measurable in SR membrane vesicles and myofibrils isolated from normal porcine myocardium. We studied also the effect of [+]EMD 60263 on the Ca^{2+} responsiveness of the Mg^{2+} ATPase of myofibrils isolated from stunned and the adjacent not-stunned myocardium in the *in situ* porcine model. Moreover, the effectiveness of [+]EMD60263 on myofibrils isolated from hypertrophic septum of patients with cardiomyopathy was tested. For comparison, we also measured the effects of the thiadiazinone derivative [+]EMD 57033, a Ca^{2+}sensitizer with weak PDE III inhibitory activity and [-] EMD

57439 a "pure" PDE III inhibitor with no Ca^{2+} sensitizing action. The results show that [+]EMD 60263, at concentrations (1-3 μM) close to the plasma concentrations that were found to be effective in the *in situ* porcine model [12], sensitizes the Mg^{2+}-ATPase of isolated myofibrils to Ca^{2+} and had no effect on the Ca^{2+} stimulated Mg^{2+}-ATPase of isolated SR membrane vesicles. Consistent with the observations of our previous *in vivo* experiments [12] is the finding that in the stunned myocardium the Ca^{2+} sensitizing effect of [+]EMD 60263 on isolated myofibrils was potentiated.

Materials and Methods

Materials The pure enantiomers [+]EMD 60263 and [-]EMD 60264 (5-[1-(α-ethylimino-3,4-dimethoxybenzyl)-1,2,3,4-tetrahydro-6-quinolyl]-6-methyl-3,6-dihydro-2H-1,3,4-thiadiazin-2-one) and [+]EMD 57033 and [-]EMD 57439 (5-[1-(3,4-dimethoxybenzyl)-1,2,3,4-tetrahydro-6-quinolyl]-6-methyl-3,6-dihydro-2H-1,3,4-thiadiazin-2-one) were supplied by E. Merck, Darmstadt, Germany. Fig. 1 depicts the chemical structures. The only difference between the enantiomers is the position of the methyl group (CH_3) bound to the C-atom beside the S-atom. Because of the asymetrical configuration of that C-atom, the [+]-enantiomer deflects the plane of light to the right direction, whereas the [-]-enantiomer deflects it to the left direction [17]. Stock solutions (0.2 mM) of [+]EMD 60263 and [-]EMD 60264 were made in destilled water and those (0.2 mM) of [+]EMD 57033 and [-]EMD 57439 in dimethylsulfoxide and prepared on the day of the experiment. All solutions contained an equivalent amount of water or dimethylsulfoxide which had no effect on the Ca^{2+} responsiveness and activities of cardiac SR and myofibrillar Mg^{2+}-ATPases. Control experiments also demonstrated that these thiadiazinone derivatives had no effect on the assay used to measure inorganic phosphate (P_i) formation. Leupeptin, aprotinin, pepstatin and thapsigargin were from Sigma Chemical Company (St Louis, USA). All other chemicals were obtained from either E. Merck (Darmstadt, Germany), Boehringer (Mannheim, Germany) or Sigma Chemical Company (St Louis, USA).

Subcellular preparations Sarcoplasmic reticulum (SR) vesicles and myofibrils were isolated from porcine ventricular muscle and hypertrophic septum of cardiomyopathic patients undergoing open heart surgery. Stunned and not-stunned was obtained from 4 anesthetized open-chest pigs in which the distribution territory of the left anterior descending coronary artery (LADCA) was stunned by two sequences of 10 min coronary artery occlusions and 30 min reperfusion. The not-stunned myocardium was obtained from the distriction territory of the left circumflex coronary artery (LCXCA) which was not occluded. For further details of this *in situ* porcine model is referred to [12]. The cardiac muscle specimen (from pigs about 3 g and from humans not more than 1 g) were minced and mixed with 4 volumes 10 mM $NaHCO_3$ and 1 mM dithiothreitol (DTT) and homogenized with a Polytron PTX 10 (Kinematica, GmbH, Luzern,

Switserland). The homogenate was centrifuged at 9000 g_{av} for 20 min at 4°C and the supernatant was centrifuged again at 9000 g_{av} for 20 min. The final supernatant was further subfractionated for isolation of enriched SR vesicles as described [18,19] (see below). The combined pellets were used for the isolation of the purified myofibrils according to the method described by Murphy and Solaro [20]. The pellets were resuspended in 4 volumes solution containing 10 mM EGTA, 8.2 mM $MgCl_2$, 14.4 mM KCl, 60 mM imidazole, 5.5 mM ATP, 22 mM creatinephosphate, 10 $U.ml^{-1}$ creatine kinase, 1 % Triton X100, 5 $\mu g.ml^{-1}$ leupeptin, 10 $\mu g.ml^{-1}$ pepstatin, 10 $\mu g.ml^{-1}$ aprotinin, 1.7 $mg.ml^{-1}$ phenylmethane-sulfonylfluoride (PMSF) in a glass-Teflon homogenizer, and left on ice for 30 min and thereafter centrifuged for 15 min at 1100 g_{av}. The supernatant was discarded and the myofibrillar pellet washed twice with 2 volumes 30 mM KCl, 30 mM imidazole and 2 mM $MgCl_2$, pH 7.0 and finally resuspended in this buffer containing 50 % glycerol up to a protein concentration of 10 $mg.ml^{-1}$. The myofibrillar suspension was stored in aliquots at -80° C.

For the isolation of SR vesicles the initial 9000 g_{av} supernatant was centrifuged for 30 min at 35000 g_{av} for 30 min at 4°C. The supernatant was discarded and the pellet resuspended in 3 ml 0.6 M KCl, 20 mM 4-morpholino-propane sulfonic acid (MOPS), 1 mM DTT, pH 6.8, again centrifuged at 35000 g_{av}. The purified SR vesicles were resuspended in 10 mm Tris, 0.3 M sucrose, 0.5 M KCl and 1 mM DTT, pH 7.0 up to a protein concentration of 5-10 $mg.ml^{-1}$. The SR vesicle suspension was stored in aliquots at -80°C. SR and myofibrillar protein (yields were about 1 and 20 mg protein/g myocardium, respectively) was determined with the method of Bradford [21].

Assay of the Mg^{2+}-ATPase The ATPase activities were determined by measuring the formation of P_i according to the method of Lanzetta *et al* [22]. Briefly, aliquots of the myofibrillar suspension were thawed and the glycerol-containing storage buffer was removed by centrifugation 15 min at 2000 g_{av} (4°C). The myofibrillar pellet was washed twice with 60 mM KCl, 30 mM imidazole and 2 mM $MgCl_2$, pH 7.0 and finally resuspended in a solution containing 60 mM KCl, 30 mM imidazole, 2 mM $MgCl_2$, 1 mM DTT and 1.7 $mg.ml^{-1}$ PMSF. SR vesicles (5 µg protein) and myofibrils (40 µg protein) were incubated at 30°C in a total volume of 200 µl solution containing 60 mM KCl, 2.5 mM $MgCl_2$, 1 mM DTT, 25 mM MOPS, pH 7.0, 2 mM ethylene glycol bis(ß-aminoethylether) N,N,N',N'-tetraacetic acid (EGTA), 2 mM ATP, 5 mM NaN_3, 0.5 µM A23187 and various amounts of Ca^{2+}. Different levels of free Ca^{2+} were achieved by varying the Ca^{2+}/EGTA ratio, keeping the total EGTA concentration constant. Free Ca^{2+} in the buffer was calculated using Fabiato's SPECS computer program [23] as described earlier [24].

Determination of plasma concentrations of [+]EMD 60263 in the in situ pig model To 600 µl of plasma obtained from blood samples taken at various time points during the course of the previous *in vivo* experiments with [+]EMD 60263 in pigs [12], 500 µl of water saturated

ethylether were added and mixed well. The organic and aquous phases were separated in an Eppendorf table centrifuge. The organic top layer was removed and collected in an Eppendorf vial. This extraction procedure was repeated 5 times. The ether phases were collected separately. Thereafter the ether was evaporated in a speed-vac centrifuge. The residuals were resuspended and dissolved in 300 µl acetonitril. The amount of [+]EMD 60263 in a given plasma sample was determined on a HPLC system: 30 µl of the acetonitril solutions were injected on a LiChrosorb RP 8 (5µm) RT 125-4 column (E. Merck, Darmstadt), which was equipped with a Hiber LiChroCart 4-4 precolumn (E. Merck, Darmstadt). 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. The elution was monitored at a wavelength of 320 nm. The concentration of [+]EMD 60263 was deduced from the area of the peaks eluting at the appropiate time from the column by comparison with the values determined for identically treated standard samples. The plasma concentration of a given blood sample was determined by adding the peak areas of the various ether extraction samples.

Statistics The results are given as mean ± SEM. The pCa-Mg^{2+} ATPase data were fitted to a sigmoid function by nonlinear regression analysis. The data normalized to maximum activity, after subtracting basal ATPase activity, were fitted to the Hill equation ($P = P_o/(1 + Q/[Ca^{2+}]^n)$ in which P_o is the maximal Ca^{2+}-stimulated Mg^{2+}-ATPase, P is the level of Ca^{2+}-stimulated Mg^{2+}-ATPase less than maximum, Q a constant and *n* is the Hill value as described [25,26]. For the Hill equation only data points were used which fulfilled the condition $0.1P_o \leq P \leq 0.9P_o$. The equation was solved for P, Q and *n*. The pCa_{50} (at 50 % of the maximal Ca^{2+}-stimulated Mg^{2+}-ATPase activity), was determined, by using *n* and the Q calculated from the Hill equation [25]. The pCa (i.e. -log[Ca^{2+}]) corresponding to 50 % activation of Ca^{2+}-stimulated Mg^{2+}-ATPase was -(1/*n*)logQ. Data were evaluated for statistical significance by the Student t-test and significance was accepted at $P < 0.05$. The pCa_{50} shifts caused by the presence of [+]EMD 60263 in myofibrils isolated from the stunned and the adjacent not-stunned myocardium was assessed by two-way ANOVA with repeated measuring and Bonferroni's adjustment (BMDF, Stastical Software Inc).

Results

Characterization of the Ca^{2+}-stimulated Mg^{2+}-ATPases in the subcellular fractions Before testing the effect of the EMD enantiomers on the Ca^{2+} responsiveness of the Mg^{2+} -ATPases of the isolated SR and myofibrils, we characterized a specific property of the SR Ca^{2+} pumping ATPase to exclude the possible cross-contamination of the isolated myofibrils and SR fraction. Contamination of the myofibrils by SR membrane vesicles was unlikely because before the last precipitation step during isolation the myofibrils were always treated with 1 % Triton X100.

Table 1

Characteristics of the Ca^{2+}-stimulated, Mg^{2+}-dependent ATPases of porcine ventricular sarcoplasmic reticulum (SR) membrane vesicles and myofibrils.

	pCa	Mg^{2+}-ATPase (nmol P_i.min^{-1}.mg^{-1})	
		Control	+ Thapsigargin
SR	7	62 ± 17	55 ± 10
	5	430 ± 52	48 ± 3
Myofibrils	7	21 ± 2	24 ± 2
	5	42 ± 4	44 ± 4

The Mg^{2+}-ATPase activities at pCa 7 of SR membrane vesicles and the myofibrils are always close to the basal activity measured in the absence of Ca^{2+} (compare Figs. 2 and 4) and can therefore be subtracted from the activity at pCa 5 to obtain the activities of the Ca^{2+}-timulated, Mg^{2+}-dependent ATPases. The thapsigargin concentration was 1 μM. Results are presented as mean ± S.E.M. for 3 experiments with different preparations of SR membrane vesicles and myofibrils.

Thapsigargin, which is a specific inhibitor of the SR Ca^{2+} pump, was tested on Ca^{2+} stimulated Mg^{2+}-dependent ATPases of both subcellular fractions (Table 1). Thapsigargin (1 μM) completely blocked the Ca^{2+} stimulated part of the ATPase activity in the SR fraction whereas there was no effect on the Ca^{2+} stimulated portion of the Mg^{2+}-ATPase activity of the myofibrils. The same results were obtained using the myofibrils isolated from human myocardium (unpublished observations). The results demonstrate that SR membrane impurities associated with myofibrils are efficiently eliminated by the pretreatment with Triton X100 and contamination of SR membrane by myofibrillar protein is also negligible.

Plasma concentrations of [+]EMD 60263 at its maximal inotropic effect in the in situ porcine model Prior to the studies of the *in vitro* effects of [+]EMD 60263 on the myofibrillar and SR Ca^{2+} stimulated, Mg^{2+} dependent ATPases, we needed to know the plasma concentrations at which the compound was effective in our previous *in vivo* experiments [12]. When the effects of two consecutive doses of [+]EMD 60263 (0.75 and 1.5 $mg.kg^{-1}$ intravenously, n=7), administered at 15-minute intervals, on segment shortening, external work and mechanical efficiency in anaesthetized pigs were measured, plasma samples had been taken for HPLC analysis of the [+]EMD 60263 concentration. Each dose was infused over a 3 min period, and the second higher dose was infused 15 min later. The mean concentrations of [+]EMD 60263 at the end of the infusion period were 4.5 ± 0.4 μM (mean ± S.E.M, n=7; lower dose) and 8.1 ± 2.0 μM (higher dose); they declined within 15 min to 0.14 ± 0.06 μM and 0.45 ± 0.12 μM, respectively. Thus, submicromolar plasma concentrations of [+]EMD 60263 have been measured at times when myocardial contractility was determined and found to be elevated which indicates

that the drug may be already intracellularly effective at these low concentrations [12,13].

In vitro effects of EMD enantiomers on myofibrillar Mg^{2+}-ATPase isolated from normal ventricle of swine Fig. 2 shows that [+]EMD 60263 increased the submaximally activated (at pCa 6.1) porcine cardiac myofibrillar Mg^{2+}-ATPase in a concentration range of 1-10 μM. The maximal effect was reached at drug concentrations ≥ 20 μM. The differential effects of the [+]EMD 60263 on the Ca^{2+} activation of porcine cardiac myofibrillar Mg^{2+}-ATPase are shown at three drug concentrations (1, 3 and 10 μM) in Fig. 3. The enhanced response to Ca^{2+} involved two effects: (1) a leftward shift of the relation between pCa and myofibrillar ATPase activity. This is indicated by the parameters listed in the legend to Fig. 3, the pCa at half-maximal activation (pCa_{50}) was shifted to the left by [+]EMD 60263 depending on its concentration (1, 3 and 10 μM) from 6.00 ± 0.05 under control conditions to respectively 6.10 ± 0.08, 6.30 ± 0.03 and 6.67 ± 0.05 ($P < 0.05$ *versus* control pCa_{50} at 3 and 10 μM [+]EMD 60263); (2) an increase of the

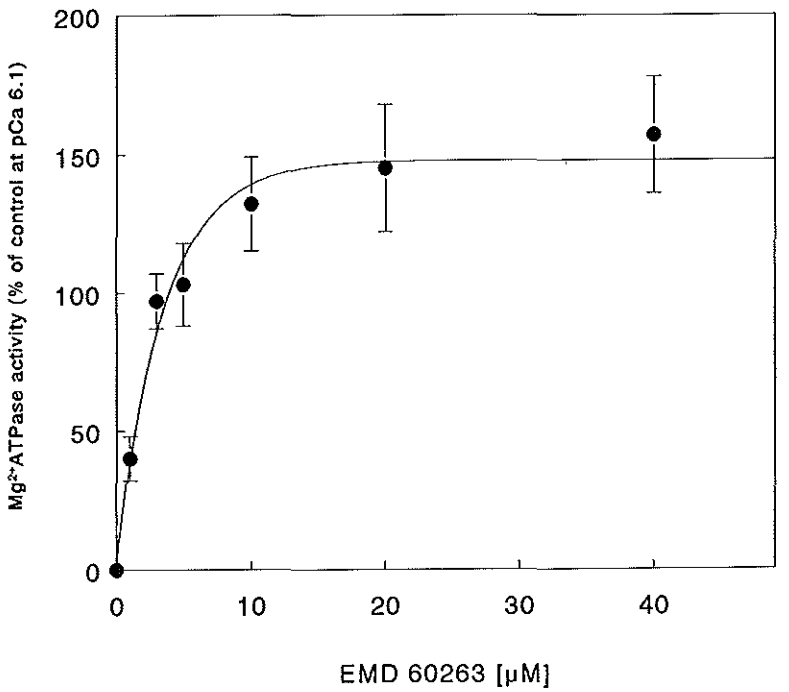

Fig. 2 Effect of varying concentrations of [+]EMD 60263 on submaximally (pCa 6.1) activated Mg^{2+}-ATPase of porcine ventricular myofibrils. Stimulated ATPase activities in the presence of [+]EMD 60263 are expressed as % increase taking the activity in the absence of the drug as 100% See materials and methods for further details. Results are presented as mean ± S.E.M. for 3 experiments with different preparations of myofibrils.

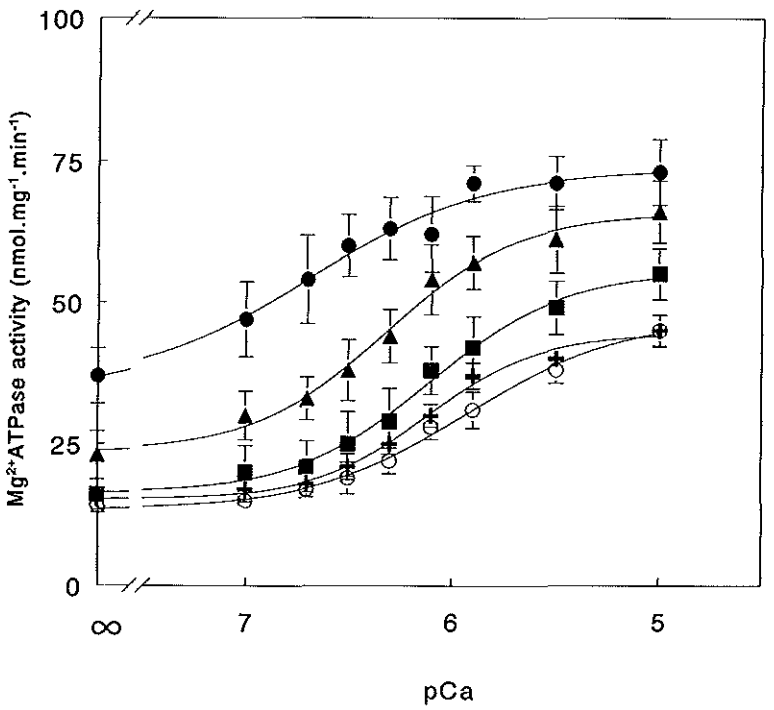

Fig. 3 Graphs showing the relation between pCa^{2+} (-log M)and the Mg^{2+}-ATPase activity (in nmol P_i. mg protein^{-1}. mg^{-1}) in porcine ventricle myofibrils under control conditions (○ , n=6), in the presence of 1 μM (■ , n=3), 3 μM (▲ , n=3) and 10 µM [+]EMD 60263 (● , n=3) and in the presence of 10 µM [-]EMD 60264 (✚ , n=3). For further details see materials and methods. Results are presented as mean ± S.E.M. for 3 experiments with separate preparations of myofibrils. Data normalized to maximum activity after subtracting basal Mg^{2+}- ATPase activity were fitted to the Hill equation giving the following parameters: under control conditions: pCa_{50} = 6.00 ± 0.05 (n = 1.46 ± 0.12); in the presence of 1, 3 and 10 µM [+]EMD 60263: 6.10 ± 0.08 (n = 1.32 ± 0.09), 6.30 ± 0.03 (n = 1.23 ± 0.12) and 6.67 ± 0.05 ($P < 0.05$ *versus* control pCa_{50} at 3 and 10 µM [+]EMD concentration) (n = 1.19 ± 0.18), respectively ; in the presence of 10 µM [-]EMD 60264: pCa_{50} = 6.10 ± 0.04 (n = 1.44 ± 0.25).

maximal myofibrillar Mg^{2+}-ATPase activity. This increasing effect on the maximal Mg^{2+} -ATPase appears to be independent of the effect on the increased Ca^{2+} responsiveness since it was measured at saturating Ca^{2+} concentrations, so that a change in Ca^{2+} responsiveness of the myofilaments should be without an effect. This increase in the maximal Mg^{2+} ATPase activity, however, seems to level off already between 3 and 10 µM [+]EMD 60263, while the increase in the Ca^{2+} responsiveness was further increased, as can be seen from the left- and upwards displacement of the Mg^{2+} ATPase/pCa^{2+}-curve in comparison to the control curve. The average Hill coefficients, reflecting the steepness of the fitted curves at pCa_{50}, remained the same at each concentration of [+]EMD 60263 tested (legend to Fig. 3). The negative enantiomer EMD 60264 (10 µM) had no significant effect on the Ca^{2+} activation pattern of the porcine cardiac

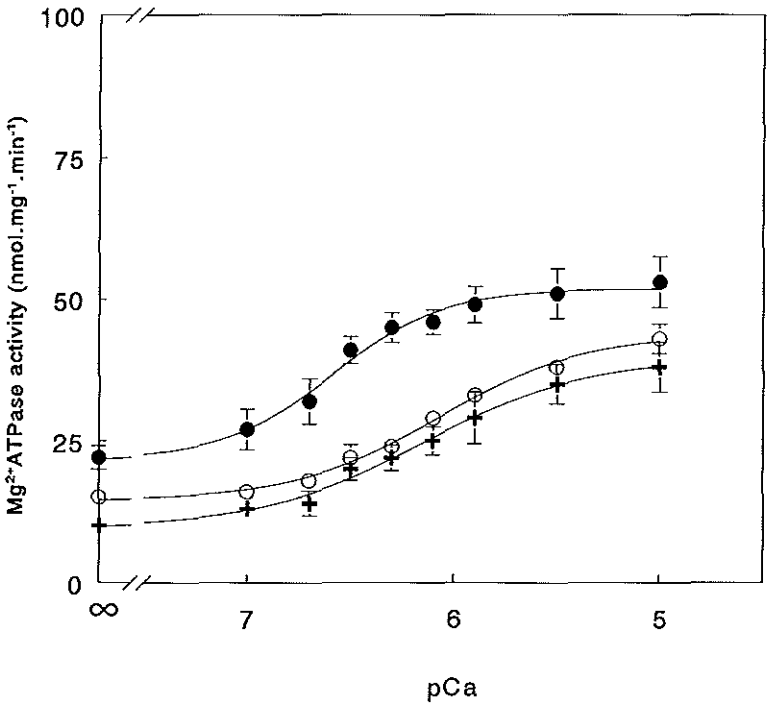

Fig. 4 Graphs showing the relation between pCa^{2+} (-log M) and Mg^{2+}-ATPase activity (in nmol P_i. mg protein^{-1}. mg^{-1}) in porcine ventricle myofibrils under control conditions (O) and in the presence of 10 μM EMD 57033 (●) and 10 μM [-]EMD 57439 (✚). See materials and methods for further details. Results are presented as mean ± S.E.M. for 3 experiments with different preparations of myofibrils. Data normalized to maximum activity after subtracting basal Mg^{2+}-ATPase activity were fitted to the Hill equation giving the following parameters: under control conditions: pCa_{50} = 6.04 ± 0.06, n = 1.35 ± 0.08; in the presence of 10 μM [+]EMD 57033: pCa_{50} = 6.55 ± 0.04 ($P < 0.05$ *versus* control pCa_{50}), n = 1.35 ± 0.09; in the presence of 10 μM [-]EMD 57439: pCa_{50} = 6.11 ± 0.03; n = 1.23 ± 0.25.

myofibrillar Mg^{2+}-ATPase (Fig. 3).

For comparison, Fig. 4 shows the effects of the known optical isomers, [+]EMD 57033 and [-]EMD 57439, on porcine heart myofibrillar Ca^{2+} stimulated Mg^{2+} dependent ATPase. Similar to [+]EMD 60263 (10 μM), but to somewhat less extent, [+]EMD 57033 (10 μM) produced a leftward shift (pCa_{50} changed from 6.04 ± 0.07 under control conditions to 6.55 ± 0.04 ($P < 0.05$ *versus* control pCa_{50}, n=3) of the Ca^{2+} activation curve of the myofibrillar Mg^{2+}-ATPase without changing the Hill coefficient. [+]EMD 57033 (10 μM) also stimulated, even though less potently than [+]EMD 60263, the maximal Mg^{2+}-ATPase activity of the myofibrils. As expected, the negative enantiomer EMD 57439 had no effect on the Ca^{2+} activation pattern of the myofibrillar Mg^{2+}-ATPase (Fig 4).

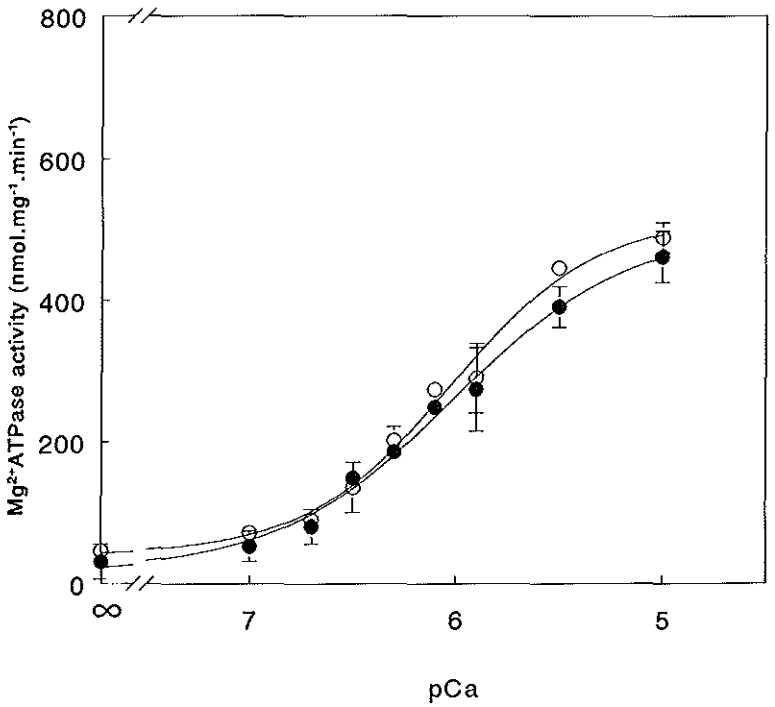

Fig. 5 Graphs showing the relation between pCa^{2+} (-log M) and Mg^{2+}-ATPase activity (in nmol P_i. mg protein^{-1}. min^{-1}) in porcine ventricular SR under control conditions (○) and in the presence 10 µM [+]EMD 60263 (●). See materials and methods for further details. Results are presented as mean ± S.E.M. for 3 experiments with different preparations of SR membrane vesicles.

In vitro effect of [+]EMD 60263 on the SR Mg^{2+}-ATPase form normal ventricle of swine Ca^{2+}-stimulated Mg^{2+}dependent ATPase was measured in SR vesicles isolated from porcine ventricle. In the assay of the ATPase Ca^{2+} ionophore A23187 (0.5 µM) was always present to uncouple the vesicular Ca^{2+} uptake process from the ATP hydrolysis to avoid possible inhibitory effects of the build-up electrochemical Ca^{2+} gradient on the Ca^{2+} stimulated, Mg^{2+}-dependent ATPase of the SR as we observed previously [18,27]. In contrast to its dramatic effects on the myofibrillar ATPase, [+]EMD 60263 (10 µM) did neither affect the pCa_{50} nor the maximum Ca^{2+} stimulated Mg^{2+}-ATPase of the SR (Fig. 5).

In vitro effect of [+]EMD 60263 on the pCa^{2+}/Mg^{2+}-ATPase activity relationships of myofibrils isolated from stunned and not-stunned myocardium Previously we showed that [+]EMD 60263 increased systolic segment shortening of both stunned and not-stunned porcine myocardium but the effect on the stunned was more pronounced than on the not-stunned myocardium [12]. Therefore, four experiments were carried out with anesthetized open-chest pigs in which the

Table 2 In vitro effects of 3 µM [+]EMD 60263 on the pCa^{2+}/Mg^{2+} ATPase activity relationship measured in myofibrils isolated from stunned and not-stunned myocardium of open-chest anesthetized pigs.

	Maximal Ca^{2+}-stimulated Mg^{2+} ATPase activity ($nmol\ P_i.mg^{-1}.min^{-1}$)						pCa_{50} (-log M)					
	Stunned			Not-stunned			Stunned			Not-stunned		
Pig no.	-	+EMD	ΔATPase	-	+EMD	ΔATPase	-	+EMD	ΔpCa_{50}	-	+EMD	ΔpCa_{50}
1	35	56	+21	23	49	+26	5.84	6.35	+0.49	5.94	6.30	+0.36
2	29	47	+18	37	49	+12	5.73	6.41	+0.68	5.83	6.34	+0.51
3	41	45	+4	40	44	+4	6.10	6.67	+0.57	6.09	6.56	+0.49
4	43	50	+7	38	47	+9	6.09	6.64	+0.55	6.18	6.51	+0.33
mean	37	50	+13	35	47	+13	5.94	6.52	+0.57*	6.01	6.43	+0.42
SEM	3	2	4	4	1	+5	0.09	0.08	0.04	0.08	0.06	+0.05

Stunned and not-stunned myocardium was obtained from 4 anesthetized open-chest pigs in which the distribution territory of the LADCA was stunned by 2 sequences of 10 min coronary artery occlusion and 30 min reperfusion. The not-stunned myocardium was obtained from the distribution territory of the LCXCA which was not occluded. The normalized pCa^{2+}/Mg^{2+} ATPase activity curves in the absence and presence of 3 µM [+]EMD 60263, measured at the pCa^{2+} values as these were also chosen in Figs. 3,4 and 6, were fitted to a sigmoid function by non-linear regression analysis. The ATPase activities normalized to maximum activity were fitted to the Hill equation as described in Methods, which analysis gave the calculated pCa_{50} values.
* significantly different ($P < 0.05$) from the [+]EMD 60263-induced ΔpCa_{50}'s in the not-stunned myocardium.

distribution territory of the LADCA was stunned by two sequences of 10 min coronary artery occlusion and 30 min reperfusion. The results of the individual experiments, including the mean data are shown in Table 2. The normalized pCa/Mg^{2+}-ATPase activity curves, obtained from the myofibrils isolated from the stunned and not-stunned myocardium, were fitted to the Hill equation ($P = P_o/(1 + Q/[Ca^{2+}]^n)$ and the pCa^{2+} corresponding to 50 % activation of the Ca^{2+}-stimulated Mg^{2+}-ATPase was calculated from (-1/n)log Q. No differences between not-stunned and stunned myocardium was seen as to the maximal activity of the Ca^{2+}-stimulated Mg^{2+}-ATPase (Table 2). In 3 out of the 4 pigs a rightward shift of the pCa^{2+}/Mg^{2+}-ATPase activity curve of stunned myocardium was observed which followed from the decrease in pCa_{50}'s (Table 2). More experiments have to be carried out to establish whether the tendency of myofibrils from stunned myocardium to desensitize to Ca^{2+} is a reproducible finding. No differences were seen between the stunned and not-stunned myocardium in the increasing effect of [+]EMD 60263 on the maximal Ca^{2+}-stimulated Mg^{2+}-dependent ATPase. However, the compound induced in each of the pigs a leftward shift of the pCa^{2+}/Mg^{2+}-ATPase activity curve ($\Delta pCa_{50} = + 0.57 \pm 0.04$ in stunned versus $\Delta pCa_{50} = + 0.42 \pm 0.05$ in not-stunned myocardium. The latter finding is consistent with our previous observations on the effects of [+]EMD 60263 on systolic segment shortening in the *in situ* porcine model.

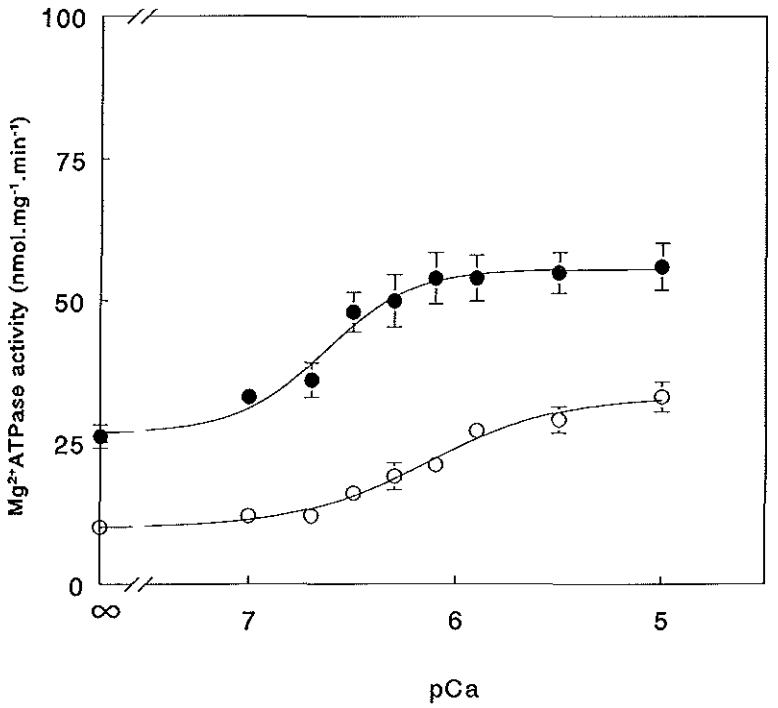

Fig. 6 Graphs showing the relation between pCa^{2+} (-log M) and Mg^{2+}-ATPase activity (in nmol P_i. mg $protein^{-1}$. mg^{-1}) in myofibrils isolated from human hypertrophic septum specimen measured under control conditions (○) and in the presence 10 µM [+]EMD 60263 (●). See materials and methods for further details. Results are presented as mean ± S.E.M. for 5 experiments with different preparations of myofibrils. Data normalized to maximum activity after subtracting basal Mg^{2+}-ATPase activity were fitted to the Hill equation giving the following parameters: under control conditions: pCa_{50} = 6.06 ± 0.09, n = 1.55 ± 0.22; in the presence of [+]EMD 60263: pCa_{50} = 6.53 ± 0.10 ($P < 0.05$ *versus* control pCa_{50}), n = 1.61 ± 0.31.

In vitro effects of [+]EMD 60263 on the myofibrillar Mg^{2+}-ATPase of human hypertrophied septum Because no data were available on the effects of [+]EMD 60263 on human myocardium, we tested the drug on myofibrils isolated from the hypertrophic septum of cardiomyopathic patients (Fig. 6). Like its effect on porcine cardiac myofibrils, the pCa_{50} shifted markedly to the left by 10 µM [+]EMD 60263 from 6.06 ± 0.09 under control conditions to 6.53 ± 0.10 ($P < 0.05$ *versus* control pCa_{50}). [+]EMD 60263 (10 µM) also increased the maximally activated Ca^{2+} stimulated Mg^{2+}-ATPase of isolated human myofibrils.

Discussion

The thiadiazinone [+]EMD 60263 has been described as the first Ca^{2+} sensitizing agent devoid of PDE inhibitory activity [28]. In chemically skinned ventricular fibers of pig heart, [+]EMD 60263 (3 μM) shifted the EC_{50} of Ca^{2+} for the contractile activation from 2.41 mM to 0.73 mM, whereas the optical isomer [-] EMD 60264 was ineffective [28]. The concentrations (IC_{50}) of [+]EMD 60263 and [-]EMD 60264 to reach half-maximal inhibition of PDE III activity of guinea pig ventricles are > 30 and 12 μM, respectively (personal communication with N. Beier). The results of the present experiments also provide evidence for a direct stereoselective activating effect of [+]EMD 60263 on the Ca^{2+} stimulated myofibrillar actomyosin Mg^{2+}-ATPase of pig and human myocardium. There were two effects of [+]EMD 60263: (1) the relation between pCa and the actomyosin Mg^{2+}-ATPase activity was shifted leftward indicative for increased responsiveness of the Mg^{2+}-ATPase to Ca^{2+}; (2) the maximal Ca^{2+} stimulated Mg^{2+} dependent ATPase was increased pointing towards a drug action on the kinetics of the ATPase. Analogous results were obtained using the [+] and [-] enantiomers of EMD 53998. The present observations with [+]EMD 57033 and [-]EMD 57439 on pig and human cardiac myofilaments are in agreement with those obtained in myofibrillar preparations from guinea pig heart [11] and canine ventricle [29]. In the former reports changes in steepness of the pCa/Mg^{2+}-ATPase curves were not observed at 10 μM [+]EMD 57033, but at 30 μM [11,29]. Likewise, in the present study Hill coefficients, reflecting the slopes of the fitted activation curves at pCa_{50} were neither changed by 10 μM [+]EMD 57033 nor by 10 μM [+] 60263. The results provide evidence for two stereoselective effects of the thiadiazinones on the contractile apparatus of heart muscle. The first, Ca^{2+} sensitization of myofilaments and second, increase in kinetics of the actomyosin Mg^{2+}-ATPase. The stereoselectivity of the effects of the thiadiazinones provides strong evidence that these agents affect a specific domain important in determining the state of activation of the myofilaments. Initially it was believed that the mechanism of Ca^{2+} activation of cardiac actomyosin Mg^{2+}-ATPase by the racemic EMD 53998 might involve an effect on Ca^{2+} binding to troponin C [10,30-32]. In subsequent studies, however, Solaro *et al* demonstrated that the Ca^{2+} sensitization by [+] EMD 57033 appears not to involve the binding of Ca^{2+} to troponin C but involves a myofilament domain other than troponin C itself [29]. The studies of Solaro *et al* [29] on desensitized myofibrils and on preparations of pure myosin and actin filaments provided evidence that [+]EMD 57033 acts by stimulating the turnover of the actin-crossbridge reaction. *In vitro* motility assays were performed by Solaro *et al* [29] by adhering monomeric cardiac myosin to nitrocellulose-coated glass coverslips. Unregulated actin filaments were allowed to interact with the myosin-coated surface in the presence of Mg^{2+}-ATP and various concentrations of [+]EMD 57033. The velocity of the actin motion significantly increased with increasing concentrations of [+]EMD 57033 while, [-]EMD 57439 had no effect. This proved the effect to be on the turnover of the cyclic cross-bridge. It has been questioned how the effects of [+]EMD 57033 on the kinetics of the actomyosin interaction relate to the drug's Ca^{2+} sensitizing activity

[29]. The relation between the two activities is indicated by the same stereoselectivity of the two effects. Leijendekker and Herzig [33] estimated turnover rates of cross-bridges by biochemical (Mg^{2+}-ATPase activity) and mechanical (tension development) characteristics of skinned porcine right ventricle. They concluded that the turnover rate of the myocardial cross-bridges was reduced in the presence of the racemate EMD 53998 at low Ca^{2+} ($pCa \geq 6.25$), but not at high Ca^{2+} ($pCa \leq 5.85$).

The relatively long lasting myocardial contractile dysfunction after brief periods of ischemia has been termed stunning. The molecular mechanism of this phenomenon is still poorly understood, but possible candidates are a decreased Ca^{2+} delivery to and a decreased Ca^{2+} reponsiveness of the myofilaments [15,34-36]. Recent data from our laboratory have shown in a model of stunned porcine myocardium that the rate of SR calcium uptake slightly increases [16,36]. Therefore it is unlikely that a change in the active Ca^{2+} transport by the SR is the principal cause of contractile dysfunction of stunned myocardium. Other reports have demonstrated that the intracellular Ca^{2+} transient remains the same in stunned myocardium [34,35]. Korbmacher *et al* [36] performed experiments investigating the effects of [+]EMD 57033 on isolated stunned rabbit hearts and showed that the thiadiazinone derivative acted as a potent positive inotropic agent. Recently, we reported on the *in vivo* cardiovascular effect of [+]EMD 60263 in pigs with or without stunned myocardium [12,13]. In these studies the effect of [+]EMD 60263 on regional myocardial function was assessed by studying the systolic segment shortening of stunned and not-stunned myocardial segments. It was shown that [+]EMD 60263 increased systolic segment shortening of both the stunned and not-stunned myocardium and that the effect on the stunned was much more pronounced than on the not-stunned myocardium. No effects were observed with [-]EMD 60264 (unpublished observations). Consistent with the observations of our previous *in vivo* experiments [12] is the present finding that in stunned- compared to not-stunned myocardium the Ca^{2+} sensitizing effect of [+]EMD 60263 on isolated myofibrils was potentiated. Moreover, in 3 out of 4 pigs we found a slight desensitization to Ca^{2+} in the stunned myocardium. These slight decreases of pCa_{50} observed apparently caused by stunning might be related to the more pronounced positive shifts of pCa_{50} produced by [+]EMD 60263 in stunned- compared to not-stunned myocardium. A recent study determined the Ca^{2+} sensitivity of isometric tension of skinned myocytes obtained from endomyocardial biopsies taken from the LADCA-perfused bed (preischemic- versus stunned myocardium) of anesthetized open-chest pigs [37]. After more severe ischemia, a reduction of myofilament Ca^{2+} sensitivity of the isolated skinned myocytes was found which finding is in agreement with our *in vitro* observations comparing the Ca^{2+} sensitivities of the Mg^{2+}-ATPase of myofibrils from stunned and not-stunned myocardium. Moreover, it was concluded in this report that not only the decreased Ca^{2+} sensitivity of the myofilaments but also the decreased cycling rates of the cross-bridges likely forms the basis of stunning [37]. Similar results were recently obtained with ventricular trabeculae from control and stunned rat myocardium [38].

At present, we report the plasma concentrations at which [+]EMD 60263 was effective in one of our previous *in vivo* studies [12]. The effects of intravenous [+]EMD 60263 infusions (0.75 and 1.50 $mg.kg^{-1}$, n=7) on myocardial functions (systolic segment shortening, external and mechanical efficiency) were determined 15 min after drug infusion when the plasma concentration of [+]EMD 60263 were 0.2 and 0.5 μM, respectively. The stereoselective *in vitro* effect of [+]EMD 60263 on the Ca^{2+} responsiveness of the Mg^{2+}-ATPase of myofibrils isolated from either normal, not-stunned, or stunned porcine myocardium became evident at concentrations (1-3 μM) that appeared to be close to the plasma concentrations required for the maximum increase in systolic segment shortening of stunned and not-stunned myocardial segments [12]. It is also noteworthy that we observed no effects of [+]EMD 60263 on the Ca^{2+} responsiveness of the Mg^{2+}-ATPase of isolated porcine cardiac SR membrane vesicles which finding implies that the SR is not involved in the positive inotropic action of [+]EMD 60263 on swine myocardium. Moreover, by the present data additional support [12,13] is provided for the hypothesis that a decreased sensitivity of the myofilaments to Ca^{2+} and not a decreased SR Ca^{2+} pumping activity is involved in the mechanism of stunning [15,16,34,35].

[+]EMD 60263 caused not only a leftward shift in the myofibrillar pCa^{2+}/Mg^{2+} ATPase relationship, but stimulated also the maximal Ca^{2+}-stimulated Mg^{2+}-ATPase (Figs. 3 and 4 and Table 2). Thus even at the lowest Ca^{2+} concentrations the myofibrillar Mg^{2+} -ATPase is still slightly increased by [+]EMD 60263 and this may indicate the occurrence of relaxation disturbances *in vivo*. We have reported on the diastolic effects of [+]EMD 60263 in stunned porcine myocardium [39]. A bolus injection of 1.5 mg/kg returned systolic segment shortening to baseline value but had no effect on the diastolic segment function. When the dose was increased to 3.0 mg/kg systolic segment shortening in both stunned and not-stunned regions increased beyond baseline value. Diastolic segment lengthening, which in control animals and at lower doses of EMD 60263 started to lengthen immediately after segment shortening reached minimal length, was delayed at high dose. The mean velocity of segment lengthening was unchanged compared to baseline. However, these *in vivo* effects are observed at lower plasma concentrations of [+]EMD 60263 (approximately at < 1 μM) than the concentrations to which the myofibrils were directly exposed in the *in vitro* experiments. Recently, Sunderdick *et al* [40] compared the effects of Ca^{2+} sensitizers [+]EMD 60263 and [+]EMD 57033 in isolated blood-perfused rabbit heart. They used similar doses as our *in vivo* studies and indeed reported that at the relatively high dose of 10 μM [+]EMD 60263 had major detrimental effects on the relaxation and also the contractile function, but not at the lower dose of 3 μM.

In summary, the results show that [+]EMD 602363 at concentrations (1-3 μM) close to the plasma concentrations that were found to be effective in the *in situ* porcine model, sensitizes Mg^{2+}-ATPase of isolated porcine and diseased human cardiac myofibrils to Ca^{2+} and had no effect on the Ca^{2+} stimulated Mg^{2+}-ATPase of isolated porcine cardiac SR membrane vesicles.

We conclude from these data that the positive inotropic action of the thiadiazinone [+]EMD 60263 in the *in situ* porcine model is primarily due to myofilament sensitization to Ca^{2+} and that this compound may have a similar action on the diseased human myocardium.

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Chapter 7

Fish Oil: A Modulator of Experimental Atherosclerosis

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Fish Oil: A Modulator of Experimental Atherosclerosis

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Populations consuming large amounts of fish (n-3 fatty acids) are reported to have a lower incidence of ischemic heart disease, which is attributed to a reduction in atherosclerosis by n-3 fatty acids. The effects of n-3 fatty acids on the various biochemical variables are well documented. Yet it remains unclear how these effects of n-3 fatty acids affect the *in vivo* process of development, progression and regression of lesions. Studies in animal models on n-3 fatty acids and atherosclerosis showed, in contrast to the results from epidemiological studies, conflicting results, which may be attributed to the diversity in animal species and experimental designs. In this paper we reviewed the results from our own studies and relate them to those found in the literature. Our studies were performed in experimental model based on the pig, that underwent dietary intervention with high cholesterol and either endothelial denudation or veno-arterial by-pass grafting for induction of atherosclerotic lesions. The effects of n-3 fatty acids on atherosclerosis was studied during the induction period and in a subsequent period after removal of cholesterol from the diet. We conclude that n-3 fatty acids affect several, but not all parameters routinely used to assess the severity of atherosclerosis in experimental models. The duration of the induction period and therefore the composition of the lesions appear to be important in the few studies that reported on regression by intervention with n-3 fatty acids. **(In: n-3 Fatty Acids: Prevention and Treatment in Vascular Disease, Eds: S.D. Kristensen, E.B. Schmodt, R. De Caterine, S. Endres, Springer Verlag, London, United Kingdom 1995: 55-75.)**

In the early seventies Bang and Dyerberg reported that in the Inuit population (Eskimos living on the West coast of Greenland) the prevalence and mortality of cardiovascular disease was lower compared to an equivalent population of Danes.[1] They also found that these Greenland Eskimos had a favorable plasma lipid profile, with low levels of triglycerides, plasma cholesterol and very low density lipoproteins (VLDL) and high levels of high density lipoproteins (HDL). From this epidemiologic study it was concluded that the diet of the Inuit population, which contains high quantities of fish and seal could contribute to these findings. Fish and seal are rich in long chain polyunsaturated n-3 fatty acids, which are presumed to be a major factor responsible for the supposedly beneficial effect of fish oil. However, one should not forget that other cardiovascular risk factors, such as hypertension and diabetes, are also uncommon among Inuit people. The report by Bang and Dyerberg[1] spurred many investigators to perform epidemiologic studies in several other countries and several of these studies confirmed that regular intake of fish oil was associated with a lower prevalence of cardiovascular events in man.[2-4] The design of these epidemiologic studies, however, does not permit to conclude that less severe atherosclerosis was indeed responsible for the decreased incidence of these cardiovascular events.

The mechanisms by which the n-3 fatty acids may exert their potentially beneficial effects are not yet fully elucidated. Epidemiologic studies have established ingestion of n-3 fatty acids bears a negative relation with levels of blood cholesterol, arterial blood pressure and platelet aggregation and thrombosis, but the possible mechanisms underlying these relationships could not be determined from these studies. Many investigators have therefore used to animal models to elucidate the mechanisms by which n-3 fatty acids may prevent or even cause regression of atherosclerosis. In these models atherosclerosis is usually induced by highly controlled diets rich in cholesterol and endothelial injury. In this chapter we will review the rationale to study the effects of fish oil on the progression and regression of experimentally-induced atherosclerosis and compare the results of our own studies performed in pigs with those of other investigators.

Biochemical processes modulated by n-3 fatty acids

Oils obtained from marine life have high concentrations of long chain polyunsaturated n-3 fatty acids. This is not surprising as the n-3 fatty acids such as eicosapentaenoic (20:n-3 or EPA), docosapentaenoic (22:5n-3 or DPA) and docosahexaenoic (22:6n-3 or DHA) acid, are mainly synthesized by algae and phytoplankton. These microorganisms then serve as food for higher marine life by which n-3 fatty acids will eventually reach humans. This pathway is responsible for the bulk of n-3 fatty acids incorporated in the human body. Synthesis can occur from conversion of α-linolenic acid (18:2n-3), but under normal conditions, when the 20- and 22-chain length n-3 polyunsaturated fatty acids are sufficiently present in the diet, the contribution of α-linolenic acid derived 20- and 22-chain n-3 polyunsaturated fatty acids is negligible. In

Table 1. Biochemical effects of n-3 fatty acids on cell functions and blood constituents involved in atherosclerosis

	INTIMAL HYPERPLASIA & LIPID INFILTRATION	PLATELET AGGREGATION	VASCULAR SMOOTH MUSCLE TONE	INFLAMMATION
PLATELETS	factor 4 ↓, ß-thromboglobulin ↓, PAF ↓, platelet survival ↑	TXA_2 ↓, TXA_3 ↑, platelet count ↓, bleeding time ↓↑	TXA_2↓, TXA_3↑	
ENDOTHELIAL CELL	PDGF ↓	PGI_3 ↑, PGI_2 preserved, EDRF effect ↑	PGI_3 ↑, EDRF effect ↑	
LIPOPROTEINS	VLDL ↓, triglycerides ↓, LDL ↓↑=, HDL ↓↑=, change in lipoprotein size; apoprotein content; physical properties; lipoprotein metabolism; lipid peroxidation			
MONOCYTE	IL-1; TNF ↓	PAF ↓		LTB_4 ↓; LTB_5 ↑
NEUTROPHIL	LTB ↓$_4$; LTB_5 ↑, free radical synthesis ↓, chemotaxis ↓			LTB_4 ↓; LTB_5 ↑, adhesion and chemotaxis ↓
VASCULAR SMOOTH MUSCLE CELL			blood pressure ↓↑ response to noradrenaline ↓	
COAGULATION FACTORS		fibrinogen =↓↑, PAI-1 ↓↑, factor VII =↑, antithrombin =↓, tPA ↑=, von Willebrand =		

For references regarding the actions of n-3 fatty acids see Sassen et al[6] (with permission from Cardiovascular Drugs and Therapy).

mammals an increased intake of n-3 fatty acids leads to a change in the function of various cell types and in the plasma concentrations of some blood constituents (Table 1).[5,6] Part of the n-3 fatty acid effect can be attributed to alteration of the eicosanoid production in cells by interference of n-3 fatty acids with the metabolism of arachidonic acid (20:4n-6). Arachidonic acid is produced from dietary linoleic acid (18:2n-6) by elongation and desaturation. N-3 fatty acids compete for the active sites of the elongation and desaturation enzymes and this explains the reduction of arachidonic acid formation in the presence of excessive n-3 fatty acids. Furthermore, the n-3 fatty acid with chain length of 20, EPA, and to a lesser extent that with chain length of 22, DHA, are not only competing with arachidonic acid for the (re)acylation of membrane (lyso)phospholipids, but also for the formation of eicosanoids by cyclooxygenase and lipoxygenase. The interference with the formation of eicosanoids occurs by two mechanisms. Firstly, the rate of conversion by cyclooxygenase and lipoxygenase is lower when these enzymes use n-3 fatty acids substrate. Secondly, the n-3 fatty acid-derived eicosanoids are different from those derived from n-6 fatty acids. Moreover, the eicosanoids derived from n-3 fatty acids are biologically less active than their n-6 fatty acid-derived counterparts. For instance, in platelets the weak agonist thromboxaneA_3 (TXA_3) is formed by cyclooxygenase from EPA at the expense of the strong agonist thromboxaneA_2 (TXA_2) derived from arachidonic acid. Thus the lower ratio of TXA_2 over TXA_3 attenuates vasoconstriction and platelet aggregation, resulting in an increase in bleeding time. In endothelial cells n-3 fatty acids slightly reduce the production of prostaglandin I_2, but increase the production of the equally biologically active prostaglandin I_3, leading to a net vasodilatory effect, which depends on the TXA/PGI-ratio. It has been shown that endothelial cells of vascular rings derived from animals fed with a n-3 fatty acid enriched diet, release more endothelium derived relaxing factor (EDRF) after a challenge with neurohumoral mediators such as adenosine diphosphate (ADP), serotonin or bradykinin. Interference with the release mechanism of EDRF was excluded as Ca^{2+} ionophores gave similar vasodilatory effects independent of n-3 fatty acid incorporation in the cell membrane.[7-9] One possible mechanism by which n-3 fatty acids could increase EDRF formation is the interference of n-3 fatty acids with the receptor-mediated signaltransduction via phospholipase C.[10,11] This enzyme turns on the phosphatidylinositol cycle, an important intracellular signalling process, which mediates extracellular stimuli, be it mechanical or neurohumoral, to the nucleus, affecting cell growth and transcription processes. Hence, it is also likely that expression of endothelial adhesion molecules and endothelial responses to mechanical stress and mitogenic responses of vascular smooth muscle cells, which are also biological functions that use the phosphatidylinositol cycle for intracellular signalling, are affected by n-3 fatty acids. Other effects of n-3 fatty acids on platelets and endothelial cells are the inhibition of the production and the release of growth factors, such as platelet-derived growth factor and fibroblast growth factor.[12] N-3 fatty acids may consequently, especially in the early phase of atherosclerosis, inhibit or retard smooth muscle cell and fibroblast proliferation.

In monocytes n-3 fatty acids reduce the production of cytokines (interleukin-1) and tissue

factors such as tumor necrosis factor and platelet aggregating factor and thus alter the interaction between platelets and the vascular wall. In leukocytes the leukotriene production is affected via substrate competition for the lipoxygenase between EPA and arachidonic acid, which results in a reduced production of leukotriene B_4 (LTB_4) and an increased leukotriene B_5 (LTB_5) level. Compared to LTB_4, LTB_5 is a relatively weak agonist, thus dietary EPA can decrease chemotaxis and adhesion of monocytes and polymorphonuclear cells, modifying not only the inflammatory response, but also the interaction between these cells and vascular wall. EPA may retard intimal hyperplasia also by these actions.

The effects of n-3 fatty acids on blood lipid and lipoprotein levels are predominantly characterized by a reduction in triglyceride levels. The dose of n-3 fatty acids critically determines the occurrence of triglyceride reduction. Furthermore, the pattern of effects on the plasma levels of triglycerides, very low density lipoprotein (VLDL), low density lipoprotein (LDL) and high density lipoprotein (HDL) is also dose-dependent. Moderate doses of n-3 fatty acids (1-3 g/day) also give rise to a decrease in plasma triglycerides and VLDL in both human normolipidemics and hyperlipidemics.[13] With higher doses of n-3 fatty acids the lowering of the triglyceride levels becomes more clear, although it should also be stressed that the effect is more pronounced when the pretreatment levels of triglycerides are greater. N-3 fatty acids attenuate postprandial hypertriglyceridemia, and this reduction of blood lipids may therefore also add to the beneficial effects of fish oil. The changes in plasma lipoprotein levels, however, are not predictable as those in triglycerides. While most investigators report a decrease in VLDL levels, the reports on the levels of LDL have been inconsistent (Table 1). The effect of n-3 fatty acids on plasma HDL cholesterol strongly depends on the dose used. Very high doses (> 10 g/day) have been reported to cause a decrease, while doses of 5-10 g/day are found to increase HDL levels.[13] The changes in plasma lipoprotein concentrations, however, may not necessarily reflect the potential anti-atherogenic effects of n-3 fatty acids on lipoproteins. N-3 fatty acids may also alter the physical properties and composition of lipoprotein particles. Changes in apoprotein content, together with n-3 fatty acid-induced modification of cellular receptor function or lipoprotein metabolizing enzymes and lipid transfer proteins may alter lipoprotein metabolism in a favorable manner regarding prevention of atherosclerosis. (See reference 5 and 6)

There are several reports, however, that n-3 fatty acids may not exert only beneficial actions, but also some detrimental effects in the process of atherosclerosis. We have shown that incorporation of dietary n-3 fatty acids increased the susceptibility of heart membranes to free radicals after short periods of ischemia.[14] Other investigators have observed that replacement of dietary polyunsaturated fatty acids with monounsaturated fatty acids reduced the susceptibility of LDL to oxidation, following an *in vitro* incubation with copper sulfate.[15,16] In some *in vitro* studies it has also been shown that after oxidative modification LDL particles are preferentially taken up by tissue macrophages.[17,18] It is, therefore, believed that the increased susceptibility of LDL to oxidative modification is implicated in the development and progression of atherosclerosis as accumulation of the oxidative modified LDL particles in macrophages and

smooth muscle cells may result in the formation of foam cells. Recently Whitman et al[19], however, showed that *in vitro* results of LDL oxidation may not reflect the *in vivo* situation. Incorporation of dietary n-3 fatty acids into LDL particles was found to increase their vulnerability to oxidative modification in the *in vitro* situation, but they did not observe an increase in the percent surface areas covered with atherosclerotic lesions in the various vessels. It is quite feasible that *in vivo* circulating anti-oxidants and pro- and anti-oxidants present in the diet affect the effect of local anti-oxidant status in the vessel wall and surroundings and thereby modify the progress of atherosclerotic processes.

Hypertension and diabetes are other risk factors for the development of atherosclerotic lesions. Most of the evidence that fatty acids may play a role in blood pressure regulation comes from studies in humans. Such studies are complicated, however, by difficulties in assessing accurately the dietary fat intake, seasonal variations in both fat intake and blood pressure and such confounding factors as alcohol consumption, physical activity, age, gender and total food intake. Beilin[20] has reviewed the evidence of the potential of fatty acids to lower blood pressure and concluded that n-3 fatty acids have a mild antihypertensive effect, which can be most clearly demonstrated in patients with untreated hypertension and in the elderly. The effect of n-3 fatty acids may be mediated by its effect on platelet- and endothelium-derived mediators in the resistance vessels. Data on the effects of n-3 fatty acids in type II diabetic patients are unequivocal and a considerable amount of research is still needed to establish whether n-3 fatty acids have a beneficial effect on glucose homeostasis. A potential mechanism could be modulation of the function of the pancreatic islets by eicosanoids, altered membrane fluidity and a change in the balance between lipid and glucose oxidation.[21] Modulation of arterial blood pressure and glucose homeostasis by n-3 fatty acids do not play a major role in most animal studies on the progression and regression of atherosclerosis, because neither hypertension or diabetes are present in the models.

Relation between n-3 fatty acids and atherosclerosis

The aforementioned effects of moderate to high doses of n-3 fatty acids are the molecular basis for the biological effects, such as the interference with inflammatory and coagulating processes, blood-vascular wall interaction, cell growth factors, cell signaling, free radical scavenging, lipoprotein metabolism and lipid infiltration of the vessel wall, which are potentially beneficial. Although it is generally accepted that fish oil can interact with the processes involved in atherosclerosis, its contribution to the development, progression and regression of atherosclerotic lesions is still a point of discussion. Most of the evidence is collected from animal studies, but comparing the results of different studies is arduous work, because of the variety of animal models (e.g. species, interventions to accelerate atherogenic processes) and diets (supplementation or replacement of n-6 fatty acids with n-3 fatty acids, duration of n-3 fatty acid feeding, cholesterol content, addition of bile acids) used. Also different parameters may be

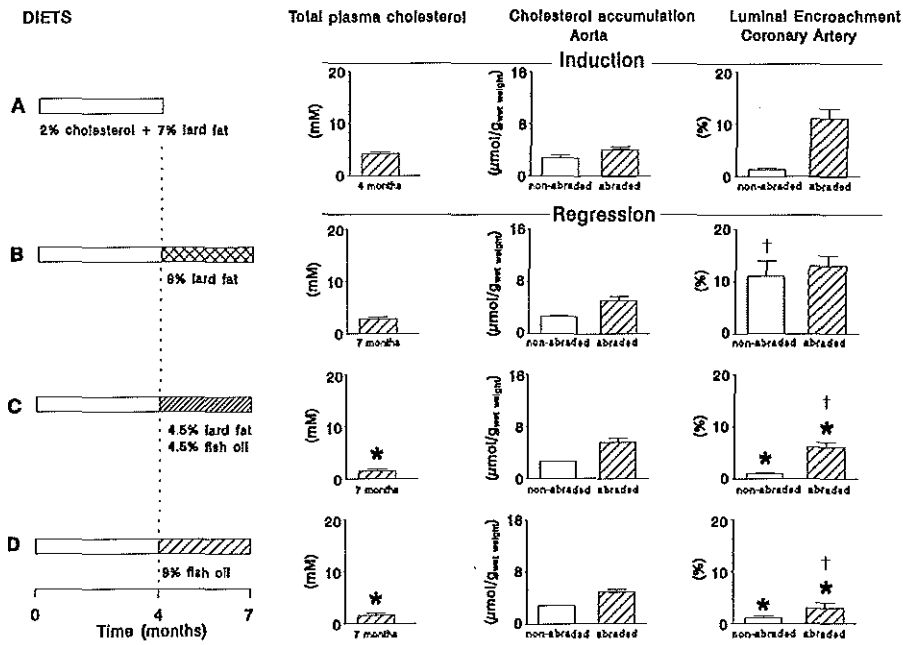

Fig 1. Effect of n-3 fatty acids (panels C and D) on total plasma cholesterol, total cholesterol (cholesterol + cholesterolester) accumulation in the aorta and luminal encroachment in the coronary artery in pigs, in which endothelial denudation was performed 2 weeks after the start of the diet. Panels on the left indicate the composition of the diets and the duration of the study. * $P<.05$ vs the comparitive value for the animals of group A. † $P<.05$ vs the comparitive value for the animals of group B. Data are mean±SEM.

studied to evaluate atherosclerosis and genetic variances may be present in the studied population (e.g. hyper- and hyporesponders within a species with respect to cholesterol feeding).[6] Thus to appreciate the effects of n-3 fatty acids on atherosclerosis it is not only necessary to investigate each possible mechanism and interaction, but preferentially these investigations should be performed in relevant models.

In order to ensure a valid and clinically useful outcome, a consensus should be reached on which animal model best resembles the human situation. Similar to man, atherosclerosis in such model should not only develop spontaneously with early lesions or fatty streaks at juvenile age progressing to advanced lesions at elder age with complication such as fibrosis, calcification, ulceration and thrombosis, but should also involve the intima of the vessel wall. Primate models by evolution closely related to man form the ideal species. They do develop diet-induced lesions, that closely resemble human atherosclerotic lesions, both anatomically and morphologically. However, animal right activists and strict legislation make it hard to use monkeys in experiments. Dogs and especially rabbits have been extensively used in atherosclerosis studies. Their biggest disadvantage is that neither of them develop atherosclerosis spontaneously. In rabbits high cholesterol feeding is necessary to induce atherosclerosis, but at the same time this

also leads to excessive cholesterol (ester) accumulation in tissues. In contrast to man, where lesions are located in the abdominal aorta and epicardial coronary arteries, the main anatomical sites of lesions in rabbits are the aortic arch and the arterioles in the myocardium.[22] The content of the lesions also differ, in that they consist mainly of fat and complicated lesions are rarely encountered. Atherosclerotic lesions are not easily obtained in dogs. Apart from feeding a high cholesterol diet, damage to the endothelial surface or even the thyroid gland is required to obtain atherosclerosis and despite a similar arterial distribution of lesions the injury primarily involves the media. In swine spontaneous development of atherosclerosis can be found and, besides being similar to those in man, it also starts at an early age (6 months).[23] However, as atherosclerosis in the swine is also a very slow process, it takes several years before mature atherosclerotic lesions can be observed. So in most studies induction of atherosclerosis is accelerated using diets with a high fat and/or cholesterol content and added bile acids, which in combination with endothelial injury can produce severe lesions. Also the high costs of strictly controlled diets and housing of large animals under experimental conditions have been major reasons that the duration of most large animal studies was short and the size of the experimental groups was relatively small. That the latter may easily lead to erroneous (both positive and negative) conclusions has been amply demonstrated by Rich et al.[24]

Apart from the above mentioned problems, concerning the chosen model, experimental design, etc., there appears to be no real consistency in the effect of n-3 fatty acids on atherosclerosis. In a recent review we scrutinized the outcome of studies investigating the effect of n-3 fatty acids on atherogenesis in hypercholesterolemic animals.[6] Although 10 out of the 21 studies showed a beneficial outcome and only 4 showed an unfavorable effect, the authors were critical about this outcome and argued that perhaps several studies yielding negative results are not submitted for publication, thereby possibly creating a bias to positive results.

Effects of n-3 fatty acids in our own studies.

Our first studies were not primarily aimed to investigate the effects of n-3 fatty acids on the progression and regression of atherosclerosis, but on the modification of plasma and cell membrane lipids and systemic hemodynamics, cardiac function, arrhythmias and ischemia-reperfusion injury by these fatty acids.[20,26] These parameters were monitored in piglets from the Landrace x Yorkshire race, which shortly after they were weaned were fed a basal diet to which 9% w/w of either lard fat or fish oil was added. The fish oil treated group showed a marked increase in the amount of n-3 fatty acids incorporated in the cell membranes of platelets and cardiac cell membranes, plasma triglycerides and both total and high density lipoprotein (HDL) cholesterol levels were reduced. These changes did not lead to changes in systemic hemodynamics, arrhythmias and cardiac response to ischemia. However, after short periods of ischemia the hyperemia in the fish oil fed animals was increased probably due to a larger vasodilatory response of the coronary vascular bed, which coincided with a decrease in

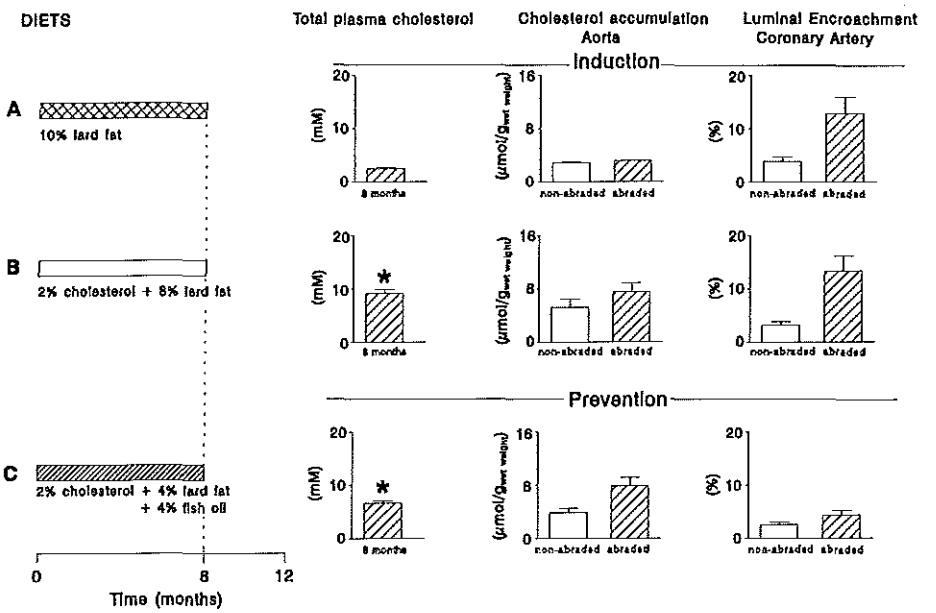

Fig 2. Effect of n-3 fatty acids (panel C) on total plasma cholesterol, total cholesterol (cholesterol + cholesterolester) accumulation in the aorta and luminal encroachment in the coronary artery in pigs, in which endothelial denudation was performed and bile acids (0.5% w/w) was added to the diet at respectively 1 and 3 months after the start of the diet. Panels on the left indicate the composition of the diets and the duration of the study. * $P<.05$ vs the comparitive value for the animals of group A. Data are mean±SEM.

thromboxane A_2/prostaglandin I_2 ratio (TXA_2/PGI_2). In the same model the postprandial responses of plasma lipids and glucose were significantly altered compared to the lard fat treated group.[26] Consistent with other reports postprandial plasma triglycerides and free fatty acids were found to be decreased by fish oil feeding. This is largely due to a decrease in very low density lipoprotein (VLDL) production rather than an increased lipolysis by lipoprotein lipase and hepatic lipase.[27,28] In a parallel series of experiments we have used a different supplement to the basal diet. Instead of 9% (w/w) fish oil, a mixture of 4.5% of fish oil and 4.5 % of lard fat was added, which is a more realistic dose of fish oil for man.[29,30] The duration for the study was increased to 16 weeks, but the responses of plasma triglycerides, total cholesterol and HDL-cholesterol at 8 and 16 weeks were identical with the lower dose of n-3 fatty acids. the same was true for the post-prandial responses at 16 weeks. The findings suggest that in the dose range between 4.5% w/w and 9% w/w there is no dose-dependent effect of fish oil on lipid levels in the normolipidemic swine model. Although not designed to investigate atherosclerosis the results of these studies do indicate that n-3 fatty acids may exert an effect on the process of atherosclerosis. It is, however, interesting to notice that not all changes point in the same direction. The decreased TXA_2/PGI_2 ratio points toward vasodilation and a reduction in thrombogenesis and thereby reducing cardiovascular riskfactors. It has been proposed that lowering of post-prandial lipid levels by n-3 fatty acids reduces the exposure time of the vessels

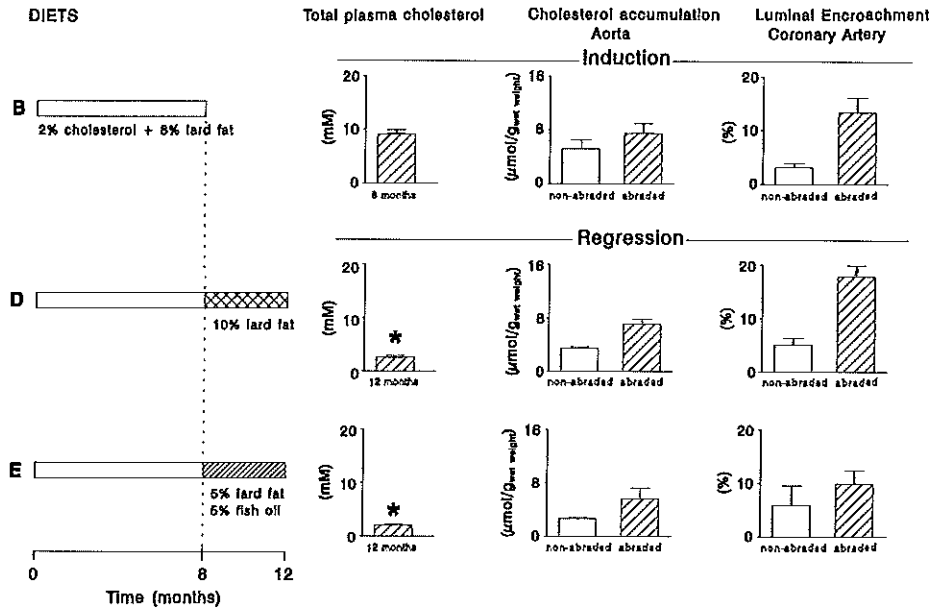

Fig 3. Effect of n-3 fatty acids (panel D) on total plasma cholesterol and the regression of atherosclerosis as assessed by total cholesterol accumulation (cholesterol + cholesterolester) in the aorta and luminal encroachment in the coronary artery in pigs, in which endothelial denudation was performed and bile acids (0.5% w/w) was added to the diet at respectively 1 and 3 months after the start of the diet. The 2% cholesterol was removed from the diet after 8 months of induction. Notice that n-3 fatty acids have no significant effect on total cholesterol accumulation in the aorta and luminal encroachment in the coronary artery. Panels on the left indicate the composition of the diets and the duration of the study. Data are mean±SEM.

and thereby the risk of atherosclerosis.[31] The same reasoning is also valid for the lower total cholesterol and triglyceride plasma levels. In contrast, HDL cholesterol and the ratio HDL/total cholesterol, which are believed to be inversely related to atherosclerosis, were reduced suggesting an increased risk of atherosclerosis.

In subsequent studies, we investigated the effect of n-3 fatty acids on atherosclerosis variables in both normolipidemic and hypercholesterolemic Landrace x Yorkshire swine. In normolipidemic animals development of atherosclerotic lesions was enhanced by implanting a Teflon constrictor with a fixed diameter of 2 mm around the left anterior descending coronary artery (LADCA).[32] Prior to surgery the animals were fed diets to which either 9% (w/w) of lard fat or 4.5% (w/w) lard fat and 4.5% (w/w) fish oil was added for a period of 2 months. The diet regime extended for another 2 months before luminal encroachment was assessed as a measure for atherosclerosis. This study showed a favorable effect as luminal encroachment at the site of the constrictor was reduced from 62±7% in the lard fat fed group to 11±4% in the group fed with fish oil.[32] Furthermore an improvement was observed in the endocardial/epicardial blood flow ratio in the LADCA-perfused myocardial region of the fish oil fed group and hence regional myocardial contractile function was found to be better preserved than in the lard fat group. This

Table 2. Effects of n-3 fatty acids on experimental atherogenesis in pigs

AUTHOR	Induction of atherosclerosis	TIME (months)	DOSE OF EPA and/or DHA	I/S	BLOOD VESSEL	ASSESSMENT OF ATHEROSCLEROSIS	EFFECT OF FISH OIL
Hill et al[44]	hypercholesterolemia + bile acids	24	6% total n-3 (weight% in diet)	I[a]	Aorta	LE[b]	=
						%lesions[b]	=
						aortic lipids	FC=;CE=
					Coronary artery	LE[b]	=
			3% total n-3 (weight% in diet)	I[c]	Aorta	LE[b]	↓
						%lesions[b]	↓
						aortic lipids	FC=;CE=
					Coronary artery	LE[b]	↓
Hill et al[45]	hypercholesterolemia + bile acids	12	3% total n-3 (weight% in diet)	I	Aorta	LE[b]	=
						%lesions[b]	=
						aortic lipids	FC=;CE=
					Coronary artery	LE[b]	=
Weiner et al[37]	hypercholesterolemia + bile acids + abrasion	8	30 ml cod liver oil per day per animal	S	Coronary artery	LE	↓
Kim et al[46]	hypercholesterolemia + bile acids	4	(330 EPA + 330 DHA)[d] - (100 EPA+100 DHA)[e] (mg/kg/day)	S	Aorta	LE	↓
					Coronary artery	LE	↓
Hartog et al[32]	normocholesterolemia + teflon constrictor	4	0.36% EPA+0.23% DHA (weight% in diet)	I	Coronary artery	LE	↓
Sassen et al[41]	hypercholesterolemia + bile acids + abrasion	8	(1040 EPA+743 DHA)[d] - (242 EPA+175 DHA)[e] (mg/kg/day)	I	Aorta	%lesions	=
						aortic lipids	=
					Coronary artery	LE	=

EPA, eicosapentaenoic acid (20:5n-3); DHA, docosahexaenoic acid (20:6n-3); [a] tallow used as control diet; [b] semiquantitative histologic grading; [c] coconut-oil used as control diet; [d] dose at the beginning of the dietary period; [e] dose at the end of the dietary period; I/S, isocaloric or supplementary administration of fish oil; %lesions stands for the percentage of the surface of the vessel that is covered with intimal lesions; LE, luminal encroachment; FC, free cholesterol; CE, esterified cholesterol; TC, total cholesterol; Effect of fish oil: =, unchanged; ↓, decrease; ↑, increase. Adapted from Sassen et al[5] (with permission from Cardiovascular Drugs and Therapy).

may indicate a better vasodilatory reserve of the endocardial layer due to n-3 fatty acids, which agrees with our earlier finding of a decreased TXA_2/PGI_2 ratio after multiple sequences of occlusion and reperfusion. Both phenomena conform with the reduced ADP-induced aggregation of platelets found in this study.

Regression of atherosclerosis in animal models have been shown in several species. [22,33-36] In a recent review on regression of atherosclerosis Schwartz et al proposed four possible targets for intervention; plaque initiation, plaque progression, plaque stabilization and reduction in plaque size and removal of constituents. Plaque initiation (formation of new lesions) and also plaque stabilization, which involves thromboresistance and thromboregulation of the atherosclerotic plaque, have already been shown to be affected by n-3 fatty acids (Table 1). However, little is known about the effects of n-3 fatty acids on plaque progression and plaque size reduction. In our subsequent studies we therefore not only focussed on the effects of n-3 fatty acids on progression, but also on regression (reduction in atherosclerotic lesions) of atherosclerosis. To this end, similar to the models used by other investigators[44-37] a high cholesterol diet and abrasion of the endothelial surface was used to accelerate the development of lesions (Table 2). In the first series of experiments atherosclerosis was induced with a diet containing 2% cholesterol and 7% lard fat for 4 months together with endothelial denudation of the LADCA and abdominal aorta in the second week.[38] At the end of the induction period plasma cholesterol level had doubled (2 mM at baseline), while cholesterol + cholesterolester content of the abraded aorta was 45% higher than in the non-abraded aorta. Similarly there was a nearly 10-fold increase in luminal encroachment in the abraded coronary artery (1.3±0.3 in the non-abraded coronary artery, Figure 1). After the induction period a low cholesterol diet supplemented with 4.5% (w/w) or 9% (w/w) of fish oil or lard fat was administered for 3 months to induce regression of atherosclerosis. In both fish oil groups, plasma cholesterol levels returned to baseline values and was significantly reduced compared to the group fed with only lard fat, without an effect on lipid accumulation in either the abraded or non-abraded aorta. In the abraded coronary artery, however, fish oil dose-dependently reduced the luminal encroachment and prevented progression of luminal encroachment in the non-abraded artery, compared to the findings in the lard fat group. The latter finding corresponds with the observation by Clarkson et al.,[39] who found that elevated levels of plasma cholesterol correlated with progression of the lesions. Plasma cholesterol levels and luminal encroachment after induction were considerably lower in our study than those reported in the literature[37,40] , however. For instance, Weiner et al reported a luminal encroachment of 44% compared to only 11% in our study at the end of the induction period.[37] This suggests a less severe atherosclerotic lesion in our study, which consequently could have contributed to our positive results obtained in the regression period. The very high plasma cholesterol levels obtained in their study (approximately 14 mM against 4 mM in our study) may underly these differences. Therefore we performed a second series of experiments in which the development and regression of atherosclerosis was investigated, in animals, which were now fed a basal diet for 8 months (instead of 4 months), to which either 10% (w/w) of lard fat, 2% cholesterol plus 8% lard fat or 2% cholesterol plus 4% lard fat and 4% fish oil were added, to induce atherosclerosis.[41] After 1 month the LADCA and abdominal aorta were denuded and in accordance with the study of Weiner et al[37] bile acids were added to the high cholesterol groups to further enhance plasma cholesterol levels. Higher plasma cholesterol levels were indeed obtained with this protocol and averaged 9 mM. In the non-abraded aorta fish oil prevented the accumulation of free cholesterol in the aortic wall compared to the cholesterol-lard fat group, but had no effect on lipid content in the abraded segment of the aorta (Figure 2). Staining the

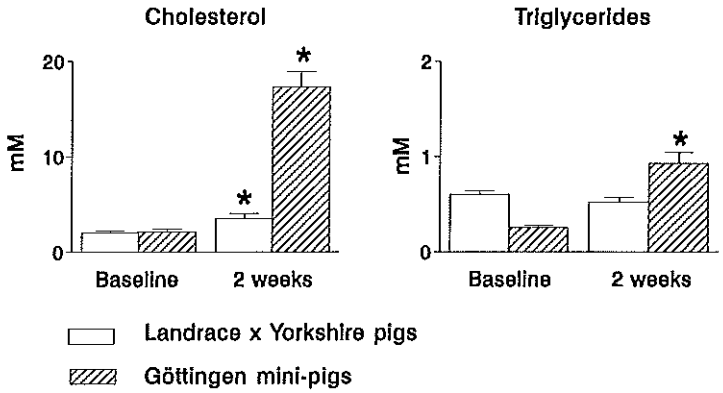

Fig 4. Comparison of the effects of 2% cholesterol feeding on plasma levels of cholesterol and triglycerides in domestic crossbred Landrace x Yorkshire pigs and Göttingen mini-pigs. Notice that in contrast to the domestic pigs the Göttingen mini-pigs respond with a marked increase in total plasma cholesterol. * $P<.05$ vs Baseline. Data are mean±SEM.

intimal surface of the aorta with Sudan IV, however, revealed no differences between lard fat and fish oil groups. Luminal encroachment of both the abraded and non-abraded coronary arteries were not affected by fish oil during the induction of atherosclerosis, in contrast to the severe of luminal encroachment reported by Weiner et al[37] (Figure 2). The higher content of bile acids and saturated fat in the diets used by Weiner et al and hence the higher plasma cholesterol level may account for the differences. Moreover, despite the longer duration of the induction period and addition of bile acids to the diet, there seems to be no difference in the diet-induced atherosclerosis as assessed by total cholesterol (cholesterol + cholesterolester) accumulation in the aorta and luminal encroachment of the coronary artery between our own two studies (Figures 1 and 3). Regression of atherosclerosis in our model was studied by removing the 2% cholesterol from the diet and substituting the 8% with 10% of lard fat and the 4% of lard fat and fish oil with 5% of each fat. After an induction period with cholesterol and lard fat the animals were fed with one of these diets for a period of 4 months (Figure 3). The results of this study revealed that, in comparison to the lard fat group, fish oil attenuated the progression of atherosclerosis leading to less lipid accumulation in the damaged aortic wall and less luminal encroachment in the coronary arteries. However, in contrast to our previous study, there was no a reduction in coronary luminal encroachment. These results are in agreement with those reported by Fincham et al (Table 3).[42] These investigators observed that after an induction period of more than one year fish oil was unable to induce regression of atherosclerosis in coronary arteries and the aorta of hypercholesterolemic non-human primates. In addition to the study of Sassen et al regression of atherosclerotic lesions has been observed by Zhu et al.[38,43] This last group of investigators reported a decrease in percent of aortic and pulmonary artery surface areas covered with atherosclerotic lesion after an induction period, which lasted only 10 weeks. The number of studies on the effect of fish oil on regression of atherosclerosis is small, but the results presented in Table 3 suggest that the duration of the induction period may be important as only the studies with induction periods lasting less than 4 months showed positive results. The longer induction

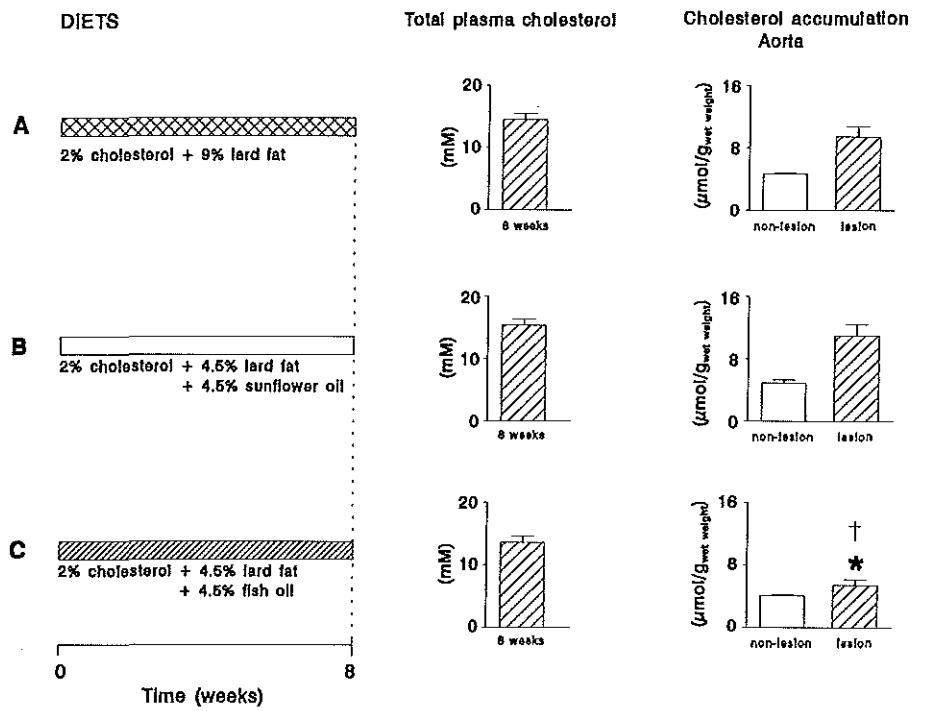

Fig 5. Comparison of the effect of n-3 fatty acids (panel C) and n-6 fatty acids (panel D) on total plasma cholesterol and total cholesterol (cholesterol + cholesterolester) accumulation in the aorta in Göttingen mini-pigs, which were fed a high cholesterol diet for 8 weeks. * $P<.05$ vs the comparitive value for the animals of group A. † $P<.05$ vs the comparitive value for the animals of group B. Data are mean±SEM.

period possibly lead to a different structure and composition of the atherosclerotic lesions, which are more resilient to regression by fish oil.

Accelerating the process of atherosclerosis with high cholesterol forage, bile acids and endothelial denudation render the foregoing studies to criticism as the induction process might affect the form and composition of the atherosclerotic lesions formed. The Landrace x Yorkshire pig race probably represents a hyporesponder to cholesterol feeding and needs very long protocols to develop atherosclerosis spontaneously. In contrast the swine breed Göttingen mini-pigs reacts more readily to a challenge with high cholesterol (Figure 4). Together with their smaller stature and a lower rate of growth, which facilitates their handling, they are more suitable for studies on atherosclerosis. Because of these advantages we have chosen for the Göttingen mini-pigs in our latest atherosclerosis study. In this study a forage with 2% cholesterol and 9% of lard fat and 4.5% of lard fat, plus either 4.5% of lard fat, sunflower oil or fish oil increased total plasma cholesterol level from about 3 mM to about 14 mM in 2 weeks time again without addition of bile acids. Furthermore after 8 weeks white-yellowish elevations on the inner surface of the abdominal aorta (fatty streak-like lesions) were observed. Compared to macroscopically normal tissue, the lesions contained more unesterified and esterified cholesterol. This model was used to study whether the protection afforded by fish oil is a direct effect of n-3 fatty acids or secondary to a fish oil induced-increase in the ratio of polyunsaturated/saturated

Table 3. Effect of n-3 fatty acids on the regression of atherosclerosis in animal models

AUTHOR	ANIMAL (Induction of atherosclerosis)	TIME (months)		DOSE OF EPA and/or DHA (mg/kg/day)	I/S	PLASMA LIPIDS	BLOOD VESSEL	ASSESSMENT OF ATHEROSCLEROSIS	EFFECT OF FISH OIL
		Induction	Regression						
Sassen et al[38]	swine (hypercholesterolemia + abrasion)	4	3	188[a]-210[b] EPA 125[a]-140[b] DHA 380[a]-396[b] EPA 209[a]-220[b] DHA	I	TC ↓, Tg ↓	Aorta Coronary artery	Lipids LE	PL=; TC=; TG= ↓ (dose dependent)
Zhu et al[43]	rabbit (hypercholesterolemia)	2.5	10	82[a]-73[b] EPA 55[a]-49[b] DHA	S	TC =, Tg =	Aorta Pulmonary artery	%lesions % lesions	↓ ↓
Fincham et al[42]	Vervet monkeys (non-human primates) (hypercholesterolemia)	24.5	20	30.1 EPA 9.7 DHA	I	TC =, Tg =	Aorta Cerebral artery Coronary artery	LE %lesions LE LE	= = = =
Sassen et al[41]	swine (hypercholesterolemia + abrasion + bile acids)	8	4	309[a]-193[b] EPA 220[a]-137[b] DHA	I		Aorta - ascending (non-abraded) - abdominal (abraded) Coronary artery	%lesion Lipids Lipids LE	= PL=; CE↓; FC=; TG= PL=; CE=; FC=; TG= =

EPA, eicosapentaenoic acid (20:5n-3); DHA, docosahexaenoic acid (20:6n-3); [a] dose at the beginning of the dietary period; [b] dose at the end of the dietary period; I/S, isocaloric or supplementary administration of fish oil; %lesions stands for the percentage of the surface of the vessel that is covered with intimal lesions; LE, luminal enchroachment; PL, phospholipids; FC, free cholesterol; CE, esterified cholesterol; TC, total cholesterol; Tg, triglycerides. Adapted from Sassen et al[6] (with permission from Cardiovascular Drugs and Therapy).

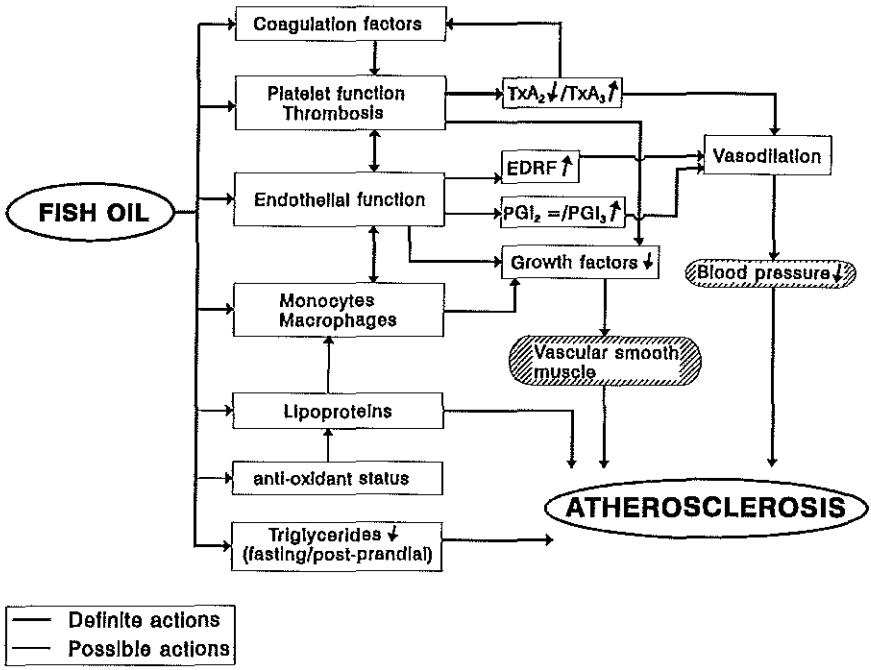

Fig 6. Proposed scheme for definite and possible mechanisms of n-3 fatty acids on atherogenesis.

fatty acid (P/S ratio). The dietary vitamin E regime was chosen such that the levels of vitamin E in the various lipoproteins did not depend on the type of fatty acid or the P/S ratio in the diet. This experimental strategy is different from what we and others have used before and is to exclude the possibility that the effects of dietary n-3 fatty acids could, at least in part, be ascribed to a changed oxidant status of the animal. Vegetable oils rich in n-6 fatty acids are also reported to protect against ischemic heart disease probably due to a beneficial effect on plasma levels of lipoproteins and cholesterol. Replacing a part of the lard fat with the sunflower oil increased the P/S ratio from 0.49 to 1.50, but with fish oil as substitute for lard fat to only 0.83. Nevertheless, only the group fed with fish oil showed a favorable effect on the fatty lesions in the aorta (total plasma cholesterol was not affected by the diets, Figure 5). Compared to both sunflower oil and lard, fat fish oil did not only reduce the number of animals in which lesions were observed, but also reduced the total cholesterol content in these lesions. It is therefore most likely that the prevention of cholesterol accumulation by fish oil is caused by a direct effect of n-3 fatty acids rather than by a higher P/S ratio. The direct effect of n-3 fatty acids on total cholesterol accumulation in the vessel wall may be attributed to the reduction in the VLDL-cholesterol level and cholesteryl ester-rich VLDL by n-3 fatty acids. Our other objective was to study the influence of the three diets on the progression of intimal thickening in saphenous veno-arterial grafts. In these grafts atherosclerosis develops in an accelerated fashion similar to that in abraded

vessels or vessels with a constrictor. It is thus quite feasible that fish oil also protects these vein-grafts against atherosclerosis. Our preliminary results showed lower occlusion rates in the fish oil treated group during the first 6 weeks after placement of the saphenous grafts in the carotid arteries. However, lipid accumulation in the graft was not affected in contrast to the spontaneous lipid accumulation in the abdominal aorta. This finding suggests that an intact endothelial layer and/or function may affect the effects of fish oil on the progression of atherosclerosis.

Summary and conclusions

We conclude from our studies that the presence of an intact endothelial layer, hypercholesterolemia and the history of the lesions determine the ultimate effect of n-3 fatty acid supplementation to the diet on the development and regression of atherosclerosis. However one should realize, that in these studies other potentially atherogenic influences such as hypertension, diabetes, aging and smoking were not taken into account. Because these parameters affect endothelial and platelet function it is feasible that they can also modify the effect of n-3 fatty acids. Therefore, while in animal models with experimentally-induced atherosclerosis n-3 fatty acids are effective in preventing the formation of new lesions and in slowing the progression of established lesions, this does not necessarily imply that the same effects will be obtained in man, in which all other riskfactors may be present.

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Chapter 8

The Effects of Dietary Fish Oil on Progression of Atherosclerosis in Vein Grafts and Aorta in Hypercholesterolemic Swine: A Comparison with Sunflower Oil and Lard Fat

Running title: Progression of atherosclerosis and fish oil

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The Effects of Dietary Fish Oil on Progression of Atherosclerosis in Vein Grafts and Aorta in Hypercholesterolemic Swine: A Comparison with Sunflower Oil and Lard Fat

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Background. The accelerated form of atherosclerosis is responsible for late failures in vein graft patency and represents a major problem in coronary bypass surgery. In earlier studies with hypercholesterolemic dogs dietary fish oil has been shown to effectively reduce intimal thickness in vein grafts. In the present study is investigated whether the effect of dietary fish oil is based on a direct effect of n-3 fatty acids or an increase in polyunsaturated/saturated fatty acid ratio. Therefore, a comparison was made between the effects of fish oil, sunflower oil or lard fat on atherosclerosis in vein grafts. The effects of the different fatty acids on atherosclerosis in a control vein, left circumflex coronary artery (LCXCA) and abdominal aorta were also investigated.

Methods and Results. Thirty-two pigs were fed a 2% cholesterol diet, which also contained by weight either 4.5% fish oil and 4.5% lard fat (FO, n=8), 4.5% sunflower oil and 4.5% lard fat (SO, n=9) or 9% lard fat (LF, n=11), for 8 weeks. After 2 weeks the animals received saphenous vein bypass grafts of both carotid arteries. A separate group of 4 animals were fed a normocholesterolemic diet to evaluate development of atherosclerosis in the abdominal aorta and changes in blood lipids. No beneficial effect of FO versus the SO and LF groups was observed in de grafts as assessed by luminal encroachment, intimal thickness and measurement of lipid accumulation. Similarly, no differences in the control vein and LCXCA were observed between the 3 hypercholesterolemic diets using the same parameters. In the abdominal aorta of the normocholesterolemic animals raised lesions developed spontaneously, which contained more cholesterolester than a macroscopically normal portion of abdominal aorta. Hypercholesterolemia increased the cholesterolester content of these lesions, but the cholesterolester content in the FO group was significantly less than in the SO and LF groups. The aortic lesions in SO and LF also contained more unesterified cholesterol than in the normocholesterolemic group. Mean plasma cholesterol increased from 2.8±0.1 mM to 14.5±0.1 mM (for all diet groups), prior to and at the end of the experimental protocol. Increased cholesterol concentrations were found in all lipoprotein density fractions. FO reduced VLDL, IDL and HDL cholesterol compared with SO. Compared to LF only VLDL cholesterol was reduced by FO. Plasma LDL cholesterol were identical for the 3 hypercholesterolemic groups. Furthermore, the chemical composition of the VLDL particles in the FO were altered. These particles contained relatively less cholesterolester and more triglycerides than SO and LF groups. Regression analysis indicated that VLDL cholesterol was related to the total cholesterol and cholesterolester contents of the aortic lesions ($r=0.77$, $p<0.001$).

Conclusion. These data suggest that dietary fish oil can inhibit the effects of hypercholesterolemia on progression of atherosclerosis and that VLDL cholesterolester may play an key role in this process. Furthermore, the anti-atherosclerotic effect of fish oil can be attributed to a direct effect of n-3 fatty acids and not to an increase in polyunsaturated/saturated fatty acid ratio.

Despite the development of interventional cardiology and new pharmacological agents, coronary artery bypass grafting (CABG) still takes a prominent place in the treatment of ischemic heart disease. The advantage of CABG is that in bypassing the original stenosis one achieves complete revascularisation independent of the severity of the primary. However, attrition rate of the grafts and thus recurrence of clinical symptoms are rather high. With the saphenous vein a conduit 12% of the grafts occlude during the first month and this has increased to 22% at the end of the first year. In the following years occlusion rates rise to 35% and to more than 50% at 5 and 10 years post operatively.[1] The internal mammary artery as bypass conduit suffers less from late failures, but the two internal mammary arteries provide insufficient material for multiple bypass grafting. Thus despite inferior longterm patency rates the saphenous vein is still the most often used conduit for CABG.[1,2,3]

During the first month of the postoperative period acute thrombosis occurs in 10% of the grafts,[2] which is likely secondary to endothelial damage of the vein graft during surgery. The medial layer is also damaged during surgery and the associated inflammatory response may trigger the fibrointimal hyperplasia, the principal cause for late luminal narrowing. Beyond the first year fibrointimal hyperplasia remains the predominant pathology in the grafts, but these abnormalities may develop into mature lipid-laden atherosclerotic plaques in the years thereafter. However, total occlusion leading to death is usually not caused by either fibrointimal hyperplasia or atherosclerosis, but by a superimposed thrombotic event. Thus the rational treatment of vein graft occlusion should focus on attenuation of progression of atherosclerosis and thrombosis.

Consumption of fish has in epidemiological studies been associated with a reduction in cardiovascular disease.[4,5] The exact mechanisms are still unknown, but the favorable effect of fish oil on the atherosclerotic process could result from alterations in plasma lipoproteins, platelet and endothelial function and inflammatory response.[4,5] These alterations may reduce thrombosis and, because thrombosis is not only related to early, but also to late vein graft failure, makes fish oil an attractive candidate for reducing vein graft occlusion. The anti-inflammatory effect of dietary fish oil may reduce neutrophil and monocyte adhesion and chemotaxis to the graft and lead to a reduction in lipid accumulation and the release of growth factors. Earlier studies have shown that fish oil can inhibit vein graft intimal thickening in hypercholesterolemic dogs.[6-8] To elucidate whether this effect is due to the n-3 fatty acids present in fish oil or to an increase of the polyunsaturated/saturated fatty acid ratio, in the present study we investigated the effects of dietary fish oil in a hypercholesterolemic swine model of arterialized saphenous vein grafts and compared them to those obtained with dietary sunflower oil and lard fat. We also studied the effects of the different fatty acids on atherosclerotic lesions in the abdominal aorta.

Table 1. Composition of the atherosclerotic base diet before addition of 4.5% 9 (w/w) of either fish oil, sunflower oil or lard fat.

	Content (g%)		
	FO	SO	LF
Ingredients			
Corn (extruded)	32	32	32
Wheat (extruded)	18	18	18
Soybean meal	14	14	14
Wheat middlings	9	9	9
Dehydrated skimmed milk powder	14	14	14
$CaHPO_4.2H_2O$	1.3	1.3	1.3
$CaCOO_3$	1.1	1.1	1.1
NaCl, iodinized	0.3	0.3	0.3
MgO	0.05	0.05	0.05
$MgSO_4$	0.05	0.05	0.05
KH_2PO_4	0.36	0.36	0.36
Choline chloride 50% (w/w)	0.18	0.18	0.18
Vitamin and trace element mixes[a]	0.7	0.7	0.7
Lard fat	4.5	4.5	9
Fish oil	4.5	-	-
Sunflower oil	-	4.5	-
Cholesterol	2.0	2.0	2.0

FO=fish oil diet; SO=sunflower oil diet; LF=lard fat diet. [a]Vitamin and trace element mixes supply the following per 100 g diet: retinol 1400 IU; cholecalciferol 140 IU; α-tocopherol 8 mg; menadione 0.2 mg; thiamine hydrochloride 1.8 mg; riboflavin 1.8 mg; pyridoxine HCl 1.4 mg; niacin 3.6 mg; vitamin C coated 20 mg; calcium D-pantothenate 3.6 mg; folic acid 0.4 mg; cyanocobalamin 0.004 mg; biotin 0.1 mg; inositol 4.5 mg; iron subcarbonate (57% Fe) 9.1 mg; $FeSO_4.H_2O$ (30% Fe) 14 mg; $Cu_2(OH)_2CO_3$ (55% Cu) 2.3 mg; ZnO (78% Zn) 11 mg; MnO (62% Mn) 9.1 mg; $Na_2Se_3H_2O$ (45% Se) 0.08 mg; $Ca(IO_3)_2$ (65% I) 0.2 mg; $CoCO_3$ (47% Co) 0.09 mg. [b]Cholesterol content was not determined in the normocholesterolemic diet. The composition is on an as fed basis.

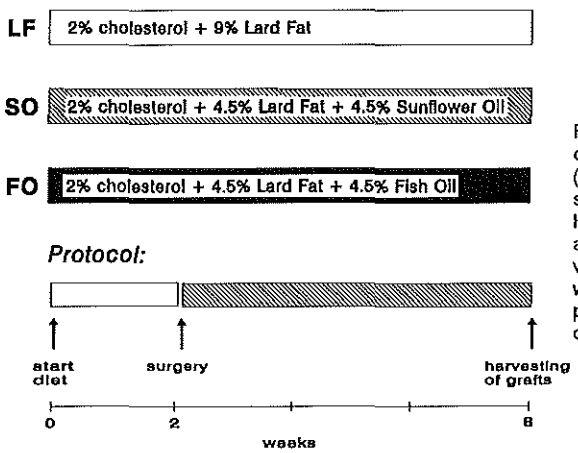

Fig 1. Schematic presentation of the three dietary groups achieved by replacing 4.5% (w/w) of lard fat with either 4.5% (w/w) of sunflower oil or 4.5% (w/w) of fish oil. Hypercholesterolemic diets were administered for 8 weeks, while saphenous veno-arterial bypass grafting took place 2 weeks after the onset of the experimental protocol. LF, lard fat group; SO, sunflower oil group; FO, fish oil group.

Materials and Methods

Animal care

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

Experimental groups and protocol

Göttingen mini-pigs (n=29, both female and castrated male), entered the study weighing between 18-20 kg and were allocated randomly to 3 groups (Fig. 1); a fish oil group (FO, n=8), a sunflower oil group (SO, n=9) and a lard fat group (LF, n=11). To obtain the 3 diet groups the animals were fed a low fat (<2%, w/w) base diet (Hope Farms BV, Woerden, The Netherlands), which contained 2% (w/w) cholesterol and either 4.5% (w/w) fish oil and 4.5% (w/w) lard fat (FO) or 4.5% (w/w) sunflower oil and 4.5% (w/w) lard fat (SO) or 9% (w/w) lard fat (LF; Table 1, Figure 1). The fatty acid profile (Table 2) of the dietary fish oil, sunflower oil and lard fat

Table 2. Fatty acid compositions of the fish oil, sunflower oil and lard fat added to the atherogenic diet, containing 2% (w/w) cholesterol and 4.5% (w/w) lard fat.

Fatty Acid	Fish Oil (mol %)	Sunflower Oil (mol %)	Lard Fat (mol %)
16:0	19.7	6.7	27.1
16:1ω7	8.5	0.1	2.2
18:0	3.7	3.9	15.0
18:1ω9	10.2	20.2	36.8
18:1ω7	4.5	0.8	2.9
18:2ω6	1.7	66.6	10.8
18:3ω3	1.1	-	1.0
20:5ω3	19.9	-	-
22:6ω3	10.6	-	-
others	2.1	1.7	4.2
PUFA/SFA ratio	1.42	6.28	0.29

PUFA=polyunsaturated fatty acids; SFA=saturated fatty acids.

resulted ultimately in a polysaturated/saturated fatty acid ratio of 0.49 for the lard fat diet, 1.50 for the sunflower oil diet and 0.83 for the fish oil diet. To compensate for the increased polyunsaturated/saturated fatty acid ratio in the diets different amounts of vitamin E (α-tocopherol) were added to the different diets. The α-, (β+γ)- and δ-tocopherol content of the fat/oils listed in Table 2 was determined by HPLC analysis and subsequent correction for relative biological activities of the different tocopherol components, yielded 0, 706 and 352 mg/kg α-tocopherol equivalents for lard fat, sunflower oil and fish oil, respectively. On basis of the

relative content of the polyunsaturated fatty acids with varying number of double bonds the minimally required α-tocopherol content was calculated to be 113, 595 and 860 mg/kg fat/oil. Because in the sunflower oil there was a 20% excess α-tocopherol, in the final calculation for matching the α-tocopherol contents a 20% excess was also provided for the lard fat and fish oil. This meant that we added 18.4, 9.2 and 45.9 mg α-tocopherol/kg in to the complete LF, SO and FO diets, respectively. It should also be noted that 80 mg/kg α-tocopherol was already present in the base diet (Table 1).

The experimental protocol lasted for 8 weeks, during which the animals received 500 g of forage per day. Before the animals were placed on their diet blood was drawn from the jugular vein for determination of total plasma cholesterol. After 2 weeks of diet, the animals received an autologous venous bypass graft in both carotid arteries and again blood samples were drawn to monitor plasma cholesterol levels. At the end of the 8-week protocol the animals were anesthetized with ketamine (700 mg) and sodium pentobarbital (10-15 mg/kg/hour), intubated and connected to a respirator. After blood samples were collected the grafts were dissected free and patency was evaluated by pulsation of the distal carotid artery or by blood flow through the graft. The left graft was perfusion fixed *in situ* with a pressure of 100 cm H_2O with 10% phosphate buffered formaline and stored until histological processing. The remaining saphenous vein from the right hindleg was taken as a negative control for the amount of intimal thickening. After the animals were killed with an overdose of sodium pentobarbital the left circumflex coronary artery and the aorta were harvested and used to assess the effect of the diets on development of atherosclerosis in normal vessels.

An additional group of 4 animals received a normocholesterolemic diet (N: Barley, 15g%; Rape seed extracted, 5g%; Tapioca, 30g%; Wheat, 12.6g%; Soybean toasted, 2.0g%; Soybean meal, 20.5g%; Meat meal, 3.9g%; Animal fat, 1.9g%; Molasses, 2.7g%; Wheat middlings, 4.2g%; $CaHPO_4.2H_2O$, 0.42g%;$CaCO_3$, 0.25g%; NaCl,iodinized, 0.35g%; Vitamin and trace element mixes, 1.00g% [see table 1]) for 8 weeks to evaluate the spontaneous development of atherosclerosis in the aorta (Table 1). Blood samples were taken from these animals, but they did not receive an autologous saphenous vein transplant.

Interposition of the saphenous vein into the carotid arteries

After overnight fasting the pigs were anesthetized with 500 mg of ketamine, intubated and connected to a respirator for ventilation with a mixture oxygen and nitrous oxide (1:2) and 2% of ethrane. The saphenous vein was extracted from the left hindleg over a length of approximately 8 cm (in situ) and carefully flushed with a saline solution containing 2% papaverine, split in half and preserved in the flush solution before implantation. Care was taken not to distend the vein graft to minimize endothelial damage. At the same time both carotid arteries were dissected free from their surroundings. After injection of 5,000 IU of heparin one carotid artery was clamped and a piece of the artery (approximately 3cm) removed. One half of the saphenous vein was interposed using end-to-end anastomoses. After this procedure was also

performed on the other artery, the animals were allowed to recover from surgery.

Plasma lipids

At the onset of the study and after 2 weeks blood samples were drawn from the subclavian vein for measuring total plasma cholesterol. After 8 weeks the blood samples were drawn from the superior caval vein and total cholesterol, free cholesterol and triglycerides, which were assayed enzymatically using kits (GHOD-PAP and GPO-PAP) from Boehringer GmbH, Mannheim, Germany. Phospholipids were assayed using the kit (PAP 150) from BioMérieux, Charbonnières, Les Bains, France.

For the male population (FO, SO and LF, n=6; N, n=4) plasma lipoproteins were separated by density-gradient ultracentrifugation into 4 fractions (VLDL, IDL, LDL and HDL).[9] The lipid composition was determined in each fraction using the same kits. Protein content of the different fractions was measured according to the method of Lowry et al. [10] The fatty acid composition (in mol%) of the phospholipids and cholesterolesters, extracted from the total plasma lipids, was determined by transmethylation with BF_3 in methanol and gaschromatography (CP-Sil 88 coated fised silicon capillary column from Chrompack, Middelburg, The Netherlands) as previously described.[11,12]

Intimal thickening of the venous bypass grafts, saphenous vein and coronary artery

The graft, the saphenous vein and the left circumflex coronary artery were imbedded in paraffine and transverse sections were made every 1 mm. The sections were routinely stained with haematoxylin-eosine (HE) and elastic von Gieson (EvG). The elastic stained slides were used to determine intimal thickening with a computer-assisted morphometric analysis system (Sigmascan/Image, Jandel Scientific GmbH, Erkrath, Germany). The area between the endothelial lining of the lumen and the internal elastic lamina (IEL) was taken as the intima area. The encroachment was defined as the ratio (x 100%) of the intima area and the corrected area within the IEL. The corrected area within the IEL was calculated from the perimeter of the IEL assuming that the IEL was a perfect circle. Mean intimal thickness was defined as the difference between the radius of the lumen and the radius of the IEL. Both radii were calculated from their respective perimeters. The lumen area was also calculated from the perimeter of the lumen. The media area was taken as the difference between the areas circumscribed by the internal and external elastic laminae.

Lipid infiltration of venous bypass graft, saphenous vein and aortic wall
Chemical analysis of the vessel wall

The graft in the right carotid artery and a part of the right saphenous vein were dissected free and frozen in liquid nitrogen before storage at -80 °C. For determining the effect of the lipid-enriched diets on lipid accumulation in spontaneous atherosclerosis the aorta was dissected

free and a longitudinal incision was made on the ventral side of the aorta. Inspection of the aorta showed the presence of macroscopic elevations (fatty streaks) in the abdominal aorta distal to the renal arteries (labelled lesions). In the absence of macroscopic elevations a representative biopsy was taken randomly from the infrarenal abdominal aorta. Samples from the suprarenal abdominal aorta served as control (non-lesions, Fig. 2). Samples of aortic lesions and non-lesions were dissected free of adventitia and directly frozen in liquid nitrogen and stored at - 80°C until analysis using the method of Bligh and Dyer for lipid extraction.[13] Briefly, tissue samples were homogenized in a Braun microdismembrator and the obtained powder was extracted with chloroform/methanol/saline (4:10:5, v/v/v). After centrifugation at 1500 g_{max} for 5 min and washing the pellet by rehomogenization in 1.9 ml of the same solvent, the two supernatants were combined and mixed with 1.5 ml chloroform and 1.5 ml saline. After vigorous vortexing and subsequent phase separation, the upper phase and the intermediate solid material were discarded. Subsequently the mixture was dried under nitrogen at 37°C and the residue dissolved in 0.2 ml 2-propranol. Cholesterol and cholesterolester, triglyceride and phospholipid contents were measured with enzymatic kits (see before). In the delipidized extracts protein and DNA contents were measured.[14]

Statistical analysis

All data are presented as mean ± standard error of the mean (SEM). The data were analyzed statistically using a one-way analysis of variance followed when appropriate by either the Student-Newman-Keuls procedure for multiple comparisons of mean values or by the Kruskall-Wallis test on ranks. Statistical significance was accepted at $P<.05$. Correlations were

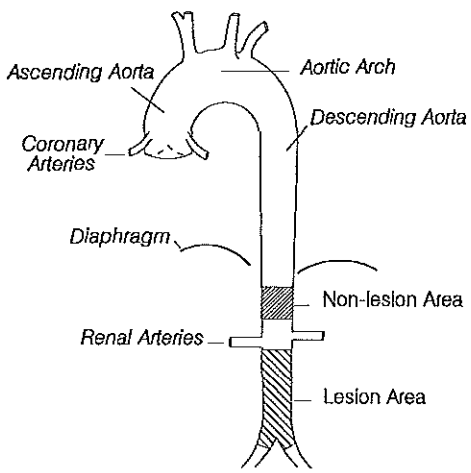

Fig 2. A schematic presentation of the location of the elevated patches on the luminal surface of the abdominal aorta (lesion area) and the macroscopically normal abdominal aorta (non-lesion area).

determined with the Spearman rank order correlation analysis.

Results

Patency of the grafts

Graft patency showed a marked difference between the groups. In the lard fat group 32% of the grafts were occluded, whereas in the sunflower and in the fish oil groups the percentages were 0% and 6%, respectively (both $P<.05$ vs lard fat group). Histological analysis showed not only that all occlusions were caused by early thrombosis of the graft, but also that, in some patent grafts, remnants of mural thrombi were present (results not shown). In 6 of the 11 grafts belonging to the lard fat group thrombosis was found, 4 of these grafts were not patent. Thrombosis was also found in 2 grafts in each of the other 2 diet groups, but these were all patent.

Fibrointimal hyperplasia

Fibrointimal hyperplasia in the vein grafts was assessed by determining luminal encroachment and intimal thickness (Table 3). No statistical difference was observed between the 3 diet groups even after exclusion of the thrombosed grafts. There were also no differences between the intima area or intimal thickness of the grafts of the different diet groups. Therefore, the tendency of a lower luminal encroachment in the fish oil treated group was more probably a refection of the larger lumen area and diameter in this group than of inhibition of fibrointimal hyperplasia.

Intimal hyperplasia was non-existent in the control vein (Table 4). To assess the effect of

Table 3. The effects of fish oil, sunflower oil and lard fat on luminal encroachment and size of the saphenous vein graft.

		Luminal encroachment (%)	Intimal Thickness (mm)	Area		
				Lumen (mm^2)	Intima (mm^2)	Media (mm^2)
Fish oil*	(n=8)	41 ± 11	-	11.2 ± 3.2	3.5 ± 0.6	2.7 ± 0.6
Sunflower oil*	(n=9)	44 ± 7	-	7.8 ± 2.5	4.7 ± 1.3	2.8 ± 0.2
Lard fat*	(n=11)	56 ± 9	-	4.4 ± 1.5	2.5 ± 0.5	1.4 ± 0.2
Without thrombosis:						
Fish oil†	(n=6)	26 ± 7	0.24 ± 0.06	14.6 ± 3.1	3.4 ± 0.7	3.2 ± 0.6
Sunflower oil†	(n=7)	39 ± 6	0.35 ± 0.04	8.7 ± 3.1	3.2 ± 0.2	2.9 ± 0.3
Lard fat†	(n=5)	29 ± 5	0.26 ± 0.06	8.7 ± 2.0	2.7 ± 0.7	2.0 ± 0.3

* Data are for all the grafts including the grafts occluded by thrombosis. † Data are for a subset of the grafts in which histological analysis revealed no signs of thrombosis. Values are mean±SEM.

the hypercholesterolemic diet and the different fatty acids on spontaneous atherosclerosis in the arterial vascular bed morphometry was also performed on the left circumflex coronary artery. In this last vessel slight intimal hyperplasia could be observed, but average luminal encroachment did not exceed 3.3±1.2% in any of the diet groups (Table 4).

Table 4. The effects of fish oil, sunflower oil and lard fat on luminal encroachment and size of the control vein and left circumflex coronary artery.

				Area	
		Luminal encroachment (%)	Lumen (mm^2)	Intima (mm^2)	Media (mm^2)
Vein					
Fish oil	(n=8)	0	0.62 ± 0.07	0	0.86 ± 0.07
Sunflower oil	(n=9)	0	0.44 ± 0.09	0	0.89 ± 0.06
Lard fat	(n=11)	0	0.51 ± 0.08	0	1.00 ± 0.09
Left circumflex coronary artery:					
Fish oil	(n=8)	2.4 ± 1.6	0.96 ± 0.10	0.02 ± 0.01	0.81 ± 0.07
Sunflower oil	(n=9)	3.3 ± 1.2	0.87 ± 0.14	0.03 ± 0.01	0.71 ± 0.04
Lard fat	(n=11)	3.2 ± 1.6	0.69 ± 0.15	0.04 ± 0.02	0.52 ± 0.10

Values are mean±SEM.

Lipid accumulation in the vein graft and the control vein

The effect of hypercholesterolemia and the different dietary fatty acids on the content of cholesterol, cholesterylesters, phospholipids and triglycerides in the graft and control vein are shown in Table 5. Grafting the saphenous vein into the arterial system produced an increase in cholesterolester content in all groups, but there was no difference between the groups in either the control vein or the graft. The triglyceride and phospholipid contents of the graft was unaffected by arterialisation in the sunflower oil and lard fat treated groups, but phospholipids in the fish oil treated group had increased ($P<.05$). The vein was unaffected by the 3 hypercholesterolemic diets. These results did not change when the lipid contents were expressed per g protein or per g DNA instead of per g wet weight.

Lipid accumulation in the aorta

Lipid accumulation was macroscopically observed as elevated patches on the luminal aspect of the abdominal aorta. All lesions were found between the renal arteries and the aortic bifurcation, but they were not detected in all animals. In the lard fat group they occurred in 8 of the 11 animals and in the sunflower oil group in 7 of the 9 animals, while in the fish oil group only 3 out of the 8 animals had these lesions ($P>.05$). In the normocholesterolemic group 3 out

Table 5. The effects of dietary fish oil, sunflower oil and lard fat on lipid accumulation ($\mu mol/g_{wet\ weight}$) in the control vein and the vein graft.

		Cholesterol	Cholesterolester	Cholesterol + Cholesterolester	Phospholipids	Triglycerides
Vein:						
Fish oil	(n=8)	4.69 ± 0.44	1.62 ± 0.45	6.31 ± 0.73	3.96 ± 0.37	3.41 ± 0.63
Sunflower oil	(n=9)	5.01 ± 0.26	1.86 ± 0.24	6.88 ± 0.41	4.40 ± 0.22	3.62 ± 0.57
Lard fat	(n=11)	5.22 ± 0.38	2.25 ± 0.34	7.47 ± 0.62	4.60 ± 0.26	8.23 ± 1.78
Vein graft:						
Fish oil	(n=8)	6.35 ± 0.51	3.26 ± 0.86*	9.61 ± 1.21	5.58 ± 0.24*	5.72 ± 1.93
Sunflower oil	(n=9)	6.55 ± 0.55	4.83 ± 0.98*	11.38 ± 1.45	5.05 ± 0.38	3.45 ± 0.88
Lard fat	(n=11)	5.72 ± 0.28	3.40 ± 0.58*	9.13 ± 0.78	4.73 ± 0.22	4.56 ± 1.71

Values are mean±SEM. *$P<.05$ vein graft versus control vein in the same diet group.

of the 4 animals presented themselves with these lesions. The occurrence of these lesions thus appeared to be independent of the cholesterol or the fatty acid content of the diets.

In the non-lesion areas no difference was observed in total cholesterol, phospholipid and triglycerides content of the aortic wall between the 4 groups (Fig. 3). Cholesterol and cholesterolester levels of the lesion areas had increased compared to the non-lesion areas in all 4 groups. However, whereas the fish oil group showed no significant differences compared to the diet with normal cholesterol, the cholesterol and cholesterolester contents of the lesions were significantly higher in both the sunflower oil and lard fat groups than in the normocholesterolemic and the fish oil diet. The phospholipid and triglyceride contents were unaffected by the diets (Fig. 3). These results did not change when the lipid contents were expressed per g protein or per g DNA instead of per g wet weight.

Fatty acid composition

Fatty acid composition of the cholesterolester and phospholipids in plasma qualitatively reflected the differences in fatty acid composition of the fish oil, sunflower oil or lard fat added to the base diet (Table 2). Addition of sunflower oil to the hypercholesterolemic diet caused the relative content of the total n-6 fatty acid pool of serum cholesterolester and phospholipids to increase at the expense of saturated and monounsaturated fatty acids. Addition of fish oil caused a relative increase in the total n-3 fatty acids, which only in the phospholipids was at the expense of n-6 fatty acids. In the grafts cholesterolester and phospolipids contained relatively more n-3 and n-6 polyunsaturated fatty acids (PUFA) in the fish oil and the sunflower oil groups, respectively. Generally, the incorporation of n-3 PUFA was at the expense of n-6 PUFA. Surprisingly, dietary sunflower oil had no effect on the relative n-6 PUFA of the phospholipid fraction of the aortic lesion, but clearly increased the relative n-6 PUFA content of the

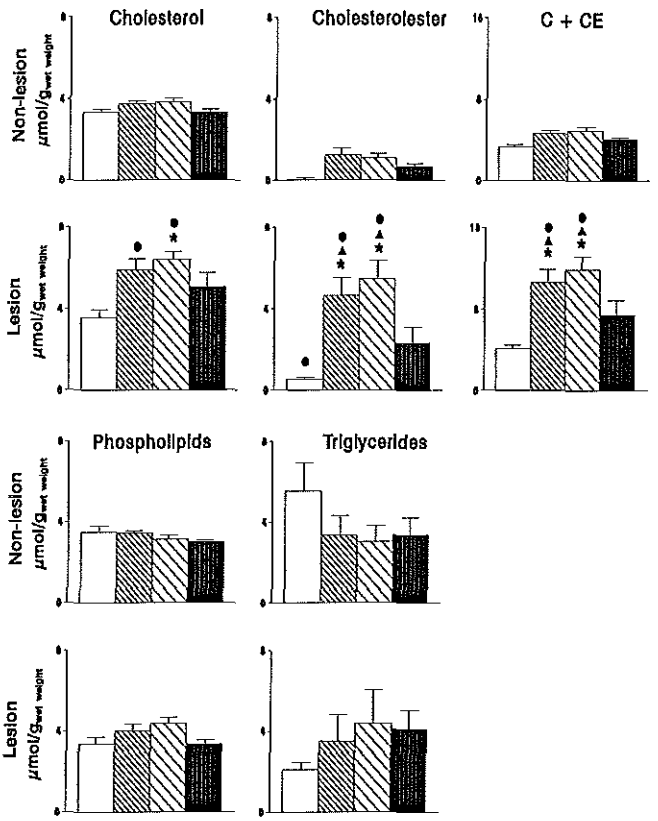

Fig 3. Bar graph showing the free cholesterol, esterified cholesterol, phospholipids and triglycerides content of the lesion and non-lesion areas of the abdominal aorta. □=Normocholesterolemic group, n=4; sunflower group, n=9; lard fat group, n=11; fish oil, n=8. Values are mean±SEM. *P<.05 vs the normolipidemic group. ▲ P<.05 vs the fish oil group. ● P<.05 lesion area vs non-lesion area within a dietary group.

phospholipids in the graft.

Plasma lipids

During high cholesterol feeding total plasma cholesterol increased more than 4-fold from 2.8±0.1 mmol/L at baseline to 14.5±0.1 mmol/L (P<.05, all animals combined). At the end of the dietary period there was no difference in total plasma cholesterol level in the 3 hypercholesterolemic groups, but the rate of increase in total plasma cholesterol was attenuated by fish oil as at 2 weeks the total plasma cholesterol level in the fish oil group was significantly lower than in the sunflower oil and lard fat group (Fig. 4).

With the normocholesterolemic diet, total plasma cholesterol was about equally distributed in the HDL and LDL lipoprotein fractions and little cholesterol was present in VLDL and IDL

Table 6. The effects of dietary fish oil, sunflower oil and lard fat on the polyunsaturated fatty acid composition (mol%) of the total lipid fraction of serum, vein grafts and aortic lesions.

	Fish Oil		Sunflower Oil		Lard Fat	
	CE	PL	CE	PL	CE	PL
Serum:						
SFA + MUFA	57 ± 1	60 ± 1	52 ± 2	57 ± 1	66 ± 1	64 ± 1
n-6 PUFA	33 ± 1	19 ± 1	48 ± 2	40 ± 1	34 ± 1	31 ± 1
n-3 PUFA	11 ± 1	21 ± 1	0 ± 1	3 ± 1	0 ± 1	5 ± 1
Vein graft:						
SFA + MUFA	71 ± 1	67 ± 1	64 ± 1	63 ± 1	78 ± 1	72 ± 2
n-6 PUFA	19 ± 1	22 ± 1	34 ± 1	35 ± 1	20 ± 1	25 ± 2
n-3 PUFA	10 ± 1	11 ± 1	2 ± 1	2 ± 1	2 ± 1	3 ± 1
Aortic lesions:						
SFA + MUFA	ND*	ND*	72 ± 2	67 ± 1	82 ± 3	68 ± 2
n-6 PUFA	ND	ND	26 ± 2	31 ± 1	17 ± 3	30 ± 2
n-3 PUFA	ND	ND	1 ± 1	2 ± 1	1 ± 1	3 ± 1

Fish oil, n=3; sunflower oil, n=3; lard fat, n=4; CE=cholesterolesters; PL=Phospholipids; SFA=saturated fatty acids; MUFA=monounsaturated fatty acids; PUFA=polyunsaturated fatty acids. ND=not detemined. * No accurate determination of fatty acids could be made of the cholesterolester in aortic lesions due to the low quantity. Values are mean ± SEM.

(Fig. 5). Feeding a 2% cholesterol diet markedly increased LDL cholesterol in all diet groups. In the fish oil group only LDL cholesterol increased significantly while in the sunflower and lard fat groups, in addition to LDL cholesterol, also VLDL and IDL cholesterol levels increased ($P<.05$ vs the normolipidemic animals). Only in the sunflower oil group there was an increase

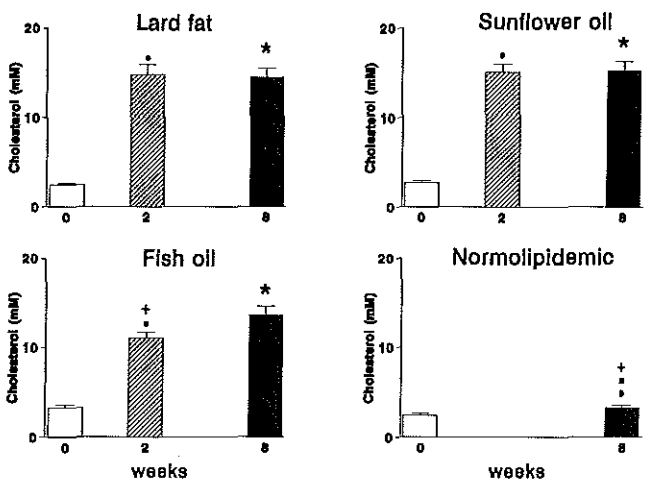

Fig 4. Plasma content of total cholesterol at the start of the experimental protocol (week 0), at the time of saphenous veno-arterial bypass grafting (week 2) and at the end of the experimental protocol (week 8) for the 3 high cholesterol diets groups and the normolipidemic diet group. Lard fat, n=11; sunflower oil, n=9; fish oil, n=8; normolipidemic, n=4. Values are mean±SEM. * $P<.05$ vs normolipidemic group. + $P<.05$ vs lard fat. ■ $P<.05$ vs sunflower oil group. ● $P<.05$ vs fish oil group.

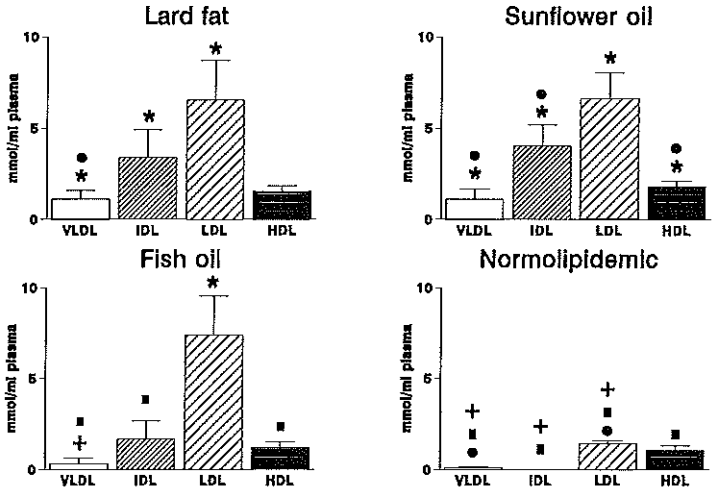

Fig 5. Bar graphs showing the distribution of plasma cholesterol between the different lipoprotein fractions of the male population (Fish oil, sunflower oil and lard fat groups, n=6; normocholesterolemic group, n=4). VLDL, very low density lipoproteins; IDL, intermediate density lipoproteins; LDL, low density lipoproteins; HDL, high density lipoproteins. Values are mean±SEM. * $P<.05$ vs normolipidemic group. * $P<.05$ vs lard fat. ■ $P<.05$ vs sunflower oil group. ● $P<.05$ vs fish oil group.

in HDL cholesterol. VLDL cholesterol was higher in the lard fat group than in the fish oil group. All lipoprotein fractions had, except for LDL, an increased cholesterol level in the sunflower group compared to the fish oil group (Fig. 5).

The chemical composition of the LDL and HDL particles was not altered by the high cholesterol diet or the different fatty acids in the hypercholesterolemic diet. The chemical composition of the IDL fraction in the 3 hypercholesterolemic diets did not differ. However, the VLDL fraction in the sunflower and lard fat groups showed a marked reduction in triglyceride content in favor of the cholesterol and cholesterolester contents. This reduction in VLDL triglyceride content was much less in the fish oil group and did not reach statistical significance when compared with the normocholesterolemic animals (Fig. 6).

Table 7. Correlations between the total cholesterol content of the different lipoprotein fractions and the total cholesterol, free cholesterol and cholesterolester content of the aortic lesions.

	AoTC		AoFC		AoCE	
Total cholesterol	r	p	r	p	r	p
VLDL	0.77	<0.001	0.77	<0.001	0.72	<0.001
IDL	0.62	0.002	0.64	0.001	0.62	0.002
LDL	0.21	0.342	0.24	0.278	0.28	0.210
HDL	0.65	<0.001	0.66	<0.001	0.68	<0.001

AoTC=total cholesterol content of the aortic lesion area; AoFC=free cholesterol content of the aortic lesion area; AoCE=cholesterolester content of the lesion area; VLDL=very low density lipoprotein; IDL=intermediate density lipoprotein; LDL=low density lipoprotein; HDL=high density lipoprotein.

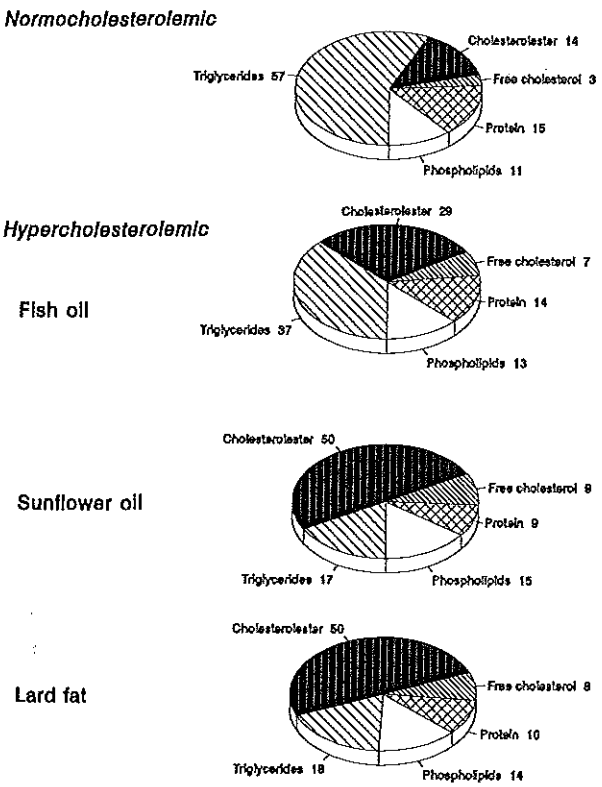

Fig 6. Pies showing the effects of dietary fish oil, lard fat and sunflower oil on the triglycerides, phopholipids, cholesterol and protein content of the VLDL fraction of the male population (Fish oil, sunflower oil and lard fat groups, n=6; normocholesterolemic group, n=4). The VLDL fraction of the normolipidemic group contained more triglycerides and less cholesterol than the VLDL fraction of the sunflower oil and lard fat groups ($P<.05$), while no difference was observed with the VLDL fraction of the fish oil group. However, there were also no difference in the phospholipd and protein composition of the VLDL fraction between the 3 hypercholesterolemic diet groups. VLDL, very low density lipoproteins.

Correlations between plasma lipids and lipid accumulation

Plasma VLDL cholesterol concentration correlated with the cholesterol content of the lesion area in the abdominal aorta (all animals combined, $r=0.77$, $p<0.001$, Table 7). HDL cholesterol levels was also correlated to the cholesterol content of the lesion area ($r=0.65$, $p<0.001$), but stepwise regression analysis showed that HDL did not decrease the variability, indicating that VLDL and HDL were dependent variables. LDL cholesterol concentration did not correlate with the cholesterol content of the aortic wall. Furthermore, the cholesterol content of the aortic lesion area showed a positive correlation with the cholesterolester content of VLDL ($r=0.75$, $p<0.001$). Because VLDL cholesterolester content showed a strong negative correlation with VLDL triglyceride content ($r=-0.98$, $p<0.001$), a significant negative correlation was found between VLDL triglyceride content and cholesterol content of the aortic lesion area ($r=-0.72$, $p<0.001$). No correlations were found between the cholesterol content of the control vein or vein graft and the cholesterol concentration in the different lipoprotein fractions.

Discussion

In the present study we observed that, while dietary fish oil (FO) and sunflower oil (SO) produced less thrombotic occlusions than lard fat (LF), there was no favorable effect of dietary fish oil on vein graft atherosclerosis as assessed with luminal encroachment, intimal thickness and the accumulation of total cholesterol, compared with sunflower oil and lard fat. However, the accumulation of total cholesterol in aortic lesions was markedly decreased by n-3 fatty acid intake. Furthermore, the aortic total cholesterol content was strongly correlated with VLDL cholesterol concentration.

Our findings in the grafts are in contrast to earlier reports on the effects of FO. In hypercholesterolemic dogs FO has consistently been found to reduce intimal thickening.[6-8] Because we feel that intimal thickness and luminal encroachment do not correctly reflect the morfometric changes in vessels, in the present study we also reported on luminal, intimal and media areas. The increase in intima area was similar for the 3 hypercholesterolemic diet groups, but luminal area in the FO group was consistently higher than with SO or LF, although statistical significance was not reached. Consequently, the average luminal encroachment and intimal thickness was lowest for the FO group compared to the SO and LF groups (see Table 3). However, this can not explain the discrepancy with earlier reports, as Sarris et al reported that the decrease in intimal thickening was accompanied by a decrease in intimal area.[6] The effect of fish oil on intimal thickening could be species related. Whereas pigs were used in the present study, all other studies were performed in dogs.[6-8] Dogs hardly developed arterial atherosclerosis even after high cholesterol feeding and the lesions usually involved the media.[5,15,16] Perhaps these

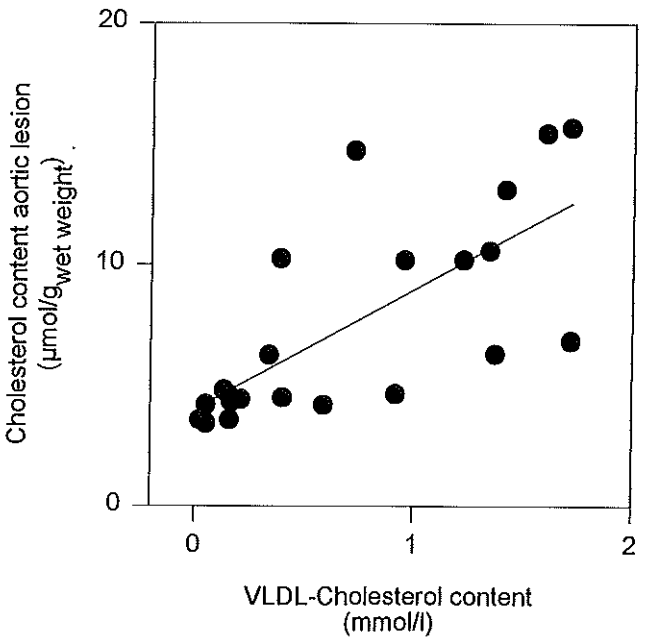

Fig 7. Graph showing the correlation between VLDL cholesterol concentration and cholesterol content of the aortic lesion in the male population (Fish oil, sunflower oil and lard fat groups, n=6; normocholesterolemic group, n=4)

characteristics also applied to vein graft atherosclerosis. Landymore et al, who also studied the vein grafts 6 weeks after implantation, reported an intimal thickness less than 40 μm,[7] while we observed a mean intimal thickness in the vein grafts between 220-300 μm. Even an induction period of 3 months and a 5% cholesterol diet, as employed by Sarris et al, resulted in an intimal thickness of only 149 ± 19 μm.[6]

Another key factor could be that in our experimental model plasma cholesterol levels were higher than reported for the hypercholesterolemic canine model.[6-8] High plasma cholesterol had been shown to positively correlate to progression of atherosclerotic lesions,[7] and could be a reason why we did not observe a favorable effect of FO in the graft. Furthermore, the type of vein used as an arterial conduit could also have played a role. We had chosen to employ the saphenous vein in accordance to clinical practice, while diaphanous veins such as femoral and jugular veins were used in the canine models. Diaphanous veins are more delicate and unaccustomed to hydrostatic pressure compared to extremity veins. The latter, therefore, have greater wall thickness and strength, and could respond differently to arterialization.[18]

The accumulation of total cholesterol in the graft was also not favorably affected by FO. Dietary intervention with FO, however, showed a significant effect in the abdominal aorta. In the normolipidemic group raised patches or lesions were macroscopically visible on the luminal aspect of the abdominal aorta, similar as those reported by Kobari et al[19] Compared to a macroscopically normal piece of aorta (non-lesion area) these lesions contained more cholesterolester. Feeding with diets containing 2% cholesterol further increased cholesterol content in the lesions, but the hypercholesterolemia-induced cholesterol accumulation in the lesion area was markedly inhibited by FO. This was likely due to a direct effect of n-3 fatty acids rather than an increase in polyunsaturated/saturated fatty acid ratio of the diet, because neither the SO nor the LF diet could emulate the favorable effect of FO. Furthermore, the cholesterol content of the aortic lesion area was positively correlated to plasma VLDL cholesterol concentration (Table 7), suggesting that VLDL cholesterol was a predicting factor for cholesterol accumulation in the aorta. This is further supported by our current finding that VLDL cholesterol was lowest in the FO group compared to LF and SO groups and that the positive correlation between VLDL cholesterol and aortic lesion cholesterol content remained even without the normocholesterolemic animals. In view of the age of the animals, which was between 10 to 12 months, these aortic lesions could be considered early atherosclerotic lesions and therefore this finding supports the report of Berenson et al, that low VLDL cholesterol levels were related to fatty streaks in the coronary arteries of persons, aged 6-30 years.[20] Our data also support the notion that the levels of cholesteryl ester rich VLDL particles relate to the severity of atherosclerotic lesions.[21] A positive correlation was also observed between plasma HDL cholesterol levels and cholesterol content of the aortic lesion area and this finding contested to some extend the protective nature of high plasma HDL cholesterol concentration. The increase in cholesterol content of the lesion area was similar to our reports on cholesterol accumulation in the abraded abdominal aorta of hypercholesterolemic swine. Sassen et al found that in

hypercholesterolemic swine endothelial denudation significantly increased the cholesterol content in the aortic wall after 8 months.[11] However, FO was unable to prevent the cholesterol accumulation in the abraded aorta. Although a different strain of pigs (Yorkshire x Landrace versus Göttingen miniature swine) and a longer induction period (8 months versus 2 months), the present favorable effect of FO in the lesion area of the aorta therefore suggests that intact endothelium is required for the effectiveness of FO in the inhibition of atherosclerosis.

The inhibition of aortic atherosclerosis in this experimental model raised the question why FO was ineffective in inhibiting lipid accumulation in the graft. During the surgical preparation the grafts were invariably traumatised,[1-3] and in response to this trauma an inflammatory reaction and wound healing would ensue with neutrophil and monocyte infiltration of the graft wall. The increased presence of macrophages together with the hypercholesterolemia may explain the increased cholesterol content of the graft. Another possibility could be the injured endothelial layer of the graft.[1-3,22,23] The PDAY (Pathobiological Determinants of Atherosclerosis in Youth) study reported that alterations of the endothelial function are implicated in the increased permeability of the endothelial barrier for lower-density lipoproteins and the development of early atherosclerotic lesions.[24,25] Ross et al posed the hypothesis that endothelial injury due to a hypercholesterolemic environment could enhance atherosclerosis.[27] In the graft endothelial injury would increase platelet aggregation. Several investigators have implicated platelet aggregation with fibrointimal hyperplasia, because anti-platelet therapy decreased fibrointimal thickening in the graft.[27-30] With the loss of the endothelial layer fish oil would loose a major point of action, such as reduction of platelet derived growth factor and increase in endothelium derived relaxing factor and prostaglandins, by which it could reduce fibrointimal hyperplasia, in the vein graft. That an intact endothelial layer could reduce intimal hyperplasia was demonstrated by Shiokawa et al, who observed an inverse correlation between the rate of re-endothelialization and intimal thickness in vein grafts.[31] In our hypercholesterolemic swine model the 6 weeks post surgery could prove to be too short for the endothelial layer, as well as endothelial function, to fully recover in the vein graft and hence for fish oil to show a marked difference in intimal hyperplasia. A longer post-operative period in the experimental protocol could therefore alter the outcome of the present study. A similar mechanism would also explain the recent finding that fish oil was effective in improving vein graft patency in patients after 1 year.[17]

We conclude that fish oil may act as a potent antiatherosclerotic agent, which action is likely to be mediated by a reduction in plasma VLDL cholesterol concentration and a change in the chemical composition of VLDL. Furthermore, the more pronounced antiatherosclerotic effect of fish oil in the aorta than in the vein graft suggests that an intact endothelium is a prerequisite for fish oil to be effective. The inhibition of atherosclerosis in the aorta is probably caused by a direct effect of n-3 fatty acids and not by an increase in the polyunsaturated/saturated fatty acid ratio in the diet.

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Chapter 9

Effect of Dietary Fish Oil on Regression of Vein Graft and Aortic Atherosclerosis: A Comparison with Sunflower Oil, Lard Fat and an Isocaloric Quantity of Carbohydrates

Running title: Regression of atherosclerosis and fish oil

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Effect of Dietary Fish Oil on Regression of Vein Graft and Aortic Atherosclerosis: A Comparison with Sunflower Oil, Lard Fat and an Isocaloric Quantity of Carbohydrates

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Background and aims of the study. Several studies have reported that in a hypercholesterolemic canine model fish oil inhibits vein graft atherosclerosis. Although in a previous study using hypercholesterolemic swine we could not show a favorable effect of fish oil on atherosclerosis in vein grafts, dietary fish oil was shown to enhance regression of atherosclerotic lesions induced by a low cholesterol diet. The effect of dietary fish oil on regression of vein graft atherosclerosis is unknown and is therefore the focus of attention in the present study. Furthermore, the effects of n-3 fatty acid-rich diet were compared with those obtained with n-6 fatty acid-rich diet, lard fat or an isocaloric quantity of carbohydrates. The effects of these diets on regression of atherosclerosis were also assessed in a control vein, the left circumflex coronary artery (LCXCA) and the abdominal aorta.

Methods and Results. Venoarterial grafts were implanted in both carotid arteries of pigs (n=47), which 2 weeks before surgery were started on a diet containing 2% cholesterol and 9% lard fat. Six weeks later the animals were randomly assigned to receive a low-cholesterol diet containing by weight either 4.5% fish oil and 4.5% lard fat (FO, n=9), 4.5% sunflower oil and 4.5% lard fat (SO, n=9), 9% lard fat (LF, n=9) or 4.5% lard fat and an isocaloric quantity of carbohydrates (CH, n=9) for an additional 8 weeks. A control group (C, n=11) were sacrificed before assignment to a low cholesterol diet. Although total cholesterol accumulation in the graft and a control vein decreased with all low cholesterol diets, luminal encroachment and intimal thickness remained unchanged. There were no differences in these parameters between the 4 low cholesterol diets either. Also the hypercholesterolemia-induced aortic lesions showed no reduction in total cholesterol content. Low cholesterol feeding decreased plasma cholesterol levels from 10.7±0.6 mM at the end of the induction period to 2.5±0.1 mM. Plasma VLDL-, IDL- and LDL-cholesterol concentrations decreased with all 4 diets, but only FO and CH had also lower HDL-cholesterol concentration. Furthermore, FO had a higher VLDL-cholesterol concentration than CH and a lower HDL--cholesterol concentration than LF. Compared to SO FO had lower LDL- as well as HDL-cholesterol concentrations. The chemical composition of the lipoproteins showed a higher free cholesterol content of the VLDL particle with FO and CH diets than with SO diet. Regression analysis indicated that VLDL-triglycerides and VLDL-total cholesterol concentration were positively correlated to cholesterol accumulation in the aorta ($r=0.77$, $p<0.001$, $r=0.62$, $p<0.01$, respectively), but did not correlate with vein graft total cholesterol.

Conclusions. The decrease in total cholesterol accumulation in the graft indicates that regression of vein graft atherosclerosis occurs with a cholesterol lowering diet, but independent of dietary fatty acids in our swine. In the present study regression has not been substantiated by a decrease in luminal encroachment or intimal thickness and thus precludes any definite conclusion about regression. The discrepancy between the efflux of total cholesterol from the aorta, vein graft and control vein may indicate that different mechanisms underly atherosclerosis in veins and arteries. Similar to our previous study high plasma VLDL concentrations can predict total cholesterol accumulation in the aortic wall, suggesting that in this animal model VLDL play a role in the development of arteriosclerosis.

The longterm patency of vein grafts is threatened by atherosclerosis in the transplanted vessels. Implicated in the development of atherosclerosis are several reactive processes. Early after coronary bypass surgery thrombosis is often found in the grafts and may trigger the atherosclerosis process, as the aggregation of platelets release amongst others growth factors. Subsequent fibrointimal hyperplasia and lipid deposition there within may eventually lead to atherosclerotic lesions, which are indistinguishable from atherosclerotic lesions in the nearby coronary arteries.[1,2] Next to prevention of graft atherosclerosis, inducing regression of graft atherosclerosis may also have a beneficial effect on longterm patency. Anti-platelet therapy has been shown to improve short term (up to 1 month) patency rates, but patency at 1 year does not benefit as much, probably because anti-platelet therapy does not affect proliferative processes.[3] Therefore, it is not likely that anti-platelet therapy will induce regression of graft atherosclerosis. Another option may be modification of plasma lipoproteins. Lowering of plasma total cholesterol has been implicated in inducing regression of atherosclerotic lesions. Blankenhorn et al showed that lowering of plasma cholesterol with colestipol-niacin therapy induces regression in coronary atherosclerosis, but only reduces progression of atherosclerosis in coronary venous bypass grafts.[4] Dietary fish oil may be more effective than cholesterol lowering agents alone, because it may target additional cellular and molecular mechanisms and thereby not only facilitate the removal of lipids from the vessel wall, but also inhibit ongoing proliferative processes. Several investigators have shown that fish oil exert an additional effect to lowering plasma cholesterol on regreession of atherosclerosis. For instance Zhu et al reported that aortic sudanophilic lesions induced by a high cholesterol diet were reduced in rabbits, when the animals were placed on a normal diet supplemented with fish oil or verapamil.[5] In swine Sassen et al showed that coronary atherosclerosis, which were induced by endothelial denudation and high cholesterol feeding, regressed on a low cholesterol diet containing fish oil.[6]

In the present study we investigated whether regression of atherosclerosis in saphenous vein grafts and in the aortic wall could be favorably influenced by dietary fish oil. Furthermore, we also studied whether altering the fatty acid profile of lipids in plasma lipoproteins and in the atherosclerotic lesions particularly as to the n-3 and n-6 fatty acids by either dietary fish oil, sunflower oil or lard fat could facilitate lipid removal from the saphenous vein graft and the aortic wall. An additional diet group, in which a portion of the dietary fat was replaced by an isocaloric amount of carbohydrates, was added to investigate whether a low fat, carbohydrate-enriched diet was also effective in regressing atherosclerosis in the studied vessels.

Materials and Methods

Animal care

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

Experimental groups and protocol

Göttingen mini-pigs (n=29, both female and castrated male), weighing 18-20 kg, were put on a diet, containing 2% (w/w) cholesterol and 9% (w/w) lard fat (Hope Farms BV, Woerden, The Netherlands). After 2 weeks the animals received an autologous venous bypass graft in both carotid arteries. For induction of atherosclerotic lesions in the graft the animals continued the same diet for another 6 weeks. Thereafter, these animals were randomized into 4 groups (Fig. 1); a fish oil group (FO, n=9), a sunflower oil group (SO, n=9), a lard fat group (LF, n=9) and

Table 1. Composition of the post-induction diets.

	Content (g%)			
	FO	SO	LF	CH
Ingredients				
Corn (extruded)	32	32	32	30
Wheat (extruded)	18	18	18	17
Soybean meal	14	14	14	13
Wheat middling	9	9	9	8
Dehydrated	14	14	14	13
skimmed milk	1.3	1.3	1.3	1.2
powder	1.1	1.1	1.1	1.0
$CaHPO_4.H_2O$	0.3	0.3	0.3	0.3
$CaCOO_3$	0.05	0.05	0.05	0.04
NaCl, iodinized	0.05	0.05	0.05	0.04
MgO	0.36	0.36	0.36	0.34
$MgSO_4$	0.18	0.18	0.18	0.17
$KH_2PO_4.2H_2O$	0.7	0.7	0.7	0.6
Choline chloride	4.5	4.5	9	4.5
50% (w/w)	4.5	-	-	-
Vitamin and trace	-	4.5	-	-
element mixes[a]	-	-	-	11
Lard fat				
Fish oil				
Sunflower oil				
Extra carbohydrates				

FO=fish oil diet; SO=sunflower oil diet; LF=lard fat diet CH=carbohydrate diet. [a]Vitamin and trace element mixes supply the following per 100 g diet: retinol 1400 IU; cholecalciferol 140 IU; α-tocopherol 8 mg; menadione 0.2 mg; thiamine hydrochloride 1.8 mg; riboflavin 1.8 mg; pyridoxine HCl 1.4 mg; niacin 3.6 mg; vitamin C coated 20 mg; calcium D-pantothenate 3.6 mg; folic acid 0.4 mg; cyanocobalamin 0.004 mg; biotin 0.1 mg; inositol 4.5 mg; iron subcarbonate (57% Fe) 9.1 mg; $FeSO_4.H_2O$ (30% Fe) 14 mg; $Cu_2(OH)_2CO_3$ (55% Cu) 2.3 mg; ZnO (78% Zn) 11 mg; MnO (62% Mn) 9.1 mg; $Na_2Se_2.5H_2O$ (45% Se) 0.08 mg; $Ca(IO_3)_2$ (65% I) 0.2 mg; $CoCO_3$ (47% Co) 0.09 mg. The composition is on an as fed basis.

a carbohydrates group (CH, n=9). For the subsequent 8 weeks the 2% cholesterol was removed from the diet and the animals were fed a diet, containing either 4.5% (w/w) fish oil and 4.5% (w/w) lard fat (FO) or 4.5% (w/w) sunflower oil and 4.5% (w/w) lard fat (SO) or 9% (w/w) lard fat (LF). In the carbohydrates group the 4.5% lard fat was replaced by an isocaloric amount of carbohydrates. The fatty acid profile (Table 2) of the dietary lard fat, sunflower oil and fish oil resulted ultimately in a polysaturated/saturated fatty acid ratio of 0.49 for the lard fat diet, 1.50 for the sunflower oil diet and 0.83 for the fish oil diet. To compensate for the increased polyunsaturated/saturated fatty acid ratio in the diets different amounts of vitamin E (α-tocopherol) were added to the different diets.The α-, (β+γ)- and δ-tocopherol content of the fat/oils listed in Table 2 was determined by HPLC analysis and subsequent correction for relative biological activities of the different tocopherol components, yielded 0, 706 and 352 mg/kg α-tocopherol equivalents for lard fat, sunflower oil and fish oil, respectively. Based upon the relative content of the polyunsaturated fatty acids with varying number of double bonds the minimally required α-tocopherol content was calculated to be 113, 595 and 860 mg/kg fat/oil. Because in the sunflower oil there was a 20% excess α-tocopherol, in the final calculation for

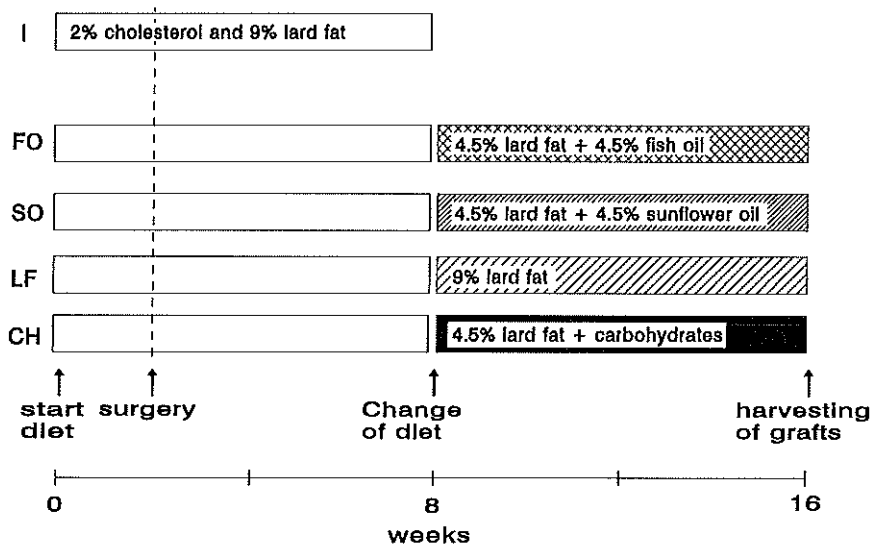

Fig 1. Schematic presentation of the experimental protocol and the diets of the different groups. A diet containing 2% cholesterol and 9% lard fat was administered for 8 weeks. Two weeks after the onset of the experimental protocol the animals received a saphenous veno-arterial bypass graft in both carotid arteries. In the post-induction period cholesterol was removed from the diet and the animals were randomly assigned to the 4 diet groups, fish oil group (FO), sunflower oil group (SO), lard fat group (LF) and a group in which 4.5% lard fat was replaced by an isocaloric quantity of carbohydrates (CH).

Table 2. Fatty acid compositions of the fish oil, sunflower oil and lard fat added to the atherogenic diet, containing 2% (w/w) cholesterol and 4.5% (w/w) lard fat.

Fatty Acid	Fish Oil (mol %)	Sunflower Oil (mol %)	Lard Fat (mol %)
16:0	19.7	6.7	27.1
16:1ω7	8.5	0.1	2.2
18:0	3.7	3.9	15.0
18:1ω9	10.2	20.2	36.8
18:1ω7	4.5	0.8	2.9
18:2ω6	1.7	66.6	10.8
18:3ω3	1.1	-	1.0
20:5ω3	19.9	-	-
22:6ω3	10.6	-	-
others	2.1	1.7	4.2
PUFA/SFA ratio	1.42	6.28	0.29

PUFA=polyunsaturated fatty acids; SFA=saturated fatty acids.

matching the α-tocopherol contents a 20% excess was also provided for the lard fat and fish oil. This meant that we added 18.4, 9.2 and 45.9 mg α-tocopherol/kg to the complete LF, SO and FO diets, respectively. For the CH diet similar amounts of α-tocopherol as in the SO diet was added. It should be noted that 80 mg/kg α-tocopherol was already present in the base diet (Table 1).

At the end of the protocol the animals were anesthetized with ketamine (700 mg) and sodium pentobarbital (10-15 mg/kg/hour), intubated and connected to a respiratory ventilator. The venous bypass grafts were dissected free and patency was evaluated by pulsation of the distal carotid artery or by blood flow through the graft. The left graft was perfusion fixed in situ with a pressure of 100 cm of H_2O with a 10% phosphate-buffered formaline and stored until histological processing. The remaining saphenous vein from the right hindleg was taken as a negative control for the amount of intimal thickening. After an overdose of sodium pentobarbital the left circumflex coronary artery (LCXCA) and the aorta were harvested. These vessels were used to determine regression of atherosclerosis in this model, which resulted from the hypercholesterolemia during the first 8 weeks of the experimental protocol. Blood samples were drawn from the jugular vein at the beginning of the protocol, just before surgery, at 8 weeks and before the animals were sacrificied to monitor plasma cholesterol and triglycerides levels.

A control group consisting of 11 animals was sacrificed after the induction period to determine the level of atherosclerosis in the vein grafts, control vein, left circumflex coronary artery and aorta, and was considered to be the baseline level to which the regresssion was rated

(induction group). These animals were also enrolled in a previous study investigating the effect of fish oil on the development of atherosclerosis in saphenous vein grafts (unpublished data).

Interposition of the saphenous vein into the carotid arteries

After overnight fasting the pigs were anesthetized with 500 mg of ketamine, intubated and connected to a respirator for ventilation with a mixture of oxygen and nitrous oxide (1:2) and 2% of ethrane. The saphenous vein was extracted from the left hindleg over a length of approximately 8 cm (in situ) and carefully flushed with a saline solution containing 2% papaverine, split in half and preserved in the flush solution before implantation. Care was taken not to distend the vein graft to minimize endothelial damage. At the same time both carotid arteries were dissected free from their surroundings. After injection of 5,000 IU of heparin one carotid artery was clamped and a piece of the artery (approximately 3cm) removed. One half of the saphenous vein was interposed using end-to-end anastomoses. After this procedure was also performed on the other artery, the animals were allowed to recover from surgery.

Plasma lipids

At the onset of the study and after 2 weeks blood samples were drawn from the subclavian vein for measuring total plasma cholesterol. After 8 weeks the blood samples were drawn from the superior caval vein and total cholesterol, free cholesterol and triglycerides, which were assayed enzymatically using kits (GHOD-PAP and GPO-PAP) from Boehringer GmbH, Mannheim, Germany. Phospholipids were assayed using the kit (PAP 150) from BioMérieux, Charbonnières, Les Bains, France.

In the male population (FO, SO, LF and CH, n=5) plasma lipoproteins were separated by density-gradient ultracentrifugation into 4 fractions (VLDL, IDL, LDL and HDL).[7] The lipid composition was determined in each fraction using the same kits. Protein content of the different fractions was measured according to the method of Lowry et al. [8] The fatty acid composition of the phospholipids and cholesterolesters, extracted from the total plasma lipids, was determined by transmethylation with BF_3 in methanol and gaschromatography (CP-Sil 88-coated fused silicon capillary column from Chrompack, Middelburg, The Netherlands) as previously described.[9,10]

Intimal thickening of the venous bypass grafts, saphenous vein and coronary artery

The graft, the saphenous vein and the LCXCA were imbedded in paraffine and transverse sections were made every 1 mm. The sections were routinely stained with haematoxylin-eosine (HE) and elastic von Gieson (EvG). The elastic stained slides were used to determine intimal thickening with a computer-assisted morphometric analysis system (Sigmascan/Image, Jandel Scientific GmbH, Erkrath, Germany). The area between the endothelial lining of the lumen and the internal elastic lamina (IEL) was taken as the intima area. The encroachment was defined as the ratio (x 100%) of the intima area and the corrected area within the IEL. The corrected area

within the IEL was calculated from the perimeter of the IEL assuming that the IEL was a perfect circle. Mean intimal thickness was defined as the difference between the radius of the lumen and the radius of the IEL. Both radii were calculated from their respective perimeters. The lumen area was also calculated from the perimeter of the lumen. The media area was taken as the difference between the areas circumscribed by the internal and external elastic laminae.

Lipid infiltration of venous bypass graft, saphenous vein and aortic wall

The graft in the right carotid artery and a part of the right saphenous vein were dissected free and frozen in liquid nitrogen before storage at -80 °C. For determining the effect of the lipid-enriched diets on lipid accumulation in spontaneous atherosclerosis the aorta was dissected free and a longitudinal incision was made on the ventral side of the aorta. Inspection of the aorta showed the presence of macroscopic elevations (fatty streaks) in the abdominal aorta distal to the renal arteries (labelled lesions). In the absence of macroscopic elevations a representative biopsy was taken randomly from the infrarenal abdominal aorta. Samples from the suprarenal abdominal aorta served as control (non-lesions, Fig. 2). Samples of aortic lesions and non-lesions were dissected free of adventitia and directly frozen in liquid nitrogen and stored at - 80°C until analysis using the method of Bligh and Dyer for lipid extraction.[11] Briefly, tissue samples were homogenized in a Braun microdismembrator and the obtained powder was extracted with chloroform/methanol/saline (4:10:5, v/v/v). After centrifugation at 1500 g_{max} for 5 min and washing the pellet by rehomogenization in 1.9 ml of the same solvent, the two supernatants were combined and mixed with 1.5 ml chloroform and 1.5 ml saline. After vigorous vortexing and

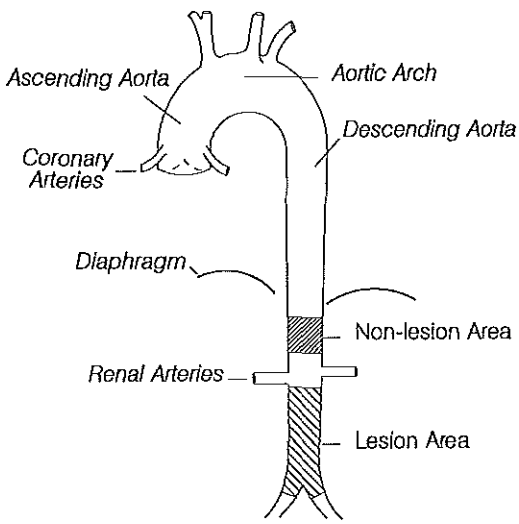

Fig 2. A schematic presentation of the location of the elevated patches on the luminal surface of the abdominal aorta (lesion area) and the macroscopically normal abdominal aorta (non-lesion area).

subsequent phase separation, the upper phase and the intermediate solid material were discarded. Subsequently the mixture was dried under nitrogen at 37°C and the residue dissolved in 0.2 ml 2-propranol. Cholesterol and cholesterolester, triglyceride and phospholipid contents were measured with enzymatic kits (see before). In the delipidized extracts protein and DNA contents were measured.[9]

Statistical analysis

All data are presented as mean ± standard error of the mean (SEM). The data were analyzed statistically using a one-way analysis of variance followed when appropriate by either the Student-Newman-Keuls procedure for multiple comparisons of mean values or by the Kruskall-Wallis test on ranks. Statistical significance was accepted at $P<.05$. Correlations were determined with the Spearman rank order correlation analysis.

Results

Patency of the grafts

In the induction group sacrificied 6 weeks post-surgery occlusion rate of the vein grafts was 32%. The removal of the 2% cholesterol from the diet with or without a partial replacement of dietary lard fat resulted in occlusion rates of 25%, 19%, 6% and 25% for the fish oil, sunflower oil, lard fat and carbohydates groups, respectively ($P>.05$).

Table 3. The effects of fish oil, sunflower oil, lard fat and carbohydrates on regression of luminal encroachment and size of the saphenous vein graft after withdrawal of cholesterol from the diet.

		Luminal encroachment (%)	Intimal Thickness (mm)	Area Lumen (mm^2)	Area Intima (mm^2)	Area Media (mm^2)
Induction:						
2% cholesterol + 9% lard fat	(n=11)	56 ± 9	0.26 ± 0.06	4.4 ± 1.5	2.5 ± 0.5	1.4 ± 0.2
Regression:						
Fish oil	(n=9)	35 ± 11	0.31 ± 0.05	6.8 ± 2.7	2.9 ± 0.5	1.9 ± 0.2
Sunflower oil	(n=9)	41 ± 10	0.21 ± 0.04	4.4 ± 1.8	1.8 ± 0.4*	1.7 ± 0.3
Lard fat	(n=9)	38 ± 8	0.36 ± 0.03	6.4 ± 2.2	3.8 ± 0.4	1.7 ± 0.1
Carbohydrates	(n=9)	41 ± 10	0.23 ± 0.04	4.9 ± 1.2	2.0 ± 0.3*	1.7 ± 0.2

Values are mean±SEM. *$P<.05$ vs. The lard fat group.

Fibrointimal hyperplasia

The effect of the different diets in the post-induction period on fibrointimal hyperplasia was assessed with luminal encroachment, intimal thickness and intima area. None of the diets showed a significant decrease versus the induction group in any of these parameters. Although no difference was observed in luminal encroachment and mean intimal thickness between the regression groups themselves, intima area in the LF group was significantly larger than that in the SO and CH groups (Table 3).

In Table 4 is shown that the control vein was not affected by the 4 regression diets compared to the induction group. Despite feeding of the low cholesterol diets luminal encroachment tended to increase in the LCXCA, but statistical significance was not reached. Surprisingly, lumen and media area of the LCXCA in the regression groups were significantly higher than those in the induction group (Table 4).

Table 4. The effects of fish oil, sunflower oil and lard fat on luminal encroachment and size of the control vein and left circumflex coronary artery.

		Luminal encroachment (%)	Lumen (mm^2)	Area Intima (mm^2)	Area Media (mm^2)
Vein					
Induction	(n=11)	0	0.51 ± 0.08	0	1.00 ± 0.09
Fish oil	(n=9)	0	0.65 ± 0.13	0	0.88 ± 0.07
Sunflower oil	(n=8)	0	0.55 ± 0.10	0	0.93 ± 0.08
Lard fat	(n=8)	0	0.34 ± 0.05	0	0.80 ± 0.08
Carbohydrates	(n=8)	0	0.29 ± 0.04	0	0.76 ± 0.05
Left circumflex coronary artery:					
Induction	(n=11)	3.2 ± 1.6	0.69 ± 0.15	0.04 ± 0.02	0.52 ± 0.10
Fish oil	(n=8)	4.4 ± 1.7	1.53 ± 0.12*	0.08 ± 0.04	1.00 ± 0.08*
Sunflower oil	(n=9)	4.6 ± 1.1	1.23 ± 0.14*	0.06 ± 0.02	0.88 ± 0.06*
Lard fat	(n=9)	10.5 ± 2.7	1.28 ± 0.09*	0.16 ± 0.05	1.03 ± 0.07*
Carbohydrates	(n=9)	6.0 ± 1.4	1.29 ± 0.09*	0.08 ± 0.02	0.91 ± 0.06*

Values are mean±SEM. *P<.05 vs. Induction group.

Table 5. The effects of dietary fish oil, sunflower oil, lard fat and carbohydrates on lipid accumulation in the control vein and the vein graft after induction of lipid accumulation with a diet containing 2% cholesterol and 9% lard fat.

		Cholesterol (μmol/g)	Cholesterolester (μmol/g)	Cholesterol + Cholesterolester (μmol/g)	Phospholipids (μmol/g)	Triglycerides (μmol/g)
Vein:						
Induction	(n=11)	5.22 ± 0.38	2.25 ± 0.34	7.47 ± 0.62	4.60 ± 0.26	8.23 ± 1.78
Fish oil	(n=9)	4.52 ± 0.70	0.00 ± 0.13*	4.50 ± 0.59*	4.39 ± 0.77	2.27 ± 0.39*
Sunflower oil	(n=9)	5.19 ± 0.74	0.00 ± 0.05*	5.17 ± 0.76*	5.43 ± 0.77	1.43 ± 0.25*
Lard fat	(n=9)	3.94 ± 0.29	0.02 ± 0.06*	3.96 ± 0.31*	4.30 ± 0.22	2.93 ± 1.12*
Carbohydrates	(n=9)	4.06 ± 0.22	0.16 ± 0.03*	4.22 ± 0.21*	3.58 ± 0.43	2.93 ± 0.54*
Vein graft:						
Induction	(n=11)	5.72 ± 0.28	3.40 ± 0.58	9.13 ± 0.78	4.73 ± 0.22	4.56 ± 1.71
Fish oil	(n=9)	3.68 ± 0.31*	0.59 ± 0.30*	4.27 ± 0.55*	3.50 ± 0.33	3.87 ± 1.78
Sunflower oil	(n=9)	4.45 ± 0.43*	0.23 ± 0.45*	4.68 ± 0.37*	4.20 ± 0.47	9.02 ± 3.57
Lard fat	(n=9)	4.24 ± 0.24*	1.28 ± 0.30*	5.53 ± 0.29*	3.65 ± 0.36	4.86 ± 2.91
Carbohydrates	(n=9)	3.73 ± 0.18*	0.55 ±0.15*	4.28 ± 0.25*	3.99 ± 0.39	6.31 ± 2.38

Values are mean±SEM. *P<.05 versus Induction group.

Lipidaccumulation in the vein graft and the control saphenous vein

The induction diet of 2% cholesterol and 9% of lard fat led to a total cholesterol content of 7.47 ± 0.62 μmol/g and 9.13 ± 0.78 μmol/g for the saphenous vein and vein graft, respectively (Table 5, Fig 3). Eight weeks after changing the diets total cholesterol of the saphenous vein decreased significantly in all regression groups, which was mainly due to a decrease in cholesterolester content. The triglycerides content of the saphenous vein also decreased compared to the induction group (P<.05). In the vein grafts total cholesterol content also decreased during the regression period of the experimental protocol. In contrast to the control vein this was due to a decrease in both free and esterified cholesterol. The cholesterolester content of the vein graft, however, remained significantly increased versus the control vein. The decreases induced by the different regression diets were not significantly different in both the saphenous vein and the vein graft.

Lipidaccumulation in the aorta

In Table 6 the effects of the induction diet and the regression diets on lipid accumulation in the abdominal aorta are shown. Dietary FO, SO and LF had no effects on lipidaccumulation in the non-lesion and lesion area compared to the induction group. Only the carbohydrates group had a lower cholesterolester content of the non-lesion area than the induction group. In all regression groups the lesion area still contained more cholesterolester than in the non-lesion area.

Fatty acid composition of the lipids in the vein grafts and aorta

The fatty acid composition of the cholesterolester and phopholipids in the vein grafts were

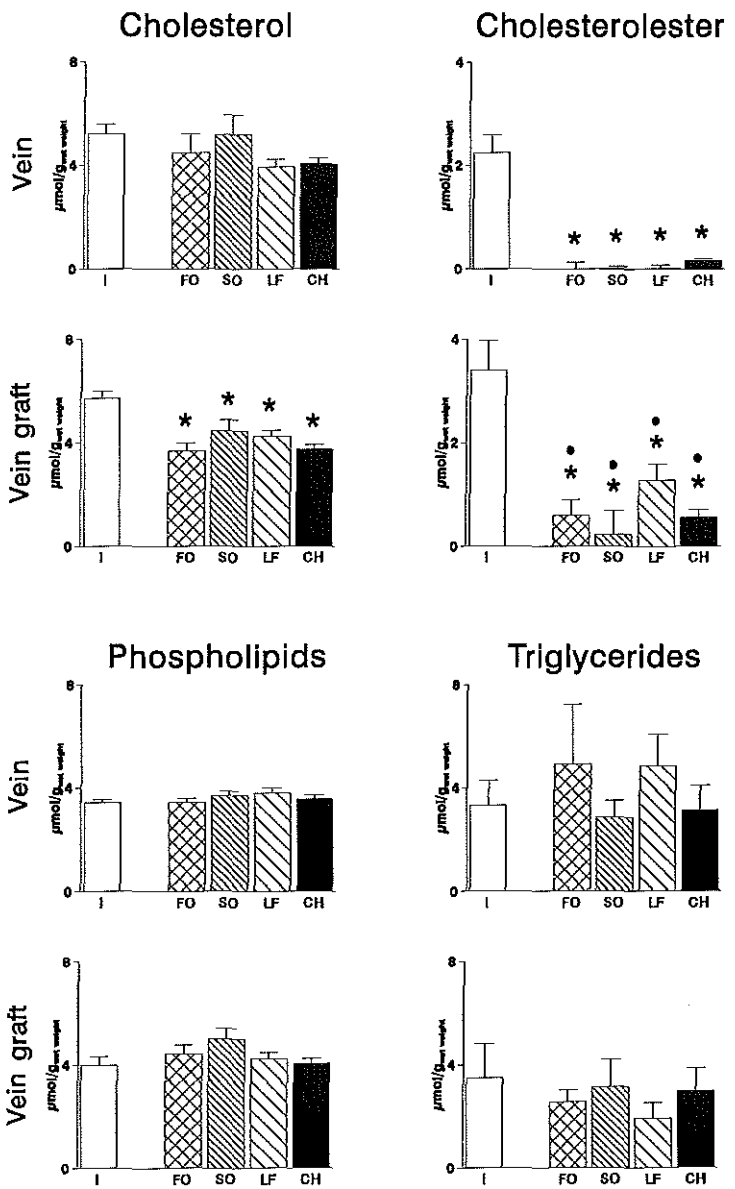

Fig 3. Bar graph showing the free cholesterol, esterified cholesterol, phospholipids and triglycerides content of the vein and the vein graft. I=induction group (n=11); FO=fish oil group (n=9); SO=sunflower oil group (n=9); LF=lard fat group (n=9); CH=carbohydrate group (n=9).Values are mean±SEM. *P<.05 vs the induction group; ● P<.05 vein graft vs vein within a dietary group.

Table 6. The effects of dietary fish oil, sunflower oil, lard fat and carbohydrates on lipid accumulation in the lesion and the non-lesion areas of the abdominal aorta after induction of lipidaccumulation with a diet containing 2% cholesterol and 9% lard fat.

		Cholesterol (µmol/g)	Cholesterolester (µmol/g)	Cholesterol + Cholesterolester (µmol/g)	Phospholipids (µmol/g)	Triglycerides (µmol/g)
Non-lesion area:						
Induction	(n=11)	3.92 ± 0.19	1.24 ± 0.35	5.16 ± 0.53	3.42 ± 0.12	3.32 ± 0.98
Fish oil	(n=9)	3.71 ± 0.27	0.33 ± 0.24	4.04 ± 0.49	3.43 ± 0.16	4.93 ± 2.32
Sunflower oil	(n=9)	4.11 ± 0.27	0.76 ± 0.18	4.86 ± 0.41	3.70 ± 0.19	2.83 ± 0.66
Lard fat	(n=9)	3.96 ± 0.17	0.50 ± 0.19	4.45 ± 0.29	3.79 ± 0.22	4.85 ± 1.23
Carbohydrates	(n=9)	3.62 ± 0.15	0.24 ± 0.07*	3.85 ± 0.18	3.54 ± 0.18	3.14 ± 0.96
Lesion area:						
Induction	(n=11)	5.46 ± 0.49	4.03 ± 0.74	9.50 ± 1.18	3.99 ± 0.35	3.50 ± 1.32
Fish oil	(n=9)	6.52 ± 0.96'	2.64 ± 1.01'	9.16 ± 1.91'	4.41 ± 0.37	2.58 ± 0.43
Sunflower oil	(n=9)	7.26 ± 0.79'	4.10 ± 0.56'	11.36 ± 1.32'	4.99 ± 0.43'	3.10 ± 1.08
Lard fat	(n=9)	5.68 ± 0.59'	2.40 ± 0.71'	8.08 ± 1.28'	4.23 ± 0.26	1.92 ± 0.60
Carbohydrates	(n=9)	4.89 ± 0.41'	1.86 ±0.42'	6.75 ± 0.79'	4.02 ± 0.23	3.00 ± 0.86

Values are mean±SEM. *P<.05 versus induction group. ' P<.05 lesion area vs non-lesion area within a diet group.

similar for the SO and LF groups (Table 7). Replacement of a half of the dietary lard fat with fish oil caused a relative increase in total n-3 fatty acids and a relative decrease in total n-6 fatty acids incorporated in the cholesterolester and phospholipids of the vein graft. No determinations in the aortic lesions of the fish oil group had been carried out. In the aortic lesions there was no difference in the n-6 fatty acid pool of the phospholipids between SO and LF. However, the fatty acid composition of the cholesterolesters showed a marked increase in n-6 polyunsaturated fatty acid incorporation at the expense of saturated and monounsaturated fatty acids.

Table 7. Polyunsaturated fatty acid content (mol%) of the lipids in vein grafts and aortic lesions.

	Fish oil (n=3)		Sunflower oil (n=3)		Lard Fat (n=3)	
	CE	PL	CE	PL	CE	PL
Vein grafts:						
SFA + MUFA	67 ± 2	71 ± 1	58 ± 3	67 ± 1	61 ± 3	67 ± 2
n-6 PUFA	29 ± 2	21 ± 1	39 ± 3	30 ± 1	37 ± 3	30 ± 2
n-3 PUFA	4 ± 1	8 ± 1	2 ± 1	3 ± 1	2 ± 1	3 ± 1
Aortic lesions:						
SFA + MUFA	-*	-*	64 ± 4	66 ± 1	82 ± 3	69 ± 1
n-6 PUFA	-	-	35 ± 4	32 ± 1	16 ± 3	29 ± 1
n-3 PUFA	-	-	1 ± 1	2 ± 1	1 ± 1	2 ± 1

CE=cholesterol ester; PL=phospholipids; SFA=saturated fatty acids; MUFA=monounsaturated fatty acids; PUFA=polyunsaturated fatty acids. - = not determined. * No determination of fatty acids was made of the cholesterolester in aortic lesions of the fish oil group. Values are mean ± SEM.

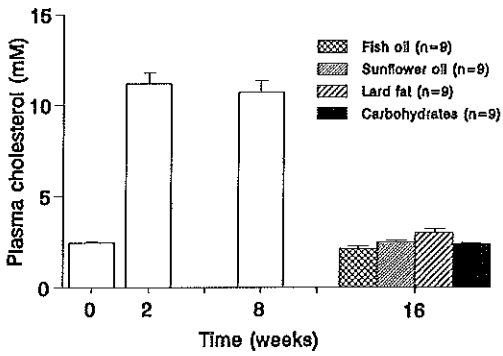

Fig 4. Bars representing the plasma cholesterol concentration before administration of the induction diet (week 0), at the time of surgery (week 2), at the end of the induction period (week 8) and at the end of the experimental protocol (week 16). The values at week 0, 2 and 8 weeks are the average for all the animals, because randomization happens at 8 weeks. In the lard fat group plasma cholesterol concentration did not completely returned to levels measured at week 0, but analysis with a paired t-test between the plasma cholesterol concentration at week 0 and week 16, but statistical significance was not reached (P<.05). Values are mean±SEM.

Plasma lipids

During the induction period total plasma cholesterol increased from 2.4±0.1 mM to 10.7±0.6 mM at baseline and at week 8, respectively. Low cholesterol feeding reduced an average total plasma cholesterol to 2.5±0.1 mM for all diets combined (Fig. 4, P<.05 vs plasma cholesterol at the end of the induction period). No difference was observed between the different regression diets.

Plasma VLDL-, IDL- and LDL-cholesterol concentrations decreased with all 4 regression diets compared to the induction group, but only the FO and CH groups had lower HDL cholesterol concentrations (Table 8). Furthermore, FO feeding resulted in higher VLDL cholesterol concentration than CH feeding and in lower HDL cholesterol concentration than LF feeding. Compared to the SO group, FO lowered LDL- and HDL-cholesterol concentrations. The HDL fraction in the CH group also contained less cholesterol than in the SO group. Between the SO and LF groups no differences were observed.

Table 8. The effects of dietary fish oil, sunflower oil, lard fat and carbohydrates on the distribution of cholesterol over the different lipoprotein fraction.

		lipoprotein fractions			
		VLDL	IDL	LDL	HDL
Induction	(n=6)	1.11 ± 0.48	3.41 ± 1.49	6.54± 2.14	1.56 ± 0.28
Fish oil	(n=5)	0.08 ± 0.05*	0.02 ± 0.01*	0.88 ± 0.15*	0.80 ± 0.24*
Sunflower oil	(n=5)	0.05 ± 0.02*	0.01 ± 0.01*	1.24 ± 0.28*	1.41 ± 0.25*‡
Lard Fat	(n=5)	0.03 ± 0.01*	0.04 ± 0.04*	1.58 ± 0.56*‡	1.23 ± 0.08*
Carbohydrates	(n=5)	0.03 ± 0.01*‡	0.01 ±0.01*	1.12 ± 0.20*	1.05 ± 0.18*

Induction=the animals, that were fed a diet containing 2% cholesterol and 9% lard fat. VLDL=very low density lipoproteins; IDL=intermediate density lipoproteins; LDL=low density lipoproteins; HDL=high density lipoproteins. *P<.05 vs induction; ‡P<.05 vs fish oil; †P<.05 vs carbohydates

The different fatty acids in the regression diet induced marked changes in the chemical composition of the VLDL fraction. In the FO and CH groups free cholesterol and phospholipid content increased compared to the SO group. Furthermore the VLDL fraction of the CH group also contained less triglycerides than that of the SO group (Fig 5). The chemical composition of the IDL, LDL and HDL fraction did not differ between the FO, SO and LF groups. The CH group contained more free cholesterol in the IDL and LDL fractions. In the HDL fraction free cholesterol of the CH group was only higher when compared to the LF group.

Correlations between plasma lipids and lipid content of the various vessel

The cholesterol accumulation in the aortic lesions and the control vein correlated with the triglyceride and cholesterol concentrations in the VLDL fraction (for all regression groups $r=0.77$, $p<0.01$ and $r=0.62$, $p<0.01$, respectively). VLDL concentration, however, did not correlate with the cholesterol content of the vein graft. Instead cholesterol content of the graft showed a better correlation with the LDL cholesterolester concentration ($r=0.48$, $P<.05$). HDL

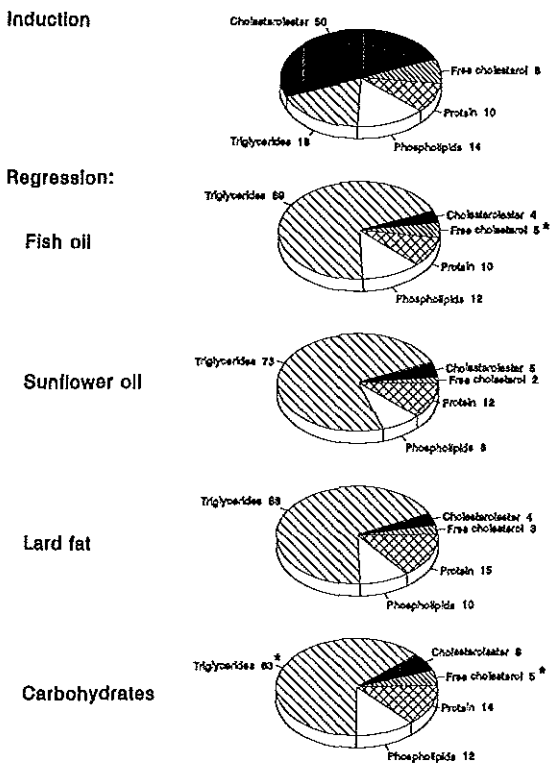

Fig 5. Pies showing the effects of the induction diet and the subsequent dietary intervention with dietary fish oil, sunflower oil, lard fat and an isocaloric quantity of carbohydrates on the triglyceride, phopholipid, free cholesterol, cholesterolester and protein content of the VLDL fraction of the male population (For all groups, n=5). *$P<.05$ vs sunflower oil.

cholesterolester concentration showed a positive correlation with the cholesterol content of the aorta, graft and control vein (Table 9).

Discussion

The most important finding in this study was that in a direct comparison with sunflower oil (SO), lard fat (LF) and an isocaloric quantity of carbohydrates (CH) fish oil (FO) did not enhance the effects of a cholesterol lowering diet on promoting regression of atherosclerosis. However, the effects of cholesterol lowering diet on of atherosclerosis in the vein graft were not consistent. While there was a marked decrease in cholesterol content of the vein graft, luminal encroachment and intimal thickness of the vessel remained unaffected and therefore did not substantiate "regression" of graft atherosclerosis. Similar observations were also made in the control vein. On the other hand in the left circumflex coronary artery (LCXCA) lumen and media area increased significantly, but luminal encroachment showed a tendency to increase during the post-induction period compared to the induction group. Total cholesterol

Table 9. Correlations between the total cholesterol content of the different lipoprotein fractions and the total cholesterol of the aortic lesions, vein grafts and control veins.

	Total cholesterol content					
	Aortic lesion		Vein graft		Control vein	
	r	p	r	p	r	p
Total cholesterol						
VLDL	0.62	<0.01	-0.06	0.81	0.57	<0.01
IDL	0.14	0.54	0.37	0.11	-0.05	0.84
LDL	0.00	0.99	0.38	0.10	-0.02	0.95
HDL	0.41	0.07	0.40	0.08	0.46	0.04
Cholesterolester						
VLDL	0.63	<0.01	0.10	0.67	0.56	0.01
IDL	0.25	0.28	0.40	0.08	0.01	0.98
LDL	0.10	0.66	0.48	0.03	0.06	0.80
HDL	0.46	0.04	0.45	0.05	0.47	0.04
Triglycerides						
VLDL	0.77	<0.001	-0.05	0.85	0.58	<0.01
IDL	0.06	0.81	0.28	0.23	0.05	0.84
LDL	-0.26	0.26	-0.37	0.11	-0.11	0.64
HDL	0.01	0.96	-0.15	0.52	0.03	0.90

VLDL=very low density lipoprotein; IDL=intermediate density lipoprotein; LDL=low density lipoprotein; HDL=high density lipoprotein.

accumulation in the lesion and non-lesion areas of the aorta remained unaffected during the post-induction period. The cholesterol content of the aortic lesion and the vein were both positively correlated to plasma VLDL-cholesterol concentration, while the cholesterol content of the graft was related to plasma LDL-cholesterolester concentration.

The reduction in total cholesterol content in the vein grafts has not led to a decrease in luminal encroachment or mean intimal thickness of the graft (Table 3). Because the atherosclerosis process in the grafts was characterized by total cholesterol accumulation and fibrointimal hyperplasia, the time course for regression may be different for the various parameters by which atherosclerosis is assessed. Donald et al reported that reduction in total cholesterol accumulation, especially the cholesterolester part, was present in the early phase of regression.[12] Regression of advanced atherosclerotic lesions, which contain a higher fibrotic component, takes much longer. In the aorta Kobari et al has found regression of fatty streaks, but not of the fibrous plaque lesions after a 9 month regression period.[13] Regression of advanced lesions has been presented by Daoud et al, but their observation followed a regression period of 14 months on a diet, low in fat and cholesterol.[14] The present finding of a lower total cholesterol content, but similar luminal narrowing may represent an early form of regression of atherosclerosis in vein grafts, provided that regression follows the same time course in grafts and in arteries. The present experimental post-induction period of 2 months may have been too short to investigate the effect of fish oil on the cellular components of vein graft atherosclerosis in pigs.

As described above luminal encroachment in the LCXCA did not show any regression of atherosclerosis in the post-induction period. In the LF group luminal encroachment even tended to increase, but statistical significance was not reached. The latter is a consistent finding in our laboratory as Sassen et al have reported the same phenomenon twice.[6,9] In those studies we observed that in the post-induction period luminal encroachment of non-abraded coronary arteries increased when by weight 9% or 10% lard fat remained present in the diet.[6,9] The higher plasma cholesterol in lard fat fed group was proposed to underly this phenomenon, because Clarkson et al reported that progression of atherosclerosis would occur when plasma cholesterol concentration did not completely return to baseline values.[15] In the present study plasma cholesterol concentration in the LF also did not completely return to mean baseline level (2.4±0.6 mM and 3.0±0.7 mM, at the onset and at the end of the experimental protocol, respectively, $P<.05$ as tested with a paired t-test), but similar to the increase in luminal encroachment statistical significance was not reached. The tendency of coronary luminal encroachment to increase was, however, accompanied by a significant increase in both lumen and media area, which was consistent in all post-induction diet groups. This finding coincides with reports that hypercholesterolemia interferes with endothelial nitric oxide production (for review see ref. 16), and may explain the narrowed lumen in the induction group. The increased media area in the post-induction period may be a response to the increased stress in the vessel wall as lumen diameter increases, suggesting some kind of remodeling of the coronary artery.

In contrast to the graft and the control vein, removal of the 2%cholesterol from the diet did not result in a lower total cholesterol content in the lesion and non-lesion area of the abdominal aorta. This finding suggests, that accumulated cholesterolester more easily leaves from the walls of veins and grafts than that of the aorta. A possible explanation for these differences may be the integrity of the endothelium of the various vessels studied. During surgery the preparation of the saphenous vein invariably causes a loss of endothelium.[1,2,17] The subsequent exposure of the vein graft to arterial blood pressures may not only hinder re-endothelialization, but also diminish the function of the remaining and regenerated endothelial cells. This is evidenced by a report of Finck et al, who found that after 6 months endothelial permeability in arterialized vein grafts was still increased, while a confluent endothelium was already present after 1 month.[18] Furthermore, vein grafts retains many of the specific characteristics of veins.[2] One of the characteristics is that veins have a more permeable endothelium than arteries.[19] The latter coincides with our finding that in the control veins total cholesterol content decreases due to a lowering in plasma cholesterol.Thus, not only the absence of a functional endothelial layer, but also the presence of venous endothelium may result in a higher susceptibility of the vein grafts for changes in plasma cholesterol level.

For the post-induction groups the cholesterol content in the aortic lesions showed a positive correlation with VLDL-triglyceride as well as VLDL-cholesterol concentrations. This finding is in apparent contrast with our previous finding in hypercholesterolemic swine that VLDL triglycerides are negatively correlated to the cholesterol content of the aortic lesions (unpublished data). This may be related to the change in chemical composition of the VLDL

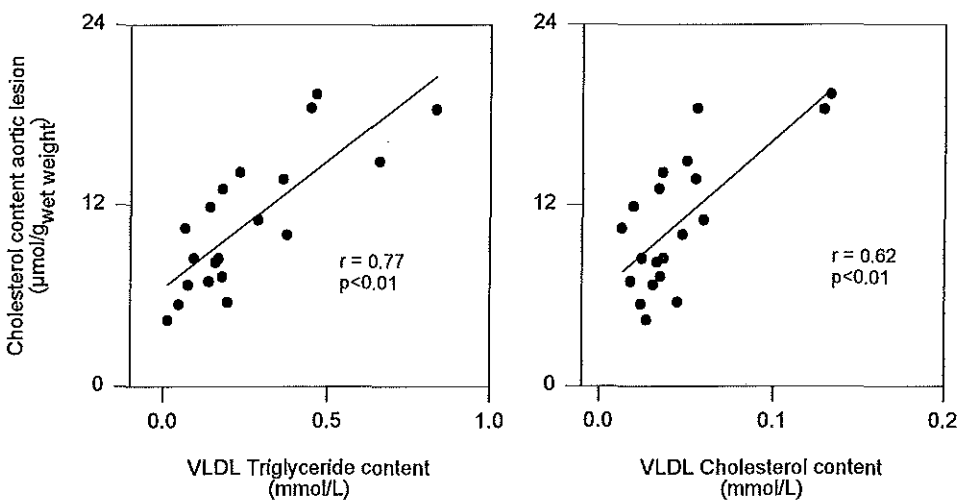

Fig 6. Graphs showing the individual points used for determination of the relation between the cholesterol content of the aortic lesion area and plasma VLDL triglyceride and cholesterol concentration. VLDL=very low density lipoprotein.

fraction. Relative to VLDL of animals in the hypercholesterolemic induction group, in the post-induction period VLDL contained more triglycerides and less cholesterolester in all dietary groups (Fig. 5). A cholesterol-rich VLDL particle undoubtedly is more atherogenic than a triglyceride-rich VLDL particle. It is therefore likely that cholesterol content in the aortic lesions correlates with the major lipid constituent of VLDL. This also means that in swine VLDL is a predicting factor for aortic total cholesterol infiltration.

We conclude that a low cholesterol diet can effectively reduce the cholesterol content of vein grafts irrespective of the presence of fish oil, sunflower oil, lard fat or an isocaloric quantity of carbohydrates in the diet. However, the duration of the present study precludes any conclusions on the beneficial effect of fish oil on regression of fibrointimal hyperplasia in vein grafts. The higher susceptibility of the vein grafts to changes in plasma cholesterol levels than the aorta suggests a different endothelial function in the vein graft compared to that in the aorta.

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Chapter 10

General discussion

In this thesis 2 facets of cardiovascular disease and their therapeutic modulation are discussed. The first concerns myocardial stunning and its pharmaceutical treatment. The second concerns the problems following revascularisation of the ischemic myocardium by venous coronary artery bypass grafting.

Since Heyndrickx et al first described the phenomenon of prolonged post-ischemic contractile dysfunction of the myocardium, several investigators had searched for ways to recruit contractile function.[1] Smith et al were the first to report that contractile function can be recruited with β-adrenergic receptor agonists.[2] Many investigators had since confirmed these report (chapter 2). However, they all used either systolic segment shortening or wall thickening to assess recovery of systolic function. The problem with these variables of contratile function is that they are highly dependent on the loading conditions of the left ventricle and in chapter 2 several drugs are described which improve contractile function of stunned myocardium by reducing afterload of the left ventricle. In chapter 3 we have used the end-systolic left ventricular pressure-segment length relationship, which provides a load-independent index of regional myocardial contractility,[3] to assess recruitment of contractile function with dobutamine in left ventricular stunned myocardium. Because this method is independent of loading conditions, it may also serve to compare stunning in left and right ventricles and their response to chronotropic and inotropic stimulation with dobutamine. Brief occlusion of the left anterior descending coronary artery decreased both load-dependent and load-independent variables of contractility. Subsequent chronotropic stimulation did not affect contractility, but infusion of dobutamine improved both load-dependent and load-independent variables of contractility in stunned myocardium of both ventricles (chapter 3).

The main concern of inotropic stimulation with β-adrenergic receptor agonists is that the increase in contractility is accompanied by an increase in myocardial oxygen demand and thus coronary blood flow. In the presence of stenosis of the coronary artery this may induce an oxygen deficit of the myocardium and worsen contractility in stunned myocardium. Furthermore it is doubtful whether β-adrenergic receptor agonists targets the underlying mechanism of stunned myocardium. Kusuoka and Marban have proposed that the likely mechanism underlying the prolonged contractile dysfunction of stunned myocardium is a decrease in the calcium sensitivity of the myofibrils.[4,5] Recently the group of Marban provides further evidence that the myofibrillar sensitivity to calcium has been decreased in stunned myocardium and they also report that stunned myocardium copes poorly with an increased calcium load warrants against excessive inotropic stimulation such as β-adrenoceptor agonists or phosphodiesterase inhibitors.[6,7] The rational approach to treat myocardial stunning will be to restore the calcium sensitivity of the myofibrils.

Until now most alleged calcium sensitizers used to improve contractile function also possesses phosphodiesterase inhibitory properties. EMD 60263 is a newly developed thiadiazinone derivative which sensitizes the myofilaments to calcium, but possesses only

minimal phosphodiesterase inhibitory properties.[8] Thiadiazinone derivatives increases calcium sensitivity by increasing the myofibrillar Mg^{2+} ATPase activity.[9] In chapter 6 is shown that *in vitro* the effects of EMD 60263 are specific for myofibrillar Mg^{2+} ATPase activity and that EMD 60263 has no effect on sarcoplasmic reticular Mg^{2+} ATPase activity. In chapter 4 we have investigated the effects of EMD 60263 on contractile function in an *in vivo* model of stunned myocardium. In this model we have previously demonstrated that the maximal calcium pump activity of the sarcoplasmic reticulum had not decreased during stunning.[10] Administration of EMD 60263 dose-dependently increases systolic segment shortening and total recovery of systolic segment shortening is achieved at a dose of 1.5 mg/kg. However, the improvement is accompanied by a decrease in heart rate, which can be attributed to blockade of the delayed rectifying current. Bradycardia may also improve systolic segment shortening, but this mechanism has been excluded, because neither the specific bradycardic agent zatebradine nor the isomeric enantiomer EMD 60264, which is identical to EMD 60263 except for the calcium sensitizing properties, could emulate the improvement in systolic segment shortening induced by EMD 60263. Other mechanisms have also been excluded, because the effects of EMD 60263 on systolic segment shortening are unaffected by α- and β-adrenergic receptor blockade. These results also support the hypothesis that a decrease in myofibrillar sensitivity to calcium is the underlying mechanism of stunned myocardium.

Some investigators, however, fear that increasing the calcium sensitivity of the myofibrils may have detrimental effects on diastolic function, because under pathologic conditions such as myocardial stunning and heart failure increased intracellular calcium have been observed.[11] Impairment of diastolic relaxation and thus left ventricular filling may subsequently hamper systolic function. At a dose of 1.5 mg/kg of EMD 60263, which already fully recovers systolic segment shortening, we observe no detrimental effect of increasing myofibrillar calcium sensitivity on diastolic function as assessed with diastolic segment length changes. At higher doses of EMD 60263 (3.0 mg/kg) increases both systolic segment length and stroke volume to above baseline level (chapter 5). However, concurrently the onset of diastolic segment length lengthening has been delayed, perhaps indicating diastolic dysfunction. Subsequent atrial pacing at heart rate levels before stunning to exclude the effects of EMD 60263-induced bradycardia returns systemic hemodynamics and regional contractile function to baseline values. Furthermore atrial pacing does mitigate the delay in the onset of segment lengthening in stunned myocardium, but not in the not stunned myocardium. The different response to atrial pacing suggests that despite a restoration in systolic segment shortening there is still a difference in the calcium sensitivity of the myofibrils of stunned and not stunned myocardium. To compare the effects of EMD 60263 with those of dobutamine the hearts have been paced at 30 beats per minute above stunning level. At this heart rate level systemic hemodynamics are impaired, because of diastolic relaxation problems as end-diastolic segment length decreased and end-diastolic left ventricular pressure increased. That diastolic function may be impaired at higher doses of EMD 60263 can also be deducted from our findings in the *in vitro* study (chapter 6). Myofibrillar Mg^{2+} ATPase

activity at low calcium levels and high doses of EMD 60263 (>10 μM) are shown to be higher than those at high calcium levels without EMD 60263. Recently Sunderdick et al have also shown that in isolated rabbit hearts high doses of EMD 60263 (3 and 10 μM) deteriorates systolic function, due to relaxation abnormalities.[12] In the same model EMD 57033, which has more phosphodiesterase inhibitory properties than EMD 60263, have also been tested, but in contrast to EMD 60263 improves not only systolic, but also diastolic function.[12]

Calcium sensitizers are promising inotropic agents, but warrant further investigations. The possible diastolic relaxation problems with high doses of EMD 60263 perhaps serve as a caution against the use and development of pure calcium sensitizers. Development of calcium sensitizing agents that also possess some phosphodiesterase inhibitory activity such as EMD 57033 may be a better alternative. Unpublished data from our laboratory obtained in conscious pigs with normal myocardium have shown that EMD 57033 increases systolic contractile function by increasing the myofibrillar calcium sensitivity, but in contrast to EMD 60263 does not impair diastolic relaxation. Investigating the effects of EMD 57033 in a similar animal model of stunned myocardium may reveal whether this is the correct future direction of treating myocardial stunning.

Instead of treating the consequences of myocardial ischemia such as stunned myocardium, treatment can be implemented at an earlier stage. Progression of atherosclerosis in the epicardial coronary arteries may necessitate revascularisation to prevent further cardiovascular events. One of the techniques is coronary artery bypass grafting. The shortterm results of CABG is favorable as both mortality and clinical symptoms are reduced.[13] However, these grafts are more susceptible to atherosclerosis, which therefore progresses in an accelerated fashion compared to native coronary atherosclerosis. The exact mechanism is unknown, but damage to the endothelial layer of the graft and the ensueing thrombosis and wound healing are likely to be involved in the genesis of graft atherosclerosis.

Various treatments have been proposed to reduce atherosclerosis in the grafts. Fuster and Chesebro have shown that anti-platelet therapy effectively reduces early graft failures, but the beneficial effect on late graft failures is less striking.[14] They believe that anti-platelet therapy may prevent a complicating thrombus superimposed on intimal hyperplasia in the graft, but that anti-platelet therapy is ineffective in preventing the primary occlusive proliferative disease. Angelini and Newby proposed lowering of plasma cholesterol because the predominant underlying mechanism of late graft failures is accumulation of lipids within the fibrointimal hyperplasia and graft wall.[15] The latter have since been confirmed by Blankenhorn et al, who have found that colestipol-niacin therapy inhibited progression of atherosclerosis in coronary bypass grafts.[16] A favorable effect of fish oil on experimental arteriosclerosis have been reported by several investigators (chapter 7). Because the process behind late graft failure resembles atherosclerosis in nearby coronary arteries, fish oil may also excert a favorable effect on vein graft atherosclerosis. In hypercholesterolemic dogs fish oil have been shown to effectively inhibit intimal thickening in veno-arterial bypass grafts.[17-19] However according to Sarris et al,

differences in lipoprotein metabolism and fibrinolytic system between dogs and humans may alter the pathogenesis of vein graft atherosclerosis.[17] Thus, whereas in dogs modulation of growth factors[17] and platelet function[18,19], but not modulation of plasma lipoproteins[17], by fish oil are the predominant factors determining graft atherosclerosis, in humans modulation of plasma lipoproteins may play a more important role.

Therefore, we investigated in a hypercholesterolemic swine model of veno-arterial bypass grafting the effects of fish oil on progression and regression of graft atherosclerosis. In contrast to the studies in hypercholesterolemic dogs, we did not observe a favorable effect of fish oil on atherosclerosis in the grafts as assessed with cholesterol accumulation and morfometry (luminal encroachment and intimal thickness). The control vessels, the right saphenous vein and the left circumflex coronary artery, did also not differ between the fish oil, sunflower oil and lard fat groups. Fish oil, however, did excert a favorable effect in the hypercholesterolemia-induced fatty streaks of the abdominal aorta, which had a lower cholesterolester content than those in the sunflower oil or lard fat group. The differences could not be explained with total plasma cholesterol, which was similar in the three hypercholesterolemic groups. The distribution of cholesterol between the different lipoprotein fractions was significantly altered by fish oil. VLDL-cholesterol was lower with fish oil than in the other 2 groups, while HDL-cholesterol in the fish oil group was lower than in the sunflower oil group. Furthermore, the chemical composition of the VLDL particles was altered by fish oil, containing more triglycerides and less cholesterolester than in the other 2 groups. Between sunflower oil and lard fat groups there were no differences. Stepwise regression analysis for all animals revealed that VLDL-cholesterol correlated to the cholesterol content of the fatty streaks in the abdominal aorta ($r=0.77$, $p<0.001$). These results suggested that VLDL-cholesterol was a predicting factor for the hypercholesterolemia-induced lipid accumulation in normal aortic tissue. The difference in response to fish oil between the abdominal aorta and the graft suggested that the presence of an intact endothelial layer is a prerequisite for fish oil to excert a favorable effect on progression of atherosclerosis.

In chapter 9 the effects of fish oil on regression of graft atherosclerosis are discussed. Several investigators have shown that fish oil can potentiate the effect of lowering plasma cholesterol on regression of arteriosclerosis, but the question remains whether this also apply to venous bypass grafts. The results in chapter show that lowering plasma cholesterol can decrease cholesterolester content of the graft, but that this effect is independent of the fatty acid present in the diet. Because the morfometric parameters have not changed it seems that regression depends on the method used to assess atherosclerosis. The difference in response to lowering plasma cholesterol between veins and vein grafts and aorta suggests that different mechanisms underly the atherosclerosis process in veins and arteries. Similar to our findings in chapter 8 a positive correlation between VLDL concentration and total cholesterol content in the aortic fatty streaks. It is possible that VLDL concentration play a major role in modulating total cholesterol accumulation in the aortic wall.

The studies regarding fish oil in this thesis show no beneficial effect of fish oil on atherosclerosis in venous bypass grafts. The effects of fish oil in the abdominal aorta as observed in chapter 8 are the most promising, but the question remains whether fish oil should be administered to the whole population to prevent atherosclerosis. In our hypercholesterolemic swine model in which the development of atherosclerosis has been accellerated fish oil is able to mitigate the development and progression of atherosclerosis. This finding coincides with the report of Bang et al in the Inuit population.[20] The lower incidence of cardiovascular disease in the Inuit population compared to a comparable age-group of Danes may also imply that fish oil inhibits rather than totally prevent the development of atherosclerosis. Furthermore the results in chapter 8 suggest that the endothelial layer may play an important role in mediating the effects of fish oil on atherosclerosis, but because we have not investigated this aspect in this thesis no definite conclusions can be made. In chapter 9 we have observed that lowering of plasma cholesterol decreases total cholesterol content of the vein grafts. This may ultimately result in a reduction of fibrointimal thickening and thus reduce late graft failures. While we have not found any evidence in our present experiments that this might happen, other investigators have shown that regression of fibrous lesions can be obtained using longer post-induction periods. However, longer experimental protocols may also alter the present findings in both chapter 8 and 9. A consistent finding in both chapter 8 and 9 is the correlation between cholesterol content of the aortic wall and VLDL concentration. Also in the PDAY study a correlation has been found between VLDL-cholesterol concentration and the severity of the atherosclerotic lesions in the aorta and coronary artery.[21] VLDL concentration may therefore be used in the clinical setting as a predicting factor of development of atherosclerosis. Furthermore, lowering of plasma VLDL concentration may result in a reduction of atherosclerosis and thus represent a future direction of treating coronary atherosclerosis and ischemic heart disease.

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Chapter 11

Samenvatting

Dit proefschrift heeft betrekking op ontstaan en behandeling van ischemie van het hartspierweefsel, het myocard. Echter, voordat ingegaan wordt op de verschillende therapeutische mogelijkheden van myocard ischemie, is het belangrijk om eerst nader in te gaan op de term myocard ischemie en de achtergronden daarvan.

Myocard ischemie houdt in dat er een onbalans bestaat tussen energie vraag en energie aanbod in het hart weefsel. Het hart is van nature een aeroob orgaan, wat inhoudt dat het hart weefsel continu zuurstof nodig heeft om voldoende energie te genereren om in leven te blijven en om de pompfunktie van het hart te kunnen garanderen. Onder normale omstandigheden haalt het hart vrijwel al het zuurstof uit het aangeboden bloed. Dit betekent dat wanneer er een toename onstaat in de energie en daarmee dus van de zuurstof vraag van het hart, deze alleen voldaan kan worden door een toename in bloeddoorstroming van het myocard. Een belangrijke rol hierbij spelen de kransslagaderen (coronair vaten) van het hart. Gezonde coronair vaten kunnen zelfs tijdens hevige inspanning zonder moeite voldoen aan de toename in energie vraag. De disbalans in energie vraag en aanbod ontstaat wanneer er onvoldoende bloed kan worden aangevoerd via de coronair vaten bijvoorbeeld als gevolg van een vernauwing van het bloedvat, de aanwezigheid van een bloedstolsel of een combinatie van beide.

Onder invloed van ischemie verandert in het myocard de stofwisseling en daarmee de pompfunktie van het hart. Door het tekort aan zuurstof gaat de aerobe stofwisseling over in anaerobe stofwisseling, die gekenmerkt wordt door melkzuur produktie en een lagere energie opbrengst. Hierdoor kan het hart weefsel niet voldoende energie genereren om contractie of homeostase van de hartcel te kunnen waarborgen, waardoor de pompfunktie van het hart en uiteindelijk ook de levensvatbaarheid van de hartcellen in geding komt. Het uiteindelijke resultaat van myocard ischemie is echter afhankelijk van de duur en de ernst van ischemie. Wanneer men praat over een volledige afsluiting van een coronair vat en een gezond myocard dan heeft een ischemie duur van minder dan 2 minuten geen blijvende nadelige gevolgen voor het myocard. Opheffing van de afsluiting leidt dan ook tot een direkt en volledig herstel van contractiele funktie van het myocard. Duurt de afsluiting langer dan 20 minuten dan beginnen hartcellen af te sterven en herstel van de bloeddoorstroming zal leiden tot een vertraagd en onvolledig herstel van de contractiele funktie. Is de duur van de ischemie tussen de 2 en 20 minuten dan blijven de hartcellen in leven, maar na normalisatie van de bloedvoorziening kan het uren tot dagen duren voordat er een volledig herstel van de contractiele funktie heeft plaats gevonden. Dit fenomeen, ook wel "myocardial stunning" genoemd, is voor het eerst beschreven door Heyndrickx et al. Zij hebben in een diermodel gevonden dat na een korte periode van ischemie met een duur van minder dan 20 minuten regionale myocard funktie sterk verminderd bleef, ondanks normale bloeddoorstroming en afwezigheid van celdood.

Myocardial stunning van een deel van de linker hartkamer (linker ventrikel) heeft op zich geen behandeling nodig, omdat funktie herstel spontaan zal plaats vinden. Dit geldt echter alleen maar, indien de rest van de linker ventrikel normaal funktioneert en de pompfunktie van het hart

gewaarborgd blijft. Komt de pompfunktie wel in het gedrang dan is behandeling noodzakelijk. Farmacologische behandeling van myocardial stunning is gericht op het voorkomen ervan, of op verbeteren van de contractiele functie van het gestunde myocard. In hoofdstuk 2 van dit proefschrift zijn de diverse behandelingsmogelijkheden beschreven, maar in het kader van de samenvatting zal alleen worden ingegaan op de behandeling, die gericht is op het verhogen van de contractiele funktie.

Van β-adrenerge receptor agonisten zoals adrenaline en noradrenaline is bekend dat ze de contractiliteit (inotropie) van het myocard verhogen. Deze en verwante stoffen zijn dan ook als eerste geprobeerd om de contractiele funktie in het gestunde myocard te verhogen. Smith et al hebben als eerste laten zien, dat isoprenaline in staat was de contractiele funktie van het gestunde myocard volledig terug te brengen. Sindsdien hebben meerdere onderzoekers dit bevestigd, maar de effekten van deze stoffen zijn tot nu toe alleen onderzocht voor de linker ventrikel. Over stunning van de rechter ventrikel en de behandeling daarvan is weinig bekend. Pompfunktie van de rechter ventrikel is belangrijk voor de vulling van de linker ventrikel en een falen van de rechter ventrikel zou de pompfunktie van de linker ventrikel nadelig kunnen beïnvloeden. Hiermee verband houdend is het ook van belang om te weten of stunning van de rechter ventrikel op dezelfde manier zal reageren op contractiliteit verhogende farmaca als de linker ventrikel. In hoofdstuk 3 hebben we deze aspecten onderzocht en vonden dat de contractiele funktie na stunning procentueel hetzelfde afnam in de rechter en linker ventrikel. Verder reageert de gestunde rechter ventrikel op dezelfde manier als de gestunde linker ventrikel op zowel hart frequentie verhoging als op inotrope stimulatie met dobutamine.

Hoewel β-adrenerge receptor agonisten en ook fosfodiesterase remmers de contractiele funktie van het gestunde myocard kunnen verhogen, blijft de vraag of dit wel de meest aangewezen therapie is. Een rationele therapie zou zijn om de onderliggende mechanismen van de afname in contractiele funktie direkt aan te pakken. Vele mechanismen zijn voorgesteld als de onderliggende oorzaak, maar in een recent overzicht van de literatuur laat Bolli zien dat vrije zuurstof radicalen en/of een verstoring van de calcium homeostase binnen de cel de meest waarschijnlijke oorzaken zijn. De huidige mening is, dat tijdens de occlusie en in het begin van de daarop volgende herstel van de bloeddoorstroming vrije zuurstof radicalen worden gevormd, die op hun beurt de calcium gevoeligheid van de contractiele elementen van de hartspiercel (myofibrillen) verlagen. De β-adrenerge receptor agonisten, fosfodiesterase remmers en verhoging van extracellulair calcium verbeteren de contractiele funktie van het gestunde myocard door de calcium transient te verhogen, met andere woorden de amplitude van de intracellulaire calcium concentratie in de cel neemt toe. In alle drie de gevallen wordt de verminderde calcium gevoeligheid van de myofibrillen eigenlijk behandeld met een overmaat aan calcium en een betere therapie zou kunnen zijn het verhogen van de calcium gevoeligheid met zogenaamde calcium sensitizers.

Tot voor kort was er geen bewijs dat calcium sensitizers de contractiele funktie van het gestunde myocard *in vivo* kunnen herstellen, omdat de gebruikte calcium sensitizers vaak ook

nog phosphodiesterase inhibiterende eigenschappen bezitten. In de drie volgende hoofdstukken van dit proefschrift zijn de effecten onderzocht van een nieuw ontwikkelde calcium sensitizer EMD 60263, die nauwelijks meer fosfodiesterase remmende eigenschappen bezit. Hoofdstuk 4 behandelt de effecten van EMD 60263 op de contractiele funktie van normaal en gestund myocard. In een varkensmodel verloor het myocard na stunning ongeveer 50% van zijn oorspronkelijke contractiele functie, bepaald met regionale segment lengte verkorting. Toediening van EMD 60263 herstelde dosis-afhankelijk de segment lengte verkorting in het gestunde gebied en met een dosis van 1,5 mg/kg was er zelfs een volledig herstel van de segment lengte verkorting. Opvallend was het feit dat het effect in het gestunde gebied beduidend hoger was dan in het aanliggende gebied. Ook de mechanische efficientie of wel de hoeveelheid arbeid per eenheid verbruikte zuurstof, die was afgenomen tijdens stunning, werd weer volledig hersteld. In het niet-gestunde gebied was er geen toename van de mechanische efficientie. Deze effecten gingen echter gepaard met een afname van de hart frequentie. Toediening van een specifiek hartfrequentie verlagende stof, zatebradine, leidde niet tot een verbetering van segment lengte verkorting. Andere mechanismen, die zouden kunnen leiden tot verbetering van de contractiele funktie werden uitgesloten, omdat toediening van α- and β-adrenerge receptor blokkers de effecten van EMD 60263 op het gestunde myocard niet blokkeerden.

Volgens sommige onderzoekers kan verhoging van de calcium gevoeligheid, naast verbetering van de contractiele funkie, ook leiden tot een verslechtering van de relaxatie van het myocard. Een vertraagde relaxatie zou de vulling van de ventrikels nadelig kunnen beïnvloeden, hetgeen de pompfunktie van het hart doet afnemen. In hoofdstuk 5 hebben we aan de hand van hetzelfde varkensmodel als we in hoofdstuk 4 gebruiken de effecten van EMD 60263 onderzocht op regionale segment lengte verlenging, als maat voor relaxatie. Bij een dosering van 1,5 mg/kg vonden we wederom een volledig herstel van segment lengte verkorting, hetgeen echter niet gepaard ging met een afwijkende segment lengte verlenging. Verhoging van de dosering tot 3,0 mg/kg vertraagde het begin van segment lengte verkorting in zowel het gestunde als het niet-gestunde myocard. Dit hield in dat de contractie duur van het myocard verlengd was vergeleken met de situatie onder normale condities of na toediening van 1,5 mg/kg EMD 60263. Om onderscheid te kunnen maken tussen de effecten van EMD 60263-geinduceerde hartfrequentie verlaging en van calcium gevoeligheid verhoging op de segment lengte verlenging, werd de hartfrequentie verhoogd tot het niveau van vlak voor de toediening van EMD 60263. Hartfrequentie verhoging door middel van elektrische prikkeling van de rechter atrium (atrial pacing) leidde tot een normalisatie van segment lengte verlenging in het gestunde gebied, maar niet in het aanliggende normale gebied. Aangezien atrium stimulatie niet alleen de calcium transient doet stijgen, maar ook de calcium gevoeligheid verlaagt, suggereert dit verschil van reactie in segment lengte verlenging, dat na toediening van EMD 60263 er nog steeds een verschil bestaat in de calcium gevoeligheid tussen het gestunde en het niet-gestunde myocard. In hoofdstuk 6 hebben we onderzocht of EMD 60263 inderdaad de calcium gevoeligheid verhoogd van de myofibrillen en niet van andere intracellulaire strukturen in een *in vitro*

opstellingen de bevindingen bevestigen de resultaten van de hoofdstukken 4 en 5.

Voor de toekomst is het onzeker of de ontwikkeling van pure calcium sensitizers zoals EMD 60263 wel geïndiceerd is, vanwege de mogelijke relaxatie stoornissen. Misschien vormen de calcium sensitizers, die ook nog enige fosfodiesterase remmende eigenschappen bezitten, een beter alternatief, omdat fosfodiesterase remming de relaxatie van het myocard kan bevorderen. EMD 57033 is een calcium sensitizer, die meer fosfodiesterase remmende eigenschappen bezit dan EMD 60263. Recente resultaten (nog niet gepubliceerd) uit ons laboratorium laten zien dat EMD 57033 de contractiele funktie verhoogt door een toename in de calcium gevoeligheid van de niet-gestunde myofibrillen. Echter, in tegenstelling tot EMD 60263 zijn er met EMD 57033 geen relaxatie stoornissen waarneembaar. EMD 57033 zal binnenkort getest worden in hetzelfde dierexperimentele model voor myocardiale stunning, zoals beschreven in hoofdstuk 6.

In de behandelde hoofdstukken heeft de nadruk voornamelijk gelegen op behandeling van de gevolgen van ischemie en in het bijzonder van myocardiale stunning. Echter, behandeling kan ook meer stroomopwaarts plaats vinden. De oorzaak van ischemie, een vernauwing van het coronair vat, kan worden opgeheven door revascularisatie van het myocard. Ondanks nieuwe ontwikkelingen in de interventie cardiologie, zoals ballon dilatatie en het plaatsen van endovasculaire prothesen neemt coronaire omleiding (bypass) chirurgie nog steeds een belangrijke plaats in bij het herstellen van de bloeddoorstroming naar het myocard. Bij een dergelijke operatie wordt een vene of een arterie van elders uit het lichaam gebruikt om een omlegging om de coronaire vernauwing te maken. Het resultaat van een bypass operatie is vaak gunstig, zoals gemeten aan overleving en vermindering van klinische symptomen. Echter, vernauwing en zelfs volledige occlusie van dergelijke bypass transplantaten komen veelvuldig voor. Hiermee keren de klinische symptomen van myocard ischemie terug, die weer leiden tot een nieuwe ingreep. De precieze mechanismen die tot occlusie van de graft leiden zijn onbekend, maar schade aan de endotheellaag van het transplantaat en de daarmee gepaarde thrombose en wondheling kunnen een versnelde vorm van atherosclerose (aderverkalking) op gang brengen.

In het begin van de jaren zeventig beschreven Bang et al dat in de Inuit populatie (een eskimo stam aan de westkust van Groenland) de mortaliteit ten gevolge van hart- en vaatziekten lager was dan bij een vergelijkbare populatie Denen. Zij brachten deze bevinding in relatie met een verhoogde visconsumptie. Sindsdien hebben vele onderzoekers de effecten van visolie bestudeerd op atherosclerose. Hieruit bleek dat het gunstige effect van visconsumptie wordt veroorzaakt door de visolie. In hoofdstuk 7 wordt ingegaan op welke wijze visolie atherosclerose gunstig kan beïnvloeden. Verder worden ook de effecten van visolie op experimentele atherosclerose in diermodellen besproken. Het gunstige effect van visolie op het ontstaan en de progressie van atherosclerose gebeurt op velerlei manieren, waaronder modificatie van plasma lipoproteïnen, bloedplaatjes en endotheel functie, ontstekingsreacties en groeifactoren.

Dat visolie atherosclerose, gemeten aan intima verdikking, kan verminderen in veneuze bypass transplantaten is reeds aangetoond in hypercholesterolemische honden. In hoofdstuk 8

wordt ingegaan op de vraag of het effect van visolie op de transplantaten tot stand komt door een direkt effect van de n-3 vetzuren in visolie of door een toename in de meervoudig onverzadigd/verzadigd vetzuur ratio. Daarom zijn de effecten van visolie vergeleken met de effecten van zonnebloemolie en reuzel vet op de transplantaten. Ook is onderzocht of de samenstelling van de lipoproteïnen hierin een rol speelt. In hoofdstuk 9 worden de effecten van visolie op regressie van atherosclerose in de transplantaten besproken. In dierexperimenteel onderzoek is aangetoond dat visolie het gunstige effect van plasma cholesterol verlaging op regressie van atherosclerose kan potentiëren, maar onbekend is of dit ook geldt voor veneuze bypass transplantaten.

Deze probleemstellingen werden onderzocht in een hypercholesterolemisch varkensmodel voor veneuze bypass transplantaten. In tegenstelling tot de studies in honden, zagen we in de transplantaten geen voordeel van dieet visolie ten opzichte van dieet zonnebloemolie en reuzelvet, beoordeeld aan de hand van lipideinfiltratie en morfometrie (vaat vernauwing en intima dikte). Morfometrisch onderzoek liet ook geen verschil zien in de controle vene en de linker circumflex coronair arterie tussen de drie groepen. Een verschil tussen de drie hypercholesterolemische groepen werd gezien in de infrarenale abdominale aorta. De atherosclerotische aorta of een representatief stuk aorta uit hetzelfde gebied bevatte in de visolie groep significant minder totaal cholesterol, met name cholesterolester, dan in de zonnebloemolie en reuzelvet groepen. Verdere analyse liet zien, dat de veranderingen in cholesterol accumulatie in de aorta gerelateerd zijn aan de veranderingen in plasma VLDL-cholesterol. De waargenomen verschillen in de effecten van visolie op lipide accumulatie in de transplantaten en de aorta suggereren, dat de aanwezigheid van een optimaal functionerende endotheellaag (binnenbekleding van een vat en een belangrijk verschil tussen de recent geplaatste veneuze bypass graft en de "intacte" aorta) een vereiste is om een positief effect van visolie op atherosclerose te zien.

De resultaten in hoofdstuk 9 tonen aan, dat verlaging van het plasma cholesterol en niet de aanwezigheid van visolie, zonnebloemolie, reuzel vet of koolhydraten in het dieet een groot effect heeft op lipide accumulatie in de transplantaten. Aangezien de morfometrische grootheden niet verbeteren ten opzichte van de inductie groep, lijkt regressie van atherosclerose in de transplantaten afhankelijk te zijn van de gebruikte meetmethode. In de aorta was er geen afname in cholesterolaccumulatie te zien, maar deze bleek ook in de regressie studie gerelateerd te zijn aan de VLDL concentratie. De afnames in cholesterol accumulatie in de transplantaten en de controle vene, maar niet in de aorta, suggereren dat verschillende mechanismen ten grondslag liggen aan het verloop van het atherosclerose proces in venen en arteriën. Het is mogelijk dat ook hier de VLDL concentratie een rol speelt.

De onderzoeken inzake visolie beschreven in dit proefschrift tonen aan dat visolie geen effect heeft op atherosclerose in een veneuze bypass transplantaat. Hoewel de effecten van visolie in de aorta hoopgevend zijn, is het de vraag of visolie wel de aangewezen therapie is tegen atherosclerose in de gemiddelde populatie. In het door ons gebruikte

hypercholesterolemisch varkensmodel waarin het atherosclerose process wordt versneld heeft visolie een vertragend effect op het ontstaan en progressie van atherosclerose. Deze observatie is in overeenstemming met de bevindingen van Bang et al in de Inuit populatie. De verminderde incidentie van hart- en vaatziekten in deze populatie ten opzichte van een ook qua leeftijd vergelijkbare groep Denen zou ook kunnen wijzen op een preventieve werking van visolie. De resultaten in hoofdstuk 8 suggereren dat het endotheel hierin een belangrijke rol speelt, maar uitsluitsel kunnen we op dit moment niet geven, omdat we dit aspect niet hebben onderzocht. In hoofdstuk 9 is de belangrijkste bevinding dat verlaging van de plasma cholesterol concentratie een gunstige invloed heeft op cholesterol ophoping in de bypass grafts. Het is mogelijk dat deze invloed op de lange duur vruchten zal afwerpen en de occlusie van de transplantaten gunstig beïnvloedt. We hebben dit niet kunnen staven met ons huidige experimentele protocollen. Een langere duur van het experimentele protocol zou de resultaten zoals beschreven in de hoofdstukken 8 en 9 kunnen veranderen. Een waarneming die een direkte toepassing in de kliniek zou kunnen vinden is de relatie tussen cholesterolophoping in de aortawand en de plasma VLDL concentratie. Een verlaging van de plasma VLDL concentratie hetzij middels dieet, hetzij middels geneesmiddelen lijkt een bruikbare manier om progressie van atherosclerose te verminderen.

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Loe Kie Soei

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