Cardioprotective Effects of Exercise Training

The importance of nitric oxide

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Cardioprotective Effects of Exercise Training

The importance of nitric oxide

Beschermende effecten van inspanningstraining op het hart

Het belang van stikstof oxide

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Contents

Chapter 1

General introduction and outline of this thesis

LV remodeling and heart failure

Cardiovascular disease is one of the most important causes of morbidity and mortality in Western countries.[1] Also in the Netherlands cardiovascular disease is the main cause of death, resulting in 33% of all deaths.[2] Per year more than 45.000 people die of cardiac disease, of which 22% is caused by an acute myocardial infarction. Myocardial infarction is necrosis (death) of the heart muscle secondary to prolonged ischemia due to occlusion of coronary arteries, and results in loss of viable myocardial tissue. Pathological left ventricular (LV) remodeling which occurs in survivors in the period after a myocardial infarction is a compensatory mechanism that serves to restore LV pump function. Despite the apparent appropriateness of LV remodeling to maintain cardiac pump function early after myocardial infarction, LV remodeling is an independent risk factor for the development of congestive heart failure.[3] Heart failure is the end stage of all myocardial diseases and is the major cause of morbidity and mortality. Due to reduced post-infarct mortality and aging of the population, the incidence and prevalence of congestive heart failure is rapidly increasing.[4,5] For example, in 2004 in the Netherlands 845 patients were hospitalized per day due to cardiovascular disease, of whom 66% involved myocardial infarction. Importantly, 60% of these patients survived myocardial infarction as a result of improved acute medical interventions (i.e. drug therapy and cardiac catheterization). In contrast, in 1973 only 20% of the hospitalized patients with a myocardial infarction survived the peri-infarct period. Thus, in the last three decades the number of deaths due to a myocardial infarction has almost been halved.[6] In the Netherlands approximately 200.000 people suffer from heart failure.[2] Every year this number of patients with heart failure increases with 10%. The prognosis of heart failure is in general poor and depends partly on the aetiology of heart failure. In general, one year after diagnosis of heart failure 70% of the patients are still alive, but after 5 years this percentage is decreased to 35%. It is expected that the incidence and prevalence of heart failure will continue to increase due to increased life span after myocardial infarction along with aging of the population. At present heart transplantation is the only treatment option in end stage heart failure. However, the number of available transplant hearts is insufficient to meet the demand. Therefore, improvement of treatments by lifestyle changes,[7] cell therapy[8] or pharmacotherapy[9] after myocardial infarction is of extreme importance to interrupt and halt the progression of LV remodeling towards the development of heart failure.

The mechanisms underlying the progressive deterioration of LV dysfunction towards overt heart failure still remain incompletely understood, but may involve

loss of cardiomyocytes through apoptosis[10] and alterations in extracellular matrix leading to progressive LV dilation.[11] More recently, a primary reduction in cellular contractile function of the surviving myocardium was shown to cause degeneration of cardiac pump function.[12,13] These processes lead to a gradual decrease in end systolic pressure and/or an increase in end diastolic pressure. Eventually, this deterioration of cardiac pump function results in overt heart failure with clinical symptoms.[14]

Cardiomyocyte contractility

The cardiac pump is able to adapt immediately in response to changes in requirement of oxygenated blood supply to vital organs in the body through alterations of heart rate and stroke volume. The latter is mainly determined by myocardial contractility. Depressed cardiomyocyte contractility is an important determinant of reduced pump function observed in heart failure.[13] Moreover, the ability to increase cellular contractility with heart rate or sympathetic stimulation (contractile reserve) is severely impaired.[15-17] At the molecular level, decreased cardiomyocyte function may be due to alterations in calcium handling, altered myofilament function, changes in the myocyte cytoskeleton, or a combination of these factors. Recently, van der Velden *et al* showed that early post myocardial infarction remodeling is associated with decreased cardiomyocyte function, which may contribute to depressed left LV function.[12]

Cardiomyocytes act as a functional network of cardiac muscle cells connected by gap junctions. All the cells are excited in synchrony to ensure maintenance of cardiac pump function. Therefore, the regulation of contractile function of individual cardiomyocytes plays a critical role in adaptation of cardiac pump function in vivo.[18] Activation of the contractile apparatus in individual cardiomyocytes is initiated upon a transient rise in intracellular calcium concentration and is driven by the energy from ATP hydrolysis. Under normal physiological conditions upon activation of the cardiomyocyte calcium enters the cell. However, this is insufficient to directly activate the myofilaments, but rather serves as a trigger to release calcium from the sarcoplasmic reticulum (SR). Collectively, this process is called excitation-contraction coupling.[19] In Figure 1 a schematic drawing is shown representing the excitation-contraction coupling in a single cardiomyocyte. A single cardiomyocyte is triggered by an action potential, whereupon the cardiomyocyte depolarizes. Calcium enters the cell through voltage-dependent L-type calcium channels (dihydropyridine receptor, DHPR) located on the sarcolemma. This calcium triggers the release of calcium, which is stored in the SR via calcium release channels (ryanodine

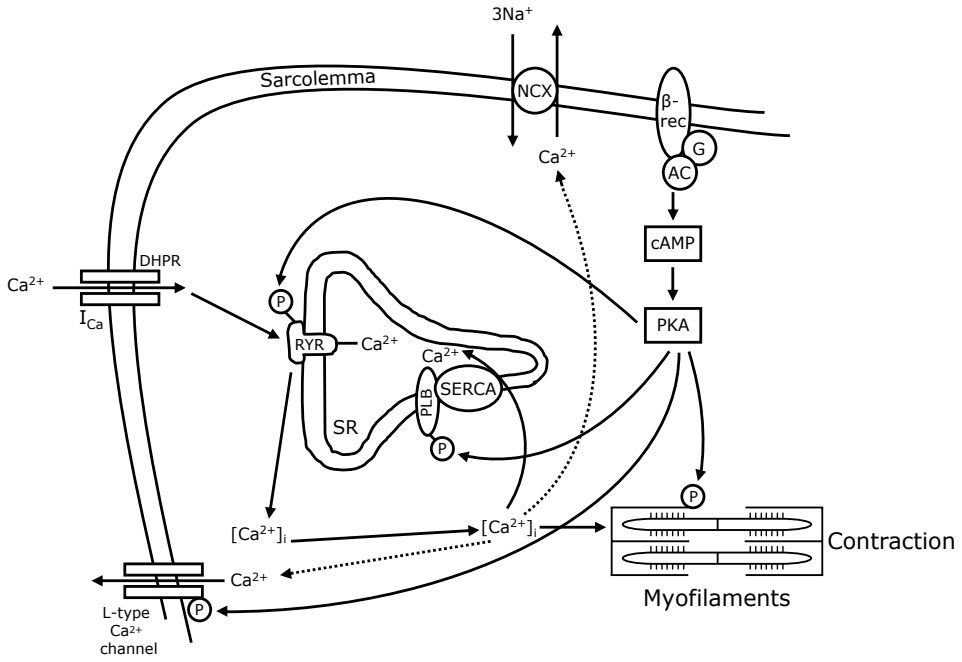

Figure 1: Cardiac calcium cycling.

Ca^{2+}: calcium, PLB: phospholamban, SR: sarcoplasmic reticulum, SERCA: SR Ca^{2+} ATPase pump, DHPR: voltage-dependent L-type Ca^{2+} channels, RYR: ryanodine receptors (SR Ca^{2+} release channels), NCX: Na^{+}/Ca^{2+} exchanger, PKA: protein kinase A, P: PKA phosphorylation site, cAMP: cyclic adenosine monophosphate, AC: adenylyl cyclase, G: G-coupled protein.

receptors, RYR). This in turn elevates the intracellular calcium concentration.

Contraction of the cardiac muscle results from the molecular interaction of two myofilament proteins; the thick myofilament, which is mainly composed of myosin, and the thin myofilament. A schematic representation of the contractile apparatus of striated muscle is shown in Figure 2.[20] The major proteins of the thick and thin filaments are indicated. The top panel reflects the sarcomeric structure during diastole and the bottom panel reflects systole. The main site for calcium regulation is the thin myofilament. The thin myofilament of cardiac muscle consists of actin, tropomyosin and troponin.[21] Troponin (cTn) consists of three subunits: troponin C (cTnC), the calcium binding protein; troponin I (cTnI), which inhibits the actin-myosin interaction and troponin T (cTnT), which transports the calcium binding signal to tropomyosin. Tropomyosin covers the myosin binding sites of actin and thereby prevents contraction. To allow the muscle cell to contract, tropomyosin must be moved to uncover the myosin binding sites on actin. When high levels of calcium diffuse through the cytosol, calcium binds to cTnC. This induces a conformational change in the tropomyosin-troponin complex such that cTnI exposes a binding site

on actin for myosin. The calcium binding to cTnC thus triggers the sliding of thin and thick filaments over each other and results in shortening of the sarcomere and cardiac force development. Subsequently, intracellular calcium levels are decreased mainly by activation of the SR calcium pump (SERCA) and sarcolemmal Na^+/Ca^{2+}-exchanger (NCX), and partly by the sarcolemmal calcium pump. Thus, the transient increase in intracellular calcium (calcium transient), which occurs subsequent to membrane excitation (the fast rising phase of an action potential) preceding force development or cell shortening, plays a key role in cardiac excitation-contraction coupling.[18] Myofilament function is determined by the expression levels of multiple isoforms of myofilament proteins, and alterations in cardiac function have been attributed to shifts in isoform composition and proteolysis of myofilament proteins. Apart from the translational changes in protein expression, post-translational modifications of myofilament proteins are essential for the regulation of cardiac function both under physiological and pathophysiological conditions. Elucidation of the functional role of post-translational protein modifications is crucial to understand the changes in myocardial performance resulting from myofilament protein alterations during cardiac pathology.[22] In this thesis we examined in chapter 2 the influence of exercise training on the MI-induced abnormalities in calcium-handling and myofilament function.

Exercise in health and disease: when and how?

Epidemiological studies over the past 50 years have unanimously shown that low physical activity and physical fitness is associated with high cardiovascular and total mortality.[23] However, recent studies also indicate that changes in physical activity, and especially changes that lead to an increase in physical fitness, can reverse this rather pessimistic scenario. Studies relating physical activity to cardiovascular disease have reported cardiovascular disease mortality as an end-point. Four of seven cohort studies found both an inverse association and a dose-response gradient between level of physical activity and risk of cardiovascular disease outcome.[24-27] One study found an inverse association within the moderately active group but less of an effect on the vigorously active group.[28] Two studies reported no relationship of physical activity with cardiovascular disease mortality.[29,30] Beneficial effects of exercise training on cardiovascular morbidity have also been reported in epidemiological studies, emphasizing the higher survival rates among people conducting physically active life-style but neglecting information of heart performance after a coronary event.[31-34] Nevertheless, vigorous physical activity can also acutely and transiently increase the risk of acute myocardial infarction

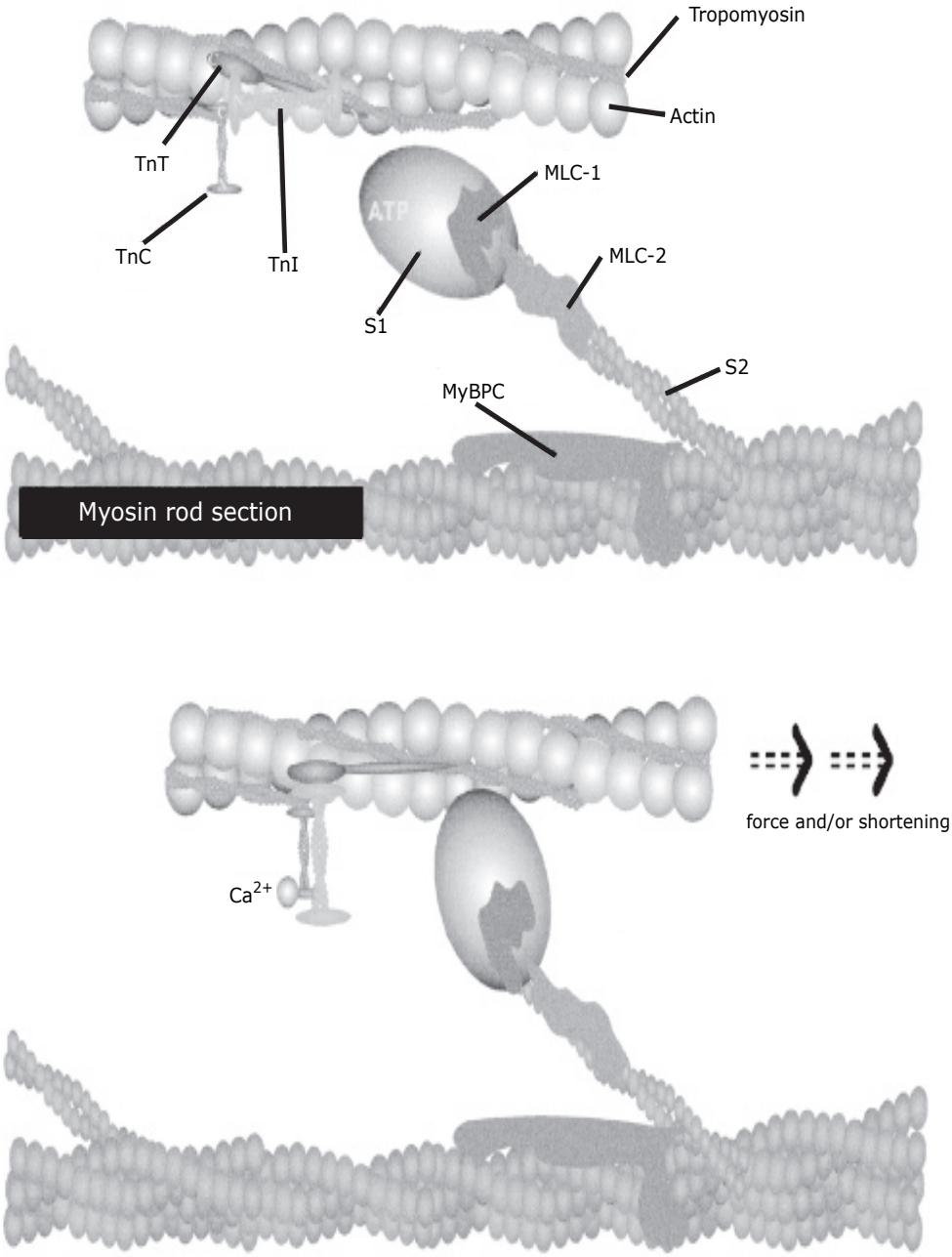

Figure 2: Sarcomere structure.

The major proteins that are involved in contractile activation and regulation are shown in diastole (top panel) and systole (bottom panel).[20] Abbreviations: TnI: troponin I, TnT: troponin T, TnC: troponin C, MLC: myosin light chain, globular head portion (S1) and hinged stalk region (S2) of myosin, MyBPC: myosin binding protein C, Ca^{2+}: calcium.

and sudden cardiac death in susceptible individuals. The Global Burden of Disease Study initiated by the World Health Organization included physical inactivity among the most important risk factors threatening global health. A sedentary lifestyle may be as detrimental to health as smoking. Encouragement of physical activity is an important and difficult task, as society is becoming increasingly successful in reducing our need to move.

In contrast to pathological LV remodeling after a myocardial infarction, LV remodeling produced by regular dynamic exercise is associated with a substantial reduced risk for coronary artery disease and heart failure.[7,35,36] Moreover, physical inactivity has been proposed to be an independent risk factor for cardiovascular disease.[37,38] Exercise training is associated with an increased myocardial perfusion capacity and with normal or even increased contractile function of the normal heart.[39,40] There is also clinical evidence that exercise after myocardial infarction has a beneficial effect on disease progression and survival.[41,42] For example, physical conditioning in patients with LV dysfunction results in an increased exercise capacity which has been ascribed, at least in part, to skeletal muscle adaptations.[43] The effects of exercise on LV remodeling and function are still incompletely understood, as several studies in humans reported contradictory effects of training on LV remodeling after a myocardial infarction.[44-53] Figure 3 represents an overview of studies mentioned below and the results from this thesis.

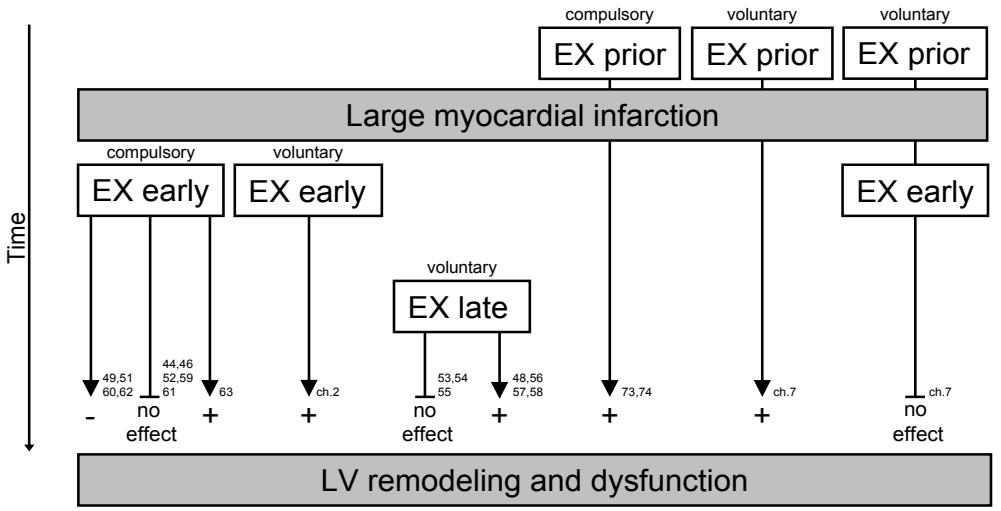

Figure 3: Schematic drawing.
Effects of compulsory or voluntary exercise training started prior to a large MI and/or started early or late after MI on LV remodeling and dysfunction. Numbers indicate the references and ch indicate the chapters in this thesis.

Careful inspection of these studies suggests that after a small myocardial infarction, exercise has no detrimental effect[46,52] or even improves[45,48,50] LV geometry and function, independent of whether exercise was started late, i.e., ~1 year,[45,48] or early, ie, <2 months,[46,50,52] after myocardial infarction. In contrast, in patients with a large myocardial infarction, exercise had either no,[53] or a beneficial[48] effect on ejection fraction (EF) and LV volumes but only when started late after myocardial infarction. However, when exercise after a large myocardial infarction is started at a time when LV remodeling is still ongoing (<3 to 4 months after myocardial infarction), the majority of studies reported that exercise has either no,[44,46,52] or even a detrimental[49,51] effect on LV volume and EF. Similar to these clinical studies, studies in rats indicate that exercise started late (>3 weeks) after a moderate to large myocardial infarction, resulting in a 35% to 50% loss of LV mass, at a time when infarct healing is complete, does not aggravate,[54,55] or even blunts[56-58] LV dilation and hypertrophy. In contrast, when started <1 week after a moderate to large myocardial infarction,[59-62] exercise resulted in variable outcomes with beneficial,[63] no,[59,61] or even detrimental[60,62] effects on LV remodeling. These rodent studies lend further support to the concern that early exercise may have detrimental effects on LV remodeling after a large myocardial infarction.

However, interpretation of these studies is hampered by the fact that late exercise studies in rats principally used treadmill running,[48,55,56,58] whereas early exercise studies predominantly used swimming.[57,59-61,63] As hemodynamic responses to swimming are markedly different from those to treadmill running the effects of exercise on LV remodeling and function may be explained by the exercise protocol rather than the timing of exercise.[64,65] In chapter 2 of this thesis we investigated the effects of exercise training through voluntary treadmill running, starting within 24 hours after a large myocardial infarction (comprising ~45% of LV mass), on LV remodeling and dysfunction in the mouse. The results indicated that exercise attenuated the myocardial infarction induced LV dysfunction, without a detrimental effect on LV remodeling. Consequently, we tested the hypothesis that exercise early after a large myocardial infarction is able to reverse the myocardial infarction induced abnormalities in β_1-adrenergic receptor and Ca^{2+}-handling protein expression, phosphorylation status of contractile proteins, Ca^{2+}-handling and myofilament function, within the non-infarcted remodeled myocardium.

There is substantially less information available as to whether prior exercise training affords any protection in situations where, despite regular exercise, a major cardiovascular event like MI does occur. Animal studies suggest that prior exercise can precondition the myocardium, thereby protecting the heart against

irreversible damage produced by ischemia-reperfusion in rats[66-69] and dogs.[70] In addition, prior exercise training may modulate post-infarct remodeling independent of any myocardial preconditioning effect. Thus, two studies in rats using a permanent coronary artery ligation (in which preconditioning cannot limit acute myocardial necrosis)[71,72] showed that infarct size and, likely as a consequence, LV remodeling were reduced by prior swim training.[73,74] The authors ascribed the reduction in infarct size to the observed increase in myocardial vascularization. An increased myocardial vascularity is indeed observed in swim trained rats.[75] In contrast, myocardial vascularization following treadmill running is not consistently associated with increased vascularization or enhanced collateral blood flow.[75]

These different effects of swimming versus running may well be explained by the markedly different hemodynamic responses in rodents to swimming versus running.[65] Furthermore, forced swim training often results in diving bradycardia and intermittent hypoxia[76] when animals are submerged, both of which are known to stimulate angiogenesis.[77,78] In contrast, studies into the effects of prior exercise training by voluntary treadmill running on LV remodeling after MI have not been performed to date. Consequently, in chapter 7 of this thesis we investigated that additional exercise training by voluntary wheel running prior to an acute myocardial infarction, i.e. a higher level of physical activity and fitness at the time of a myocardial infarction, is associated with improved survival and attenuated LV dysfunction after myocardial infarction.

NO-GC-cGMP

In 1980, Furchgott and Zawadski[79] discovered the endogenous endothelium-derived relaxation factor, and this was shown to be nitric oxide (NO) by Palmer, Ferrige and Moncada in 1987.[80] NO is produced by NO synthases (NOS) via a reaction in which the substrate L-arginine is converted to L-citrulline (Figure 4).[81] There are currently three known NOS in mammals: neuronal NOS (nNOS), inducible NOS (iNOS) and endothelial NOS (eNOS). The enzymes and the genes encoding the enzymes are also termed NOS1 (nNOS), NOS2 (iNOS) and NOS3 (eNOS).[81,82] NO activates cyclic guanosine monophosphate (cGMP), which induces smooth muscle relaxation by multiple mechanisms including: *(i)* increased intracellular cGMP, which inhibits calcium entry into the cell and decreases intracellular calcium concentrations; *(ii)* increased intracellular cGMP activates K^+-channels, which leads to hyperpolarization and relaxation, and *(iii)* stimulates a cGMP-dependent protein kinase that activates myosin light chain phosphatase, the enzyme that dephosphorylates myosin light chains, which leads to smooth muscle relaxation.

Three different enzymes, guanylyl cyclases (GC), are responsible for the synthesis of cGMP namely: soluble GC (sGC) and the particulate forms of GC (pGC A and B). sGC and pGC are classically distinguished in function by their main subcellular location (Figure 4). sGCs are mainly located in the cytosol and pGCs are integral proteins of the cell membrane. Another important difference between these enzymes is that sGCs are activated by NO and pGCs by specific peptides as atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP) and C-type natriuretic peptide (CNP).

In contrast to ANP and BNP, which are produced mainly in the cardiac atria and ventricles, CNP is mainly released in an autocrine/paracrine fashion from endothelial cells as an endothelium-derived vasodilator.[83,84] Since Wei and colleagues first confirmed the presence of CNP within the human myocardium by immunohistochemistry and radioimmunoassay,[85] accumulating evidence suggests that CNP exhibits important autocrine and paracrine functions within the heart and coronary circulation.[86] Hobbs *et al*[87] demonstrated that endothelium-derived CNP is involved in the regulation of rat coronary circulation and represents a protective mechanism against ischemia-reperfusion injury in isolated perfused hearts, since infusion of CNP, either prior to or following an ischemic insult, resulted in a 30–50% reduction in the infarct size. Furthermore, in vivo administration of CNP has been shown to improve cardiac function and attenuate cardiac remodeling after myocardial infarction in rats.[88] However, it is still not certain whether the cardiac CNP effects are dependent on the endothelium, cardiomyocytes or both. Thus, we generated transgenic mice selectively overexpressing CNP in cardiomyocytes. This in vivo model was used to investigate in chapter 6 of this thesis the effect of cardiomyocyte-specific CNP overexpression on ischemia-reperfusion injury and myocardial infarction.

Over de last 20 years, the NO literature has experienced exponential growth and has been associated with a considerable increase in knowledge. In 1998, the importance of NO was underscored by the award of Nobel prizes to three outstanding cardiovascular investigators, Furchgott, Ignarro and Murad. Apart from its vasodilator effect NO modulates several other aspects of vascular function, including leukocyte activation, platelet aggregation, interactions between the endothelium and circulating cells, vascular smooth muscle proliferation, and angiogenesis. In addition, NO appears to be capable of modulating organ and tissue oxygen consumption. These biological roles of NO have been studied classically with pharmacological strategies to inhibit NOS, e.g. via L-arginine analogues such as L-nitro-arginine (L-NA), L-nitro-monomethyl-arginine (L-NMMA) and L-nitro-arginine

methylester (L-NAME). Since these compounds lack specificity with respect to the NOS isoforms (e.g. L-NA blocks both iNOS and eNOS), animals with selective overexpression (through transgenesis) or deficiency (knockout, ko) of specific NOS isoforms are extremely useful, which allow a more targeted approach. In this thesis eNOS transgenic (eNOStg) or eNOSko mice are used to investigate the role of eNOS, which is the major isoform of NOS that is expressed in coronary vascular endothelium and cardiac myocytes,[89] in cardiovascular pathology produced by MI. For the generation of eNOStg mice, we used a DNA fragment that comprised the complete human eNOS genomic sequence, including all exons and introns as well its natural flanking sequences.[90] Therefore, our mice are different from those described by Ohashi *et al* which overexpress bovine eNOS cDNA, driven by the murine preproendothelin-1 promoter.[91] Our approach was chosen in order to preserve the natural regulation of the gene and to prevent ectopic expression. For example, the endothelium enhancer element that is located 4.9 kilo bases upstream from the transcription start site of the eNOS gene[92] is included in this construct. Since the eNOS promoter has been shown to contain essential elements for transcriptional regulation and elements responsible for the tissue distribution of eNOS expression,[92,93] we chose this approach to mimic the human situation in terms of regulation and tissue distribution of eNOS as closely as possible.[90]

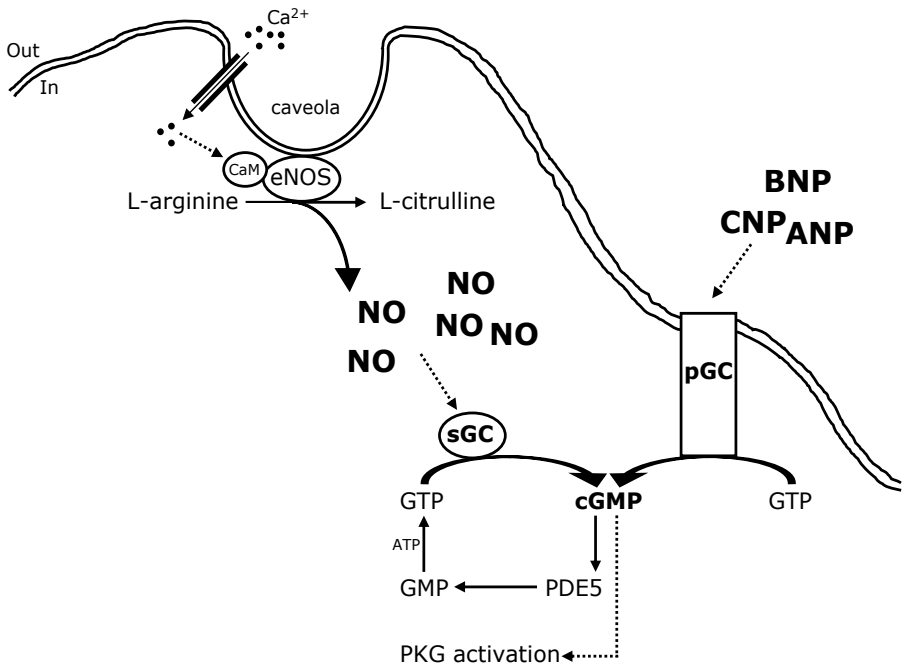

Figure 4: NO signaling.

Ca^{2+}: calcium, eNOS: endothelial nitric oxide synthase, CaM: calmodulin, sGC: soluble guanylyl cyclase, pGC: particulate gyanylyl cyclase, GTP: guanosine triphosphate, ATP: adenosine triphosphate, GMP: cyclic guanosine monophosphate, PDE5: phosphodiesterase type 5, PKG: protein kinase G, BNP, CNP, ANP: brain, cardiac, atrial natriuretic peptides.

NO and cardiac function in health and disease

Although the importance of NO was first described in the cardiovascular system, there is continuing controversy about the effects of NO in cardiovascular disease: good or bad, beneficial or deleterious, friend or foe?

The mammalian heart consists of two atrial chambers that collect blood and two ventricles that pump it out. An electrical impulse initiated in the right atrium spreads through a conduction system to coordinate myocardial contraction (excitation–contraction coupling). Two isoforms of NOS, nNOS and eNOS, are variably distributed throughout the heart.[94,95] The ventricular cardiac myocytes contain both isoforms. nNOS is expressed in the sarcoplasmic reticulum[95] (SR) and eNOS in the sarcolemma and T-tubule membranes, sites notable for their control of calcium flux, which are the chief determinants of the force and rhythm of myocardial contraction. eNOS is also found in the mitochondria,[96] and the third isoform, iNOS, is expressed under pathophysiological conditions.[97] Studies at the molecular, cellular and organism levels have firmly established the influence of NO on cardiac performance, in particular, myocardial contractility,[98,99] lusitropy,[100] chronotropy,[101] and energy production or consumption.[102] Several studies in eNOSko mice reported contradictory effects on muscarinic and β-adrenergic regulation of isolated myocyte heart rate, papillary muscle contractility, and calcium current.[101,103,104] NO has been shown to intervene with muscarinic and β-adrenergic signaling indirectly through cyclic cGMP. In the myocyte, this second messenger tends to offset the actions of cyclic AMP (cAMP). cAMP stimulates contraction, partly by activation of the L-type calcium channel. Increases in cAMP are produced by β-adrenergic coupling to adenylyl cyclase through a stimulary G-protein (Figure 1). In an alternate pathway, NO binds directly to cysteine sulfhydryls or iron centers in proteins (nitrosylation/redox signaling). The sarcolemmal L-type calcium channel,[94] the SR-calcium release channel (RYR)[105] and the SR-calcium ATPase (SERCA)[95] are all NO/redox-regulated. NO and related molecules also influence cellular respiration by binding to mitochondrial electron transport complexes. However, NO frequently exhibits dual activities. In particular, NO concentrations rise and fall in the heart on millisecond timescales. Corresponding increases in cGMP inhibit contractility, whereas S-nitrosylation-induced activation of the SR-calcium release channel and/or the L-type channel has force-enhancing effects.[106] Such opposing activities of NO during the cardiac cycle, perhaps achieved by coordinated actions of different NOS isoforms, would facilitate cardiac performance. The subtle effects of NO-related molecules reflect a permissive role in signaling, which if not obligatory, is likely to be essential for optimal cardiac function.

NO can modulate many of the processes leading to cardiac hypertrophy and remodeling. Experimental and clinical studies suggest that the vascular endothelium plays an important role in modulating the progression of ventricular and vascular remodeling in heart failure. For instance, endothelium derived NO produces systemic and pulmonary vasodilation,[107] resulting in a lower ventricular afterload. Also NO donors such as nitrates have been used in humans to treat angina pectoris and hypertensive crises, for afterload reduction in heart failure, and for the acute management of myocardial infarction. An increase in NO bioavailability has been shown to improve many of the processes perturbed in LV remodeling, including angiogenesis,[108] cardiac fibrosis,[109] and angiotensin II induced cardiomyocyte hypertrophy.[110] Although the weight of evidence suggests that eNOS activity does not modulate left or right ventricular weight in normal mice, eNOS activity appears to be protective against LV hypertrophy and remodeling in response to a pathological stimulus. Indeed, in vivo experiments in eNOS overexpressing[111] and eNOS deficient mice[112] have provided evidence that eNOS protects against LV remodeling and LV dysfunction after myocardial infarction. eNOS deficiency resulted in exaggerated LV dilation and hypertrophy and enhanced decreases in indices of systolic and diastolic LV function, 28 days after myocardial infarction produced by permanent coronary artery occlusion.[112] Conversely, LV hypertrophy produced by 7 days of isoproterenol infusion was markedly attenuated in eNOS transgenic mice, together with a reduction of ANP expression.[113] Jones *et al*[111] reported that elevated expression of the human eNOS gene in the vascular endothelium in transgenic overexpressing mice not only improved survival, but also blunted LV dysfunction and pulmonary congestion after MI. These beneficial effects were ascribed to a reduction in afterload, secondary to a lower peripheral vascular resistance,[114] thereby facilitating LV pump function, whereas global LV contractility was unchanged.

Nitric oxide and exercise training

The lack of a beneficial effect of exercise (as mentioned above), which started immediately after a myocardial infarction, in patients with a large myocardial infarction is difficult to explain but may be related to the presence of risk factors, and hence endothelial dysfunction,[115] in the majority of patients. Beneficial effects of exercise on cardiac function can be attributed, at least in part, to an exercise-induced up regulation of eNOS expression and activity.[116] Studies involving exercise training have shown local and systemic beneficial effects on agonist-mediated endothelium dependent vasodilation as well as in flow-dependent vasodilation.[117]

The increase in shear stress at the local level coupled with the systemic effects of heart rate and blood pressure have resulted in increased coronary and peripheral blood flow. These changes may be the main contributors to the significant improvement in endothelial function that follows exercise training.

There is some mechanistic insights into the molecular mechanisms underlying the enhanced endothelial vasodilator function in response to exercise.[118] Hambrecht *et al*[119] demonstrated that transcriptional and posttranscriptional activation of eNOS contribute to exercise-induced improvement of endothelial function in patients with coronary artery disease. More specifically, the authors link the effect of regular exercise training to an increase in eNOS protein expression and activation of eNOS enzyme activity via Akt-dependent phosphorylation. Inhibition of Akt (protein kinase B) blocks shear stress–induced NO synthesis as Akt activates the enzymatic activity of eNOS. If exercise results in improved endothelial function via Akt-dependent mechanisms, other interventions that activate Akt might also have demonstrable effects on endothelial function. Accordingly lipid-lowering therapy with statins not only activates the Akt-eNOS pathway[120] but also rapidly improves endothelial NO production and, most importantly, decreases cardiovascular events in patients at risk for coronary artery disease. Thus, it is reasonable to speculate that activation of the Akt-eNOS signaling pathway by exercise, as reported by Hambrecht *et al*,[119] will eventually lead to a reduced progression of atherosclerotic disease. Interestingly, however, the effects of exercise on Akt-mediated eNOS activation observed by Hambrecht *et al*[119] appear to be superimposed on the effects of statin therapy, given that 88% of the exercise trained patients studied were chronically receiving statins.[118] In chapter 5 we investigated whether eNOS overexpression mimicked cardiac responsiveness to exercise after myocardial infarction.

Aim and outline of this thesis

The general aim of this thesis is to study the effects of regular physical exercise on pathological LV remodeling and dysfunction in a mouse model of myocardial infarction, with a special emphasis on cardiomyocyte function. A second aim is to investigate the morphological, cellular and molecular basis of these effects, in particular the role of an increased NO production in the beneficial effects of exercise training.

In **Chapter 2**, the effects of regular physical exercise by voluntary treadmill running, started immediately after a large myocardial infarction, on LV remodeling and dysfunction in the mouse are assessed. In addition, we tested the hypothesis

that exercise started immediately after a large myocardial infarction is able to reverse the abnormalities induced by myocardial infarction in β_1-adrenergic receptor and Ca^{2+}-handling protein expression, phosphorylation status of contractile proteins, Ca^{2+}-handling and myofilament function, within the non-infarcted remodeled myocardium. The results indicate that exercise training after a large MI attenuated global LV dysfunction and cardiomyocyte shortening, which can be explained by the exercise-induced improvement of myofilament function.

The beneficial effects of exercise on cardiac function can be attributed, at least in part, to an exercise-induced upregulation of endogenous eNOS expression and activity.[121-123] Recent understanding regarding the importance of eNOS in controlling cardiac function, led us to investigate in **Chapter 3** the critical role of eNOS in the exercise-induced amelioration of LV dysfunction after myocardial infarction. For this purpose we used eNOS deficient mice. The results indicated that eNOS plays an obligatory role in the beneficial effects of exercise after myocardial infarction on LV remodeling and dysfunction. Consequently, the question rose whether the beneficial effects of exercise after myocardial infarction could be mimicked by increased levels of eNOS. Therefore, we first studied in **Chapter 4** the in vivo effects of increased eNOS expression on the cardiovascular system. For this purpose we created transgenic mice with elevated expression of the human eNOS gene. We used this mouse model in **Chapter 5**, to test the hypothesis that the beneficial effects of exercise after myocardial infarction, which we found in chapter 2, can be mimicked by elevated eNOS expression. The results showed that elevated eNOS expression mimicked the beneficial effects of exercise training, but only in part. Subsequently, we tested whether combining exercise and elevated eNOS-expression would provide additional protection against LV remodeling and dysfunction after myocardial infarction. The results unexpectedly indicated that exercise and elevated eNOS expression abolished the beneficial effects of either treatment alone on global cardiac function after MI. Furthermore, we investigated whether enhanced cGMP through CNP-mediated increased pGC, can also result in improved cardiac function after MI similar to eNOS-mediated increased sGC (chapter 5). **Chapter 6** shows the beneficial effect of elevated CNP expression on cardiac hypertrophy induced by myocardial infarction in transgenic mice.

There is limited information available as to whether prior exercise training affords any protection in situations where, despite exercise training, a major cardiovascular event like MI does occur. Therefore, in **Chapter 7**, additional exercise training prior to an acute MI resulting in a higher level of physical fitness at the time a myocardial infarction occurs, was studied to determine whether this is associated

with improved survival and attenuated LV dysfunction after myocardial infarction. The results showed that prior exercise training improves survival, infarct healing and LV dysfunction after myocardial infarction and in **Chapter 8**, a discussion and summary of the major findings of the studies presented in this thesis and future perspectives are presented.

References

1. Kay D, Blue A, Pye P, Lacy A, Gray C, Moore S. *Heart failure: improving the continuum of care.* Care Manag J 2006;7:58-63.
2. Jager-Geurts MH PR, van Dis SJ, Bots ML. *Hart- en vaatziekten in Nederland 2006, cijfers over ziekte en sterfte.* In: Den Haag: Nederlandse Hartstichting, ed., 2006.
3. Konstam MA, Udelson JE, Sharpe N. *Prevention and reversal of left ventricular remodeling: summation.* J Card Fail 2002;8:S506-511.
4. Hellermann JP, Jacobsen SJ, Gersh BJ, Rodeheffer RJ, Reeder GS, Roger VL. *Heart failure after myocardial infarction: a review.* Am J Med 2002;113:324-330.
5. Braunwald E. The Denolin lecture. *Congestive heart failure: a half century perspective.* Eur Heart J 2001;22:825-836.
6. Heidenreich PA, McClellan M. *Trends in treatment and outcomes for acute myocardial infarction: 1975-1995.* Am J Med 2001;110:165-174.
7. Manson JE, Greenland P, LaCroix AZ, Stefanick ML, Mouton CP, Oberman A, Perri MG, Sheps DS, Pettinger MB, Siscovick DS. *Walking compared with vigorous exercise for the prevention of cardiovascular events in women.* N Engl J Med 2002;347:716-725.
8. Lovell-Badge R. *The future for stem cell research.* Nature 2001;414:88-91.
9. Yan AT, Yan RT, Liu PP. *Narrative review: pharmacotherapy for chronic heart failure: evidence from recent clinical trials.* Ann Intern Med 2005;142:132-145.
10. Narula J, Haider N, Arbustini E, Chandrashekhar Y. *Mechanisms of disease: apoptosis in heart failure--seeing hope in death.* Nat Clin Pract Cardiovasc Med 2006;3:681-688.
11. Spinale FG. *Myocardial matrix remodeling and the matrix metalloproteinases: influence on cardiac form and function.* Physiol Rev 2007;87:1285-1342.
12. van der Velden J, Merkus D, Klarenbeek BR, James AT, Boontje NM, Dekkers DH, Stienen GJ, Lamers JM, Duncker DJ. *Alterations in myofilament function contribute to left ventricular dysfunction in pigs early after myocardial infarction.* Circ Res 2004;95:e85-95.
13. Houser SR, Margulies KB. *Is depressed myocyte contractility centrally involved in heart failure?* Circ Res 2003;92:350-358.
14. de Tombe PP. *Altered contractile function in heart failure.* Cardiovasc Res 1998;37:367-380.
15. Houser SR, Piacentino V, 3rd, Mattiello J, Weisser J, Gaughan JP. *Functional properties of failing human ventricular myocytes.* Trends Cardiovasc Med 2000;10:101-107.
16. Lefkowitz RJ, Rockman HA, Koch WJ. *Catecholamines, cardiac beta-adrenergic receptors, and heart failure.* Circulation 2000;101:1634-1637.
17. Pieske B, Maier LS, Bers DM, Hasenfuss G. *Ca^{2+} handling and sarcoplasmic reticulum Ca^{2+} content in isolated failing and nonfailing human myocardium.* Circ Res 1999;85:38-46.
18. Endoh M. *Signal transduction and Ca^{2+} signaling in intact myocardium.* J Pharmacol Sci 2006;100:525-537.
19. Bers DM. *Excitation-contraction coupling and cardiac contractile force.* Dordrecht, The Netherlands: Kluwer Academic Press, 2001.
20. de Tombe PP. *Cardiac myofilaments: mechanics and regulation.* J Biomech 2003;36:721-730.
21. Metzger JM, Westfall MV. *Covalent and noncovalent modification of thin filament action: the essential role of troponin in cardiac muscle regulation.* Circ Res 2004;94:146-158.
22. van der Velden J. *Functional significance of myofilament protein oxidation.* Eur Heart J 2006;27:764-765.
23. Booth FW, Lees SJ. *Fundamental questions about genes, inactivity, and chronic diseases.* Physiol Genomics 2007;28:146-157.
24. Kannel WB, Belanger A, D'Agostino R, Israel I. *Physical activity and physical demand on the job and risk of cardiovascular disease and death: the Framingham Study.* Am Heart J 1986;112:820-825.
25. Kannel WB, Sorlie P. *Some health benefits of physical activity. The Framingham Study.* Arch Intern Med 1979;139:857-861.
26. LaCroix AZ, Leveille SG, Hecht JA, Grothaus LC, Wagner EH. *Does walking decrease the risk of cardiovascular disease hospitalizations and death in older adults?* J Am Geriatr Soc 1996;44:113-120.
27. Paffenbarger RS, Jr., Hyde RT, Wing AL, Steinmetz CH. *A natural history of athleticism and cardiovascular health.* Jama 1984;252:491-495.
28. Lindsted KD, Tonstad S, Kuzma JW. *Self-report of physical activity and patterns of mortality in Seventh-Day Adventist men.* J Clin Epidemiol 1991;44:355-364.
29. Arraiz GA, Wigle DT, Mao Y. *Risk assessment of physical activity and physical fitness in the Canada Health Survey mortality follow-up study.* J Clin Epidemiol 1992;45:419-428.
30. Sherman SE, D'Agostino RB, Cobb JL, Kannel WB. *Physical activity and mortality in women in the Framingham Heart Study.* Am Heart J 1994;128:879-884.
31. Berlin JA, Colditz GA. *A meta-analysis of physical activity in the prevention of coronary heart disease.* Am J Epidemiol 1990;132:612-628.
32. Sandvik L, Erikssen J, Thaulow E, Erikssen G, Mundal R, Rodahl K. *Physical fitness as a predictor of mortality among healthy, middle-aged Norwegian men.* N Engl J Med 1993;328:533-537.
33. Shephard RJ, Balady GJ. *Exercise as cardiovascular therapy.* Circulation 1999;99:963-972.
34. Wagner A, Simon C, Evans A, Ferrieres J, Montaye M, Ducimetiere P, Arveiler D. *Physical activity and coronary event incidence in Northern Ireland and France: the Prospective Epidemiological Study of Myocardial Infarction (PRIME).*

Circulation 2002;105:2247-2252.
35. Gielen S, Schuler G, Hambrecht R. *Exercise training in coronary artery disease and coronary vasomotion.* Circulation 2001;103:E1-6.
36. Hakim AA, Petrovitch H, Burchfiel CM, Ross GW, Rodriguez BL, White LR, Yano K, Curb JD, Abbott RD. *Effects of walking on mortality among nonsmoking retired men.* N Engl J Med 1998;338:94-99.
37. Booth FW, Laye MJ, Lees SJ, Rector RS, Thyfault JP. *Reduced physical activity and risk of chronic disease: the biology behind the consequences.* Eur J Appl Physiol 2008;102:381-390.
38. Wannamethee SG, Shaper AG. *Physical activity and cardiovascular disease.* Semin Vasc Med 2002;2:257-266.
39. Cohen MV. *Coronary and collateral blood flows during exercise and myocardial vascular adaptations to training.* Exerc Sport Sci Rev 1983;11:55-98.
40. Moore RL, Korzick DH. *Cellular adaptations of the myocardium to chronic exercise.* Prog Cardiovasc Dis 1995;37:371-396.
41. Coats AJ. *Exercise training in heart failure.* Curr Control Trials Cardiovasc med 2000;1:155-160.
42. You Fang Z, Marwick TH. *Mechanisms of exercise training in patients with heart failure.* Am Heart J 2003;145:904-911.
43. Adamopoulos S, Coats AJ, Brunotte F, Arnolda L, Meyer T, Thompson CH, Dunn JF, Stratton J, Kemp GJ, Radda GK, *et al*. *Physical training improves skeletal muscle metabolism in patients with chronic heart failure.* J Am Coll Cardiol 1993;21:1101-1106.
44. Dubach P, Myers J, Dziekan G, Goebbels U, Reinhart W, Vogt P, Ratti R, Muller P, Miettunen R, Buser P. *Effect of exercise training on myocardial remodeling in patients with reduced left ventricular function after myocardial infarction: application of magnetic resonance imaging.* Circulation 1997;95:2060-2067.
45. Ehsani AA, Biello DR, Schultz J, Sobel BE, Holloszy JO. *Improvement of left ventricular contractile function by exercise training in patients with coronary artery disease.* Circulation 1986;74:350-358.
46. Giannuzzi P, Tavazzi L, Temporelli PL, Corra U, Imparato A, Gattone M, Giordano A, Sala L, Schweiger C, Malinverni C. *Long-term physical training and left ventricular remodeling after anterior myocardial infarction: results of the Exercise in Anterior Myocardial Infarction (EAMI) trial. EAMI Study Group.* J Am Coll Cardiol 1993;22:1821-1829.
47. Giannuzzi P, Temporelli PL, Corra U, Gattone M, Giordano A, Tavazzi L. *Attenuation of unfavorable remodeling by exercise training in postinfarction patients with left ventricular dysfunction: results of the Exercise in Left Ventricular Dysfunction (ELVD) trial.* Circulation 1997;96:1790-1797.
48. Giannuzzi P, Temporelli PL, Corra U, Tavazzi L. *Antiremodeling effect of long-term exercise training in patients with stable chronic heart failure: results of the Exercise in Left Ventricular Dysfunction and Chronic Heart Failure (ELVD-CHF) Trial.* Circulation 2003;108:554-559.
49. Jugdutt BI, Michorowski BL, Kappagoda CT. *Exercise training after anterior Q wave myocardial infarction: importance of regional left ventricular function and topography.* J Am Coll Cardiol 1988;12:362-372.
50. Koizumi T, Miyazaki A, Komiyama N, Sun K, Nakasato T, Masuda Y, Komuro I. *Improvement of left ventricular dysfunction during exercise by walking in patients with successful percutaneous coronary intervention for acute myocardial infarction.* Circ J 2003;67:233-237.
51. Kubo N, Ohmura N, Nakada I, Yasu T, Katsuki T, Fujii M, Saito M. *Exercise at ventilatory threshold aggravates left ventricular remodeling in patients with extensive anterior acute myocardial infarction.* Am Heart J 2004;147:113-120.
52. Otsuka Y, Takaki H, Okano Y, Satoh T, Aihara N, Matsumoto T, Yasumura Y, Morii I, Goto Y. *Exercise training without ventricular remodeling in patients with moderate to severe left ventricular dysfunction early after acute myocardial infarction.* Int J Cardiol 2003;87:237-244.
53. Sullivan MJ, Higginbotham MB, Cobb FR. *Exercise training in patients with severe left ventricular dysfunction. Hemodynamic and metabolic effects.* Circulation 1988;78:506-515.
54. Libonati JR. *Exercise and diastolic function after myocardial infarction.* Med Sci Sports Exerc 2003;35:1471-1476.
55. Musch TI, Moore RL, Leathers DJ, Bruno A, Zelis R. *Endurance training in rats with chronic heart failure induced by myocardial infarction.* Circulation 1986;74:431-441.
56. Orenstein TL, Parker TG, Butany JW, Goodman JM, Dawood F, Wen WH, Wee L, Martino T, McLaughlin PR, Liu PP. *Favorable left ventricular remodeling following large myocardial infarction by exercise training. Effect on ventricular morphology and gene expression.* J Clin Invest 1995;96:858-866.
57. Wisloff U, Loennechen JP, Currie S, Smith GL, Ellingsen O. *Aerobic exercise reduces cardiomyocyte hypertrophy and increases contractility, Ca^{2+} sensitivity and SERCA-2 in rat after myocardial infarction.* Cardiovasc Res 2002;54:162-174.
58. Zhang LQ, Zhang XQ, Musch TI, Moore RL, Cheung JY. *Sprint training restores normal contractility in postinfarction rat myocytes.* J Appl Physiol 2000;89:1099-1105.
59. Alhaddad IA, Hakim I, Siddiqi F, Lagenback E, Mallavarapu C, Nethala V, Mounce D, Ross PL, Brown EJ, Jr. *Early exercise after experimental myocardial infarction: effect on left ventricular remodeling.* Coron Artery Dis 1998;9:319-327.
60. Gaudron P, Hu K, Schamberger R, Budin M, Walter B, Ertl G. *Effect of endurance training early or late after coronary artery occlusion on left ventricular remodeling, hemodynamics, and survival in rats with chronic transmural myocardial infarction.* Circulation 1994;89:402-412.
61. Hochman JS, Healy B. *Effect of exercise on acute myocardial infarction in rats.* J Am Coll Cardiol 1986;7:126-132.
62. Kloner RA, Kloner JA. *The effect of early exercise on myocardial infarct scar formation.* Am Heart J 1983;106:1009-1013.
63. Oh BH, Ono S, Rockman HA, Ross J, Jr. *Myocardial hypertrophy in the ischemic zone induced by exercise in rats after*

coronary reperfusion. Circulation 1993;87:598-607.
64. Bernstein D. *Exercise assessment of transgenic models of human cardiovascular disease.* Physiol Genomics 2003;13:217-226.
65. Flaim SF, Minteer WJ, Clark DP, Zelis R. *Cardiovascular response to acute aquatic and treadmill exercise in the untrained rat.* J Appl Physiol 1979;46:302-308.
66. Brown DA, Jew KN, Sparagna GC, Musch TI, Moore RL. *Exercise training preserves coronary flow and reduces infarct size after ischemia-reperfusion in rat heart.* J Appl Physiol 2003;95:2510-2518.
67. Powers SK, Demirel HA, Vincent HK, Coombes JS, Naito H, Hamilton KL, Shanely RA, Jessup J. *Exercise training improves myocardial tolerance to in vivo ischemia-reperfusion in the rat.* Am J Physiol 1998;275:R1468-1477.
68. Yamashita N, Hoshida S, Otsu K, Asahi M, Kuzuya T, Hori M. *Exercise provides direct biphasic cardioprotection via manganese superoxide dismutase activation.* J Exp Med 1999;189:1699-1706.
69. Zhang KR, Liu HT, Zhang HF, Zhang QJ, Li QX, Yu QJ, Guo WY, Wang HC, Gao F. *Long-term aerobic exercise protects the heart against ischemia/reperfusion injury via PI3 kinase-dependent and Akt-mediated mechanism.* Apoptosis 2007;12:1579-1588.
70. Domenech R, Macho P, Schwarze H, Sanchez G. *Exercise induces early and late myocardial preconditioning in dogs.* Cardiovasc Res 2002;55:561-566.
71. Murry CE, Jennings RB, Reimer KA. *Preconditioning with ischemia: a delay of lethal cell injury in ischemic myocardium.* Circulation 1986;74:1124-1136.
72. van den Doel MA, Gho BC, Duval SY, Schoemaker RG, Duncker DJ, Verdouw PD. *Hypothermia extends the cardioprotection by ischaemic preconditioning to coronary artery occlusions of longer duration.* Cardiovasc Res 1998;37:76-81.
73. Freimann S, Scheinowitz M, Yekutieli D, Feinberg MS, Eldar M, Kessler-Icekson G. *Prior exercise training improves the outcome of acute myocardial infarction in the rat. Heart structure, function, and gene expression.* J Am Coll Cardiol 2005;45:931-938.
74. McElroy CL, Gissen SA, Fishbein MC. *Exercise-induced reduction in myocardial infarct size after coronary artery occlusion in the rat.* Circulation 1978;57:958-962.
75. Duncker DJ, Bache RJ. *Regulation of coronary blood flow during exercise.* Physiological Reviews 2008;88 (in press)
76. Baldwin KM. *Effects of chronic exercise on biochemical and functional properties of the heart.* Med Sci Sports Exerc 1985;17:522-528.
77. Hudlicka O, Brown MD, Walter H, Weiss JB, Bate A. *Factors involved in capillary growth in the heart.* Mol Cell Biochem 1995;147:57-68.
78. Hudlicka O, Price S. *The role of blood flow and/or muscle hypoxia in capillary growth in chronically stimulated fast muscles.* Pflugers Arch 1990;417:67-72.
79. Furchgott RF, Zawadzki JV. *The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine.* Nature 1980;288:373-376.
80. Palmer RM, Ferrige AG, Moncada S. *Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor.* Nature 1987;327:524-526.
81. Michel T, Feron O. *Nitric oxide synthases: which, where, how, and why?* J Clin Invest 1997;100:2146-2152.
82. Li H, Forstermann U. *Nitric oxide in the pathogenesis of vascular disease.* J Pathol 2000;190:244-254.
83. Ogawa Y, Itoh H, Nakao K. *Molecular biology and biochemistry of natriuretic peptide family.* Clin Exp Pharmacol Physiol 1995;22:49-53.
84. Walther T, Stepan H. *C-type natriuretic peptide in reproduction, pregnancy and fetal development.* J Endocrinol 2004;180:17-22.
85. Wei CM, Heublein DM, Perrella MA, Lerman A, Rodeheffer RJ, McGregor CG, Edwards WD, Schaff HV, Burnett JC, Jr. *Natriuretic peptide system in human heart failure.* Circulation 1993;88:1004-1009.
86. D'Souza SP, Davis M, Baxter GF. *Autocrine and paracrine actions of natriuretic peptides in the heart.* Pharmacol Ther 2004;101:113-129.
87. Hobbs A, Foster P, Prescott C, Scotland R, Ahluwalia A. *Natriuretic peptide receptor-C regulates coronary blood flow and prevents myocardial ischemia/reperfusion injury: novel cardioprotective role for endothelium-derived C-type natriuretic peptide.* Circulation 2004;110:1231-1235.
88. Soeki T, Kishimoto I, Okumura H, Tokudome T, Horio T, Mori K, Kangawa K. *C-type natriuretic peptide, a novel antifibrotic and antihypertrophic agent, prevents cardiac remodeling after myocardial infarction.* J Am Coll Cardiol 2005;45:608-616.
89. Massion PB, Balligand JL. *Modulation of cardiac contraction, relaxation and rate by the endothelial nitric oxide synthase (eNOS): lessons from genetically modified mice.* J Physiol 2003;546:63-75.
90. van Haperen R, de Waard M, van Deel E, Mees B, Kutryk M, van Aken T, Hamming J, Grosveld F, Duncker DJ, de Crom R. *Reduction of blood pressure, plasma cholesterol, and atherosclerosis by elevated endothelial nitric oxide.* J Biol Chem 2002;277:48803-48807.
91. Ohashi Y, Kawashima S, Hirata K, Yamashita T, Ishida T, Inoue N, Sakoda T, Kurihara H, Yazaki Y, Yokoyama M. *Hypotension and reduced nitric oxide-elicited vasorelaxation in transgenic mice overexpressing endothelial nitric oxide synthase.* J Clin Invest 1998;102:2061-2071.
92. Laumonnier Y, Nadaud S, Agrapart M, Soubrier F. *Characterization of an upstream enhancer region in the promoter of the human endothelial nitric-oxide synthase gene.* J Biol Chem 2000;275:40732-40741.
93. Govers R, Rabelink TJ. *Cellular regulation of endothelial nitric oxide synthase.* Am J Physiol Renal Physiol 2001;280: F193-206.
94. Balligand JL, Kobzik L, Han X, Kaye DM, Belhassen L, O'Hara DS, Kelly RA, Smith TW, Michel T. *Nitric oxide-dependent*

parasympathetic signaling is due to activation of constitutive endothelial (type III) nitric oxide synthase in cardiac myocytes. J Biol Chem 1995;270:14582-14586.
95. Xu KY, Huso DL, Dawson TM, Bredt DS, Becker LC. *Nitric oxide synthase in cardiac sarcoplasmic reticulum.* Proc Natl Acad Sci U S A 1999;96:657-662.
96. Bates TE, Loesch A, Burnstock G, Clark JB. *Mitochondrial nitric oxide synthase: a ubiquitous regulator of oxidative phosphorylation?* Biochem Biophys Res Commun 1996;218:40-44.
97. Balligand JL, Ungureanu-Longrois D, Simmons WW, Pimental D, Malinski TA, Kapturczak M, Taha Z, Lowenstein CJ, Davidoff AJ, Kelly RA, *et al*. *Cytokine-inducible nitric oxide synthase (iNOS) expression in cardiac myocytes. Characterization and regulation of iNOS expression and detection of iNOS activity in single cardiac myocytes in vitro.* J Biol Chem 1994;269:27580-27588.
98. Hare JM, Givertz MM, Creager MA, Colucci WS. *Increased sensitivity to nitric oxide synthase inhibition in patients with heart failure: potentiation of beta-adrenergic inotropic responsiveness.* Circulation 1998;97:161-166.
99. Hare JM, Kim B, Flavahan NA, Ricker KM, Peng X, Colman L, Weiss RG, Kass DA. *Pertussis toxin-sensitive G proteins influence nitric oxide synthase III activity and protein levels in rat heart.* J Clin Invest 1998;101:1424-1431.
100. Paulus WJ, Vantrimpont PJ, Shah AM. *Acute effects of nitric oxide on left ventricular relaxation and diastolic distensibility in humans. Assessment by bicoronary sodium nitroprusside infusion.* Circulation 1994;89:2070-2078.
101. Feron O, Dessy C, Opel DJ, Arstall MA, Kelly RA, Michel T. *Modulation of the endothelial nitric-oxide synthase-caveolin interaction in cardiac myocytes. Implications for the autonomic regulation of heart rate.* J Biol Chem 1998;273:30249-30254.
102. Suto N, Mikuniya A, Okubo T, Hanada H, Shinozaki N, Okumura K. *Nitric oxide modulates cardiac contractility and oxygen consumption without changing contractile efficiency.* Am J Physiol 1998;275:H41-49.
103. Campbell DL, Stamler JS, Strauss HC. *Redox modulation of L-type calcium channels in ferret ventricular myocytes. Dual mechanism regulation by nitric oxide and S-nitrosothiols.* J Gen Physiol 1996;108:277-293.
104. Vandecasteele G, Eschenhagen T, Scholz H, Stein B, Verde I, Fischmeister R. *Muscarinic and beta-adrenergic regulation of heart rate, force of contraction and calcium current is preserved in mice lacking endothelial nitric oxide synthase.* Nat Med 1999;5:331-334.
105. Han X, Kubota I, Feron O, Opel DJ, Arstall MA, Zhao YY, Huang P, Fishman MC, Michel T, Kelly RA. *Muscarinic cholinergic regulation of cardiac myocyte ICa-L is absent in mice with targeted disruption of endothelial nitric oxide synthase.* Proc Natl Acad Sci U S A 1998;95:6510-6515.
106. Gonzalez DR, Fernandez IC, Ordenes PP, Treuer AV, Eller G, Boric MP. *Differential role of S-nitrosylation and the NO-cGMP-PKG pathway in cardiac contractility.* Nitric Oxide 2008;18:157-167.
107. Duncker DJ, Stubenitsky R, Tonino PA, Verdouw PD. *Nitric oxide contributes to the regulation of vasomotor tone but does not modulate O(2)-consumption in exercising swine.* Cardiovasc Res 2000;47:738-748.
108. Murohara T, Asahara T, Silver M, Bauters C, Masuda H, Kalka C, Kearney M, Chen D, Symes JF, Fishman MC, Huang PL, Isner JM. *Nitric oxide synthase modulates angiogenesis in response to tissue ischemia.* J Clin Invest 1998;101:2567-2578.
109. Kim NN, Villegas S, Summerour SR, Villarreal FJ. *Regulation of cardiac fibroblast extracellular matrix production by bradykinin and nitric oxide.* J Mol Cell Cardiol 1999;31:457-466.
110. Ritchie RH, Schiebinger RJ, LaPointe MC, Marsh JD. *Angiotensin II-induced hypertrophy of adult rat cardiomyocytes is blocked by nitric oxide.* Am J Physiol 1998;275:H1370-1374.
111. Jones SP, Greer JJ, van Haperen R, Duncker DJ, de Crom R, Lefer DJ. *Endothelial nitric oxide synthase overexpression attenuates congestive heart failure in mice.* Proc Natl Acad Sci U S A 2003;100:4891-4896.
112. Scherrer-Crosbie M, Ullrich R, Bloch KD, Nakajima H, Nasseri B, Aretz HT, Lindsey ML, Vancon AC, Huang PL, Lee RT, Zapol WM, Picard MH. *Endothelial nitric oxide synthase limits left ventricular remodeling after myocardial infarction in mice.* Circulation 2001;104:1286-1291.
113. Ozaki M, Kawashima S, Yamashita T, Hirase T, Namiki M, Inoue N, Hirata K, Yasui H, Sakurai H, Yoshida Y, Masada M, Yokoyama M. *Overexpression of endothelial nitric oxide synthase accelerates atherosclerotic lesion formation in apoE-deficient mice.* J Clin Invest 2002;110:331-340.
114. van Deel ED, Merkus D, van Haperen R, de Waard MC, de Crom R, Duncker DJ. *Vasomotor control in mice overexpressing human endothelial nitric oxide synthase.* Am J Physiol Heart Circ Physiol 2007;293:H1144-1153.
115. Harrison DG. *Cellular and molecular mechanisms of endothelial cell dysfunction.* J Clin Invest 1997;100:2153-2157.
116. Sessa WC, Pritchard K, Seyedi N, Wang J, Hintze TH. *Chronic exercise in dogs increases coronary vascular nitric oxide production and endothelial cell nitric oxide synthase gene expression.* Circ Res 1994;74:349-353.
117. Kubo SH, Rector TS, Bank AJ, Williams RE, Heifetz SM. *Endothelium-dependent vasodilation is attenuated in patients with heart failure.* Circulation 1991;84:1589-1596.
118. Dimmeler S, Zeiher AM. *Exercise and cardiovascular health: get active to "AKTivate" your endothelial nitric oxide synthase.* Circulation 2003;107:3118-3120.
119. Hambrecht R, Adams V, Erbs S, Linke A, Krankel N, Shu Y, Baither Y, Gielen S, Thiele H, Gummert JF, Mohr FW, Schuler G. *Regular physical activity improves endothelial function in patients with coronary artery disease by increasing phosphorylation of endothelial nitric oxide synthase.* Circulation 2003;107:3152-3158.
120. Kureishi Y, Luo Z, Shiojima I, Bialik A, Fulton D, Lefer DJ, Sessa WC, Walsh K. *The HMG-CoA reductase inhibitor simvastatin activates the protein kinase Akt and promotes angiogenesis in normocholesterolemic animals.* Nat Med 2000;6:1004-1010.
121. Shen W, Zhang X, Zhao G, Wolin MS, Sessa W, Hintze TH. *Nitric oxide production and NO synthase gene expression contribute to vascular regulation during exercise.* Med Sci Sports Exerc 1995;27:1125-1134.
122. Woodman CR, Muller JM, Laughlin MH, Price EM. *Induction of nitric oxide synthase mRNA in coronary resistance*

arteries isolated from exercise-trained pigs. Am J Physiol 1997;273:H2575-2579.
123. Delp MD, Laughlin MH. *Time course of enhanced endothelium-mediated dilation in aorta of trained rats.* Med Sci Sports Exerc 1997;29:1454-1461.

Chapter 2

Early exercise training normalizes myofilament function and attenuates left ventricular pump dysfunction in mice with a large myocardial infarction

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Abstract

The extent and mechanism of the cardiac benefit of early exercise training following myocardial infarction (MI) is incompletely understood, but may involve blunting of abnormalities in Ca^{2+}-handling and myofilament function. Consequently, we investigated the effects of 8-weeks of voluntary exercise, started early after a large MI, on left ventricular (LV) remodeling and dysfunction in the mouse. Exercise had no effect on survival, MI size or LV dimensions, but improved LV fractional shortening from 8±1 to 12±1%, and $LVdP/dt_{P30}$ from 5295±207 to 5794±207 mmHg/s (both $P<0.05$), and reduced pulmonary congestion. These global effects of exercise were associated with normalization of the MI-induced increase in myofilament Ca^{2+}-sensitivity ($\Delta pCa_{50}=0.037$). This effect of exercise was PKA-mediated and likely because of improved β_1-adrenergic signaling, as suggested by the increased β_1-adrenoceptor protein (48%) and cAMP levels (36%; all $P<0.05$). Exercise prevented the MI-induced decreased maximum force generating capacity of skinned cardiomyocytes (F_{max} increased from 14.3±0.7 to 18.3±0.8 kN/m^2; $P<0.05$), which was associated with enhanced shortening of unloaded intact cardiomyocytes (from 4.1±0.3 to 7.0±0.6%; $P<0.05$). Furthermore, exercise reduced diastolic Ca^{2+}-concentrations (by ~30%, $P<0.05$) despite the unchanged SERCA2a and PLB expression and PLB phosphorylation status. Importantly, exercise had no effect on Ca^{2+}-transient amplitude, indicating that the improved LV and cardiomyocyte shortening were principally because of improved myofilament function. In conclusion, early exercise in mice after a large MI has no effect on LV remodeling, but attenuates global LV dysfunction. The latter can be explained by the exercise-induced improvement of myofilament function.

Introduction

Left ventricular (LV) remodeling after myocardial infarction (MI) is a compensatory mechanism that serves to restore LV pump function. Despite the apparent appropriateness of LV remodeling to maintain cardiac pump function early after MI, remodeling is an independent risk factor for the development of congestive heart failure.[1] The mechanism underlying the progression from LV remodeling to overt heart failure remains incompletely understood, but recent evidence indicates that abnormalities in myofilament function and Ca^{2+}-handling contribute to the LV dysfunction in the porcine heart, early after MI.[2]

In contrast to pathological LV remodeling after MI, LV remodeling produced by regular dynamic exercise is associated with a decreased risk for coronary artery disease and heart failure.[3] Exercise training is associated with an increased myocardial perfusion capacity and with normal or even increased contractile function in the normal heart.[4,5] There is also clinical evidence that exercise after MI has a beneficial effect on disease progression and survival.[6,7] For example, physical conditioning in patients with LV dysfunction results in an increased exercise capacity which has been ascribed, at least in part, to skeletal muscle adaptations.[8]

The effects of exercise on LV remodeling and function are still incompletely understood, as several studies in humans reported contradictory effects of training on LV remodelling after a MI.[9–18] Careful inspection of these studies suggests that after a small MI, exercise has no detrimental effect[11,13] or even improves[15,17,18] LV geometry and function, independent of whether exercise was started late, ie, ~1 year,[17,18] or early, ie, ~2 months,[11,13,15] after MI. In contrast, in patients with a large MI, exercise had either no[14], or a beneficial[18] effect on ejection fraction (EF) and LV volumes but only when started late after MI. However, when exercise after a large MI is started at a time when LV remodeling is still ongoing (~3 to 4 months after MI), the majority of studies reported that exercise has either no,[11–13] or even a detrimental[9,10] effect on LV volume and EF.

Similar to these clinical studies, studies in rats indicate that exercise started late (~3 weeks) after a moderate to large MI, encompassing 35% to 50% of LV mass, at a time when infarct healing is complete, does not aggravate,[19,20] or even blunts[21–23] LV dilation and hypertrophy. In contrast, when started ~1 week after a moderate to large MI,[24–27] exercise resulted in variable outcomes with beneficial,[28] no[24,27] or detrimental[25,26] effects on LV remodeling. These rodent studies lend further support to the concern that early exercise may have detrimental effects on LV remodeling after a large MI, although interpretation is hampered by the fact that late exercise studies in rats principally used treadmill running,[18,19,21,22] whereas

early exercise studies predominantly used swimming.[23–25,27,28] This is important because the exercise responses to swimming are markedly different from those to treadmill running.[29,30]

In light of these observations, the first aim of the present study was to assess the effects of exercise by voluntary treadmill running, started within 24 hours after a large MI, on LV remodeling and dysfunction in the mouse. The results indicated that exercise attenuated the MI-induced LV dysfunction, without a detrimental effect on LV remodeling. Consequently, we tested the hypothesis that exercise early after a large MI is able to reverse the MI-induced abnormalities in β_1-adrenergic receptor and Ca^{2+}-handling protein expression, phosphorylation status of contractile proteins, Ca^{2+}-handling and myofilament function, within the noninfarcted remodeled myocardium.

Materials and Methods

Experiments complied with The Guide for Care and Use of Laboratory Animals of the National Institutes of Health (NIH Publication No. 86-23, Revised 1996), and were approved by the Erasmus MC Animal Care Committee.

Experimental groups

A total of 147 C57Bl/6J mice of either sex (10-12 weeks old) entered the study. Animals were randomly assigned to one of four experimental groups. Sham operated mice (Sham) and mice that underwent a MI were subjected to a sedentary life-style (SH_{SED}, MI_{SED}) or to voluntary exercise training (SH_{EX}, MI_{EX}). Mice were individually subjected to either sedentary housing or voluntary exercise training (EX) for a period of eight weeks. Treadmills were custom built to allow electronic measurement of the distance run by the mice. Voluntary EX was the training of choice due to its ability to achieve uniform controlled EX in mice and to minimize stress factors, which are present during forced running and particularly during swimming.[1] C57Bl/6J mice were used because they are known to perform excellent in voluntary wheel running.[1]

In an additional group of 8 mice, 24 hours after permanent ligation of the left anterior descending coronary artery (LAD), the LV area at risk (AR) was determined using negative 1% Evans Blue staining administered via the jugular vein. Subsequently the heart was excised, quickly frozen for a few minutes and cut in five slices in the direction from apex to aorta. Infarct area (IA) was determined with negative 2% triphenyltetrazolium chloride (TTC) staining for 5 min at 37°C. After TTC staining, the area of infarction included the anterior wall and apical part

of the LV appears pallid, whereas the viable myocardium appears red. The LV was then separated from the RV and each slice was weighed. To calculate IA an AR, both sides of each of the slices were photographed, and the IA and AR for both sides of each section was determined using Sigma Scan Pro 5 software. Infarct size was calculated as IA/AR*100%.[2]

Experimental procedures

Mice were weighed, sedated with 4% isoflurane, intubated and ventilated with a mixture of O_2 and N_2O (1/2, vol/vol) with a pressure controlled ventilator (CWE, SAR-830/P) to which 2.3% isoflurane was added for anesthesia.[3] The ventilation rate was set at 90 strokes/min, with a peak inspiration pressure of 18 cm H_2O and a positive end expiration pressure (PEEP) of 4 cm H_2O. The mice were placed on a heating pad to maintain body temperature at 37°C. A thoracotomy was performed through the third left intercostal space, after which the lungs were protected with gauze and the pericardium opened. MI was produced, in two groups of animals (MI_{SED} and MI_{EX}), by permanent ligation of the LAD with a 7-0 silk suture mounted on a tapered needle (BBraun; Aesculap AG&CO. KG, Germany). Subsequently, the chest was closed with a 6-0 silk suture (BBraun; Aesculap AG&CO. KG, Germany) and the skin with suture clips (BBraun; Aesculap AG&CO. KG, Germany) whereupon the mice received 0.05 mg/kg analgesics subcutaneously (Buprenorfine-hydrochloride, Reckitt Benckiser Inc., UK) and were allowed to recover.

Eight weeks after entering the study, mice were anesthetized and ventilated as described above. In vivo trans-thoracic echocardiography of the LV was performed (ALOKA ProSound SSD-4000; Japan) using a 13-MHz linear array transducer.[3] At the start of the experiment and after placement of a 1.4F microtipped pressure transducer catheter in the LV (SPR-671, Millar Instruments, Houston, TX; calibrated prior to each experiment with a mercury manometer) echocardiograms were obtained with simultaneous ECG registration. M-mode echocardiograms were captured from short-axis 2D views of the LV at midpapillary level. LV diameters at end diastole (LVEDD) and end systole (LVESD) were measured from the M-mode images using SigmaScan Pro 5 Image Analysis software (SPSS Inc., Chicago, IL). A blinded observer analyzed three cardiac cycles for each animal in the four experimental groups and fractional shortening was calculated as FS=(LVEDD–LVESD)/LVEDD*100%. Pressure-diameter relations were obtained from M-mode images synchronized with LV pressure by simultaneous recorded ECG and constructed with a program written in MatLab (Mathworks Inc, Natick, MA). Data from four consecutive beats were averaged.

Following echocardiography, the mice were instrumented with a fluid filled polyethylene catheter (PE 10) inserted into the left carotid artery and advanced into the aortic arch for measuring mean aortic pressure and heart rate. A stretched PE 50 catheter was introduced into the left external jugular vein and advanced into the superior caval vein for infusion of Haemaccel (Hoechst Marion Roussel B.V.), to maintain fluid-balance. A 1.4F Millar Instruments pressure transducer catheter was inserted in the right carotid artery and advanced in the LV for measuring LV pressure.[3] Hemodynamic data were recorded and digitized using an on-line four-channel data acquisition program (ATCODAS, Dataq Instruments, Akron, OH), for later analysis with a program written in MatLab. Ten consecutive beats were selected for determination of heart rate, mean aortic pressure, LV systolic pressure, LV end diastolic pressure, the less afterload-sensitive contractility parameter, rate of rise of LV pressure at 30 mmHg ($LVdP/dt_{P30}$) and the relaxation parameters the time constant of LV pressure decay (Tau, τ) and the maximum rate of LV pressure decay ($LVdP/dt_{min}$).

Tissue analysis

At the conclusion of each experiment, the heart was excised and the atria removed after which the right ventricle (RV) and LV including septum were separated. The weight of RV (RVW) and LV (LVW), and tibial length (TL) were determined. Accumulation of lung fluid weight was assessed by measuring the difference between the wet- and dry lung weights, which is an indication for pulmonary edema. In 12 SH_{SED} and 12 SH_{EX} animals skeletal muscle samples were obtained for maximal citrate synthase (CS) activity[4] measurements spectrophotometrically on skeletal muscle homogenates (expressed as $\mu mol.g^{-1}$ of protein).[5] The LV was cut in two halves along the longitudinal axis. Half of the LV was fixed overnight in freshly prepared 4% paraformaldehyde in PBS and embedded in paraffin for histology, while the other half was quickly frozen in liquid nitrogen for assessment of force measurements in single skinned cardiomyocytes and analysis of the β_1-adrenergic signaling pathway and contractile, Ca^{2+}-handling and myofilament proteins.

Histology

Four micron thick paraffin LV sections were stained with Masson's trichrome for analysis of collagen volume fraction and cardiomyocyte cross-sectional area (CSA) measurements within the remote non-infarcted myocardium. Four fields were randomly selected in two sections of eight mice per group and photographed using an Olympus BH 20 microscope (Olympus Corporation, Japan) at a magnification of

x400. Within each field, segments representing connective and muscle tissue were identified and manually traced with a digitizing pad and computer image analysis software (Clemex Vision PE 3.5) to calculate the traced area. Collagen volume fraction was calculated in each field as the sum of all connective tissue areas divided by the sum of all connective tissue and muscle areas and averaged for each animal per group. Myocyte CSA was measured by tracing the outline of myocytes showing the nucleus in each field and averaged for each animal in the groups. Finally, the infarct region was demarcated and the area was measured. Endocardial and epicardial infarct circumference were demarcated and the lengths measured. Infarct thickness was measured at the shortest distance between endocardium and epicardium. The operator was blinded to the experimental group during the analysis.

Force measurements in single permeabilized cardiomyocytes

Cardiomyocytes were mechanically isolated from liquid nitrogen frozen LV samples of 5 mice (3 myocytes/animal) per group. Before mechanical isolation, tissue was defrosted in cold relaxing solution (pH 7.0; in mmol/l: free $MgCl_2$, 1; KCl, 145; EGTA, 2; ATP, 4; imidazole, 10). During the isolation, the tissue was kept on ice. Mechanically isolated myocytes were permeabilized in relaxing solution containing 0.5% Triton X-100 (5 minutes) a treatment which also removes soluble and membrane-bound kinases and phosphatases, which may alter the phosphorylation status of myofibrillar proteins. To remove Triton, cells were washed twice in relaxing solution. Subsequently, a single myocyte was attached between a force transducer and a piezoelectric motor. Isometric force measurements were performed at different $[Ca^{2+}]$, at 15°C, and a sarcomere length, measured in relaxing solution, of 2.2 μm. Rate of force redevelopment (Ktr) was determined in activating solution (pCa values ranging from 4.5 to 5.8) when a steady level of force was developed by rapidly slackening and re-stretching the cardiomyocyte by 20% of its length. Upon slackening force drops to zero and upon re-stretch force redevelopment occurs to the initial steady level. Force redevelopment was fitted to a single exponential to estimate Ktr.

The diameters of the cardiomyocyte were measured microscopically, in two perpendicular directions. Cross-sectional area was calculated assuming an elliptical cross-section. The composition of relaxing and activating solutions used during force measurements was calculated as described previously.[6,7] The pCa, –log $[Ca^{2+}]$, of the relaxing and activating solution (pH 7.1) were 9 and 4.5, respectively. Solutions with intermediate free $[Ca^{2+}]$ were obtained by mixing of the activating

and relaxing solutions. After the first control activation at saturating (maximal) [Ca^{2+}] (pCa=4.5), resting sarcomere length was readjusted to 2.2 μm, if necessary. The second control measurement was used to calculate maximal isometric tension (force divided by cross-sectional area). The next force measurements were performed at submaximal [Ca^{2+}], followed by a control measurement. Force values obtained in solutions with submaximal [Ca^{2+}] were normalized to the interpolated control values. Cardiomyocytes were stored on ice to prevent myofilament-bound kinases and phosphatases to alter Ca^{2+}-sensitivity (pCa_{50}) of force during the measurements.

After the first force–pCa series, part of the myocytes from Sham and MI hearts were incubated in relaxing solution containing the exogenous catalytic subunit of protein kinase A (3 μg/mL [100 U/mL]; Sigma, batch 35H9522) and 6 mmol/L dithiothreitol for 40 minutes at 20°C, after which a second force–pCa series was obtained. The mean parameters of the measurements before protein kinase A (PKA) treatment were consistent with the observations in the entire Sham and MI group studied. Force-pCa relations were fit to the Hill equation:[6]

$F\ (Ca^{2+})/F_0=[Ca^{2+}]^{nH}/(Ca_{50}{}^{nH}+[Ca^{2+}]^{nH})$, where F is steady-state force, F_0 denotes the steady force at saturating [Ca^{2+}], nH reflects the steepness of the relationship, and Ca_{50} (or pCa_{50}) represents the midpoint of the relation.

Myosin-Heavy-Chain composition

The myosin heavy chain (MHC) isoform composition was analyzed by one-dimensional SDS-PAGE.[8]

Myofilament proteins phosphorylation status

To determine phosphorylation status of the myofilament proteins MyBP-C, TnT, TnI, MLC-2 and desmin, LV samples (20 μg/lane) were separated on gradient gels (4-15%) and stained with Pro-Q diamond phosphoprotein gel stain (Pro-Q; Molecular Probes) in conjunction with SYPRO Ruby staining (Molecular Probes) of the gels. To preserve the endogenous phosphorylation status, frozen tissue samples were homogenized in liquid nitrogen and re-suspended in 1 mL ice-cold (-20°C) 10% trichloroacetic acid (TCA; dissolved in acetone containing 0.1% (w/v) dithiothreitol (DTT)) solution. The phosphorylation signals for myofilament proteins were normalized to the intensities of the SYPRO Ruby stained myosin binding protein C bands. The signals were analyzed using the luminescent image analyzer las-3000 and Aida image analyzer.

β_1-adrenergic signaling

cAMP. About 5-10mg of frozen left ventricular tissue was homogenized in a liquid nitrogen cooled Teflon vial with a Teflon sphere (Mikro-Dismembrator U, setting 1700rpm, 4 minutes) in the presence of 100µl frozen 0.1M HCl. The frozen powder was collected and the vial was rinsed with the remaining 40 volumes of 0.1M HCl. The homogenate was sonicated for 30 seconds at 10 micron on ice water. An aliquot was removed for protein determination. cAMP levels were measured in 5x diluted extracts using an enzyme immunoassay kit (Assay Designs, USA) according to the manufacturer's instructions.

PKA. About 5-20mg of frozen tissue was homogenized in the presence of 100µl frozen PKA extraction buffer (25mM Tris-HCl pH 7.4, 10mM 2-mercapto-ethanol, 1mM NaF, 100µM IBMX, 50µM Cantharidin and protease inhibitor mix CompleteTM (Roche, Switzerland) similarly as described above. The homogenate in totally 20 volumes of PKA extraction buffer was sonicated for 30 seconds at 10 micron on ice water and centrifuged for 5 minutes. An aliquot of the clear supernatant was removed for protein determination. PKA levels were measured using the PepTag® Assay kit (Promega, USA) with a modified reaction buffer (1mM extra ATP), 10µg of protein and 20 minutes reaction time at room temperature. After the reaction phosphorylated and unphosphorylated fluorescent proteins were separated on a 0.8% agarose gel and ratios of bands were determined using a Kodak Image Station 440CF equipped with Kodak 1D software Amounts of PKA were calculated from a standard curve of pure cAMP-dependent PKA catalytic subunit.

Western immunoblotting

Approximately 25 mg of frozen tissue was homogenized at liquid nitrogen temperature in a microdismembrator unit (B. Braun Biotech International, 1700 rpm) for 4 min in a Teflon vial with a Teflon sphere. The frozen powder was suspended in 20 volumes of cold Laemmli loading buffer, heated for 15 min at 37°C and centrifuged for 1 min at 9700•gav. Aliquots of the clear supernatants were removed for protein determination using the RCDC protein assay (Bio-Rad Laboratories) and for Western blot analysis. Proteins were separated by SDS-PAGE using 10% gels, 15% gels or 7.5%-15% gradient gels. Samples were reheated for 5 min at 37°C before use and either 5µg or 20µg (depending on the antibody to be used) of protein/lane was applied onto the gels. Following electrophoresis proteins were blotted overnight at 40V onto PVDF membranes (Immunblot, Bio-Rad). Blots were stained reversibly with Ponceau Red to check protein loading and transfer. Blots were pre-incubated in TTBS (10 mmol/L Tris-HCl pH 7.6, 150 mmol/L NaCl,

0.1% Tween-20) supplemented with 0.5% non-fat milk powder for 1 h at room temperature and incubated overnight at 4°C with diluted primary antibodies. Anti-phospholamban (PLB, mouse monoclonal) was from Affinity Bioreagents and anti-SERCA2A (rabbit polyclonal) was from Abcam. Anti-Gαi-3 (C-10, rabbit polyclonal), anti-GRK2 (C-15, rabbit polyclonal), anti-Na^+/Ca^{2+}-exchanger 1 (NCX1, P-13, goat polyclonal) and anti-β_1-adrenergic receptor (rabbit polyclonal) were from Santa Cruz Biotechnology. Phospho-specific rabbit polyclonal antibodies against a phospho-serine (P-Ser16) or phospho-threonine (P-Thr17) containing sequence of PLB were from Cyclacel. Anti-atrial natriuretic peptide was from Chemicon. A mouse atrial extract was used as positive control.

Blots were probed for 3 h at room temperature with horseradish peroxidase conjugated goat anti-rabbit or rabbit anti-goat or goat anti-mouse secondary antibody (Pierce). In between the incubations the blots were washed extensively with TBS. For SERCA2a, phosholamban and phospho-phospholamban detection a rat cardiac membrane preparation was used as positive control. For GRK2, Gαi-3 and β_1-adrenergic receptor detection a rat brain extract was used as positive control. Signals were visualized using Supersignal® West Femto Maximum Sensitivity Substrate (Pierce) and HyperfilmTM ECL (Amersham Biosciences). Signal densities were quantified using a Bio-Rad calibrated GS-800 scanner and Quantity One quantitation software (Bio-Rad).

Contractile properties of intact cardiomyocytes

In an additional group of 6 MI_{SED} and 5 MI_{EX} mice, single LV cardiomyocytes were enzymatically dissociated as previously described;[9] in each animal 3 to 5 cells were studied. Mice were heparinized and killed by an overdose of pentobarbital (100 mg/kg i.p.), and the hearts quickly excised. After cannulation of the aorta, hearts were mounted to a Langendorff perfusion set. The heart was briefly rinsed with normal Tyrode solution, containing (mM): 137 NaCl, 5.4 KCl, 0.5 $MgCl_2$, 1 $CaCl_2$, 11.8 Hepes and 10 glucose, pH 7.4. Subsequently the heart was perfused with a Ca^{2+}-free solution for 8 min. The Ca^{2+}-free Tyrode solution contained (mM): 130 NaCl, 5.4 KCl, 1.2 KH_2PO_4, 1.2 $MgSO_4$, 6 Hepes, 20 glucose, pH 7.2. Collagenase A (0.6 mg ml^{-1}, Roche Diagnostics, GmbH, Mannheim, Germany) and protease (type XIV, 0.1 mg ml^{-1}, Sigma, St Louis, MO, USA) were added to the Ca^{2+}-free solution and perfused for 10 min. The enzymes were washed out with low Ca^{2+} solution, i.e. the Ca^{2+}-free solution to which 0.18 mM $CaCl_2$ was added. The hearts were removed and the non-infarcted part of the LV, including the septum was cut into small pieces and further dissociated into single cells by gentle shaking. Cells were

stored in low Ca^{2+} solution at room temperature and used within 8 h after isolation. All experimental procedures were approved by the Ethics Committee on Animal Use of the University of Leuven.

Cells were placed in a perfusion chamber on the stage of an inverted microscope (Nikon Diaphot). Cell shortening was measured with a video-edge detector (Crescent, Salt Lake City, UT, USA) at 240 Hz frame rate. Field stimulation was done with 5 ms square pulses of constant voltage, at 20 % above threshold. The external solution was the normal Tyrode solution, bath temperature was 30°C. The absolute cell shortening is expressed as the fractional shortening, i.e. normalized to resting cell length, ΔL/L0.

$[Ca^{2+}]_i$ was measured in cells using whole-cell ruptured patch recording. The set-up for fluorescence recording, procedures for calibration to $[Ca^{2+}]_i$, and recording of membrane potential and currents were as described before.[9] The pipette solution contained (in mmol/L): 120 K-aspartate, 20 KCl, 10 K-HEPES, 5 MgATP, 10 NaCl, and 0.05 K_5-fluo-3, pH 7.2. The external solution was the normal Tyrode solution, bath temperature was 30°C. Cells were stimulated under current clamp with 5 ms square pulses at different frequencies.

Statistics

Data were analyzed using two-way ANOVA, followed by post hoc testing with Student-Newman-Keuls, or using unpaired t-testing, as appropriate. Survival was analyzed by Kaplan-Meier method and log-rank (Mantel-Cox) test. Significance was accepted when $P<0.05$. Data are means±SEM.

Results

Exercise and survival

MI_{EX} initially ran shorter distances per day compared with SH_{EX} (Figure 1), but total distance over the 8-week period and hence daily distance was similar in SH_{EX} (5.9±0.2 km/d) and MI_{EX} mice (5.2±0.2 km/d). Exercise increased skeletal muscle CS-activity in SH_{EX} (371±28 µmol-CS/g-protein) compared with SH_{SED} (290±21 µmol-CS/g-protein; $P<0.05$). MI was associated with 40% mortality; exercise had no significant effect on survival in MI mice.

LV remodeling

Twenty-four hours after LAD ligation, 88±2% of the area-at-risk had become infarcted, corresponding to 43±3% of the LV. This resulted in marked LV dilation and hypertrophy, reflected in the increased LV diameter, relative LVW (LVW/ TL), and

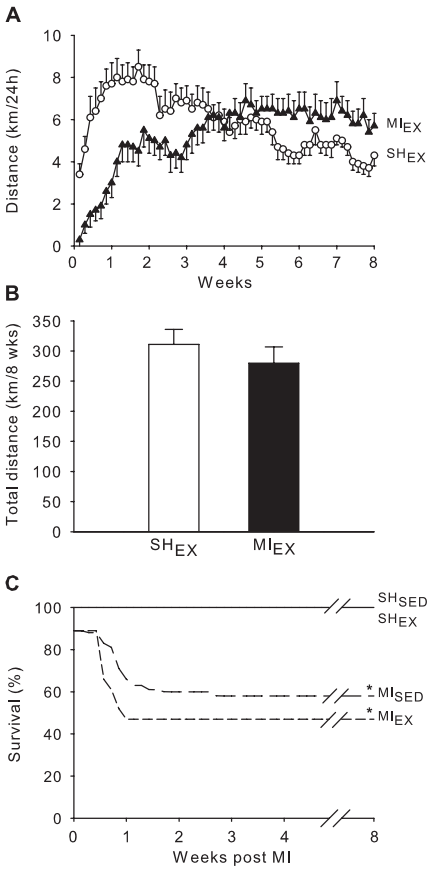

Figure 1:
Daily running distance in MI (n=21) and SH (n=18) mice that survived the entire 8-week follow-up period (A). Total distance run over 8 weeks. No significant differences were observed (B). Kaplan-Meier survival curves for all four groups. Time point zero represents the immediate postoperative survival of 89% after MI and 100% in SH mice (C). Numbers of mice entering the study: SH_{SED} (n=34), SH_{EX} (n=18), MI_{SED} (n=61), MI_{EX} (n=34). *P<0.05 versus corresponding SH.

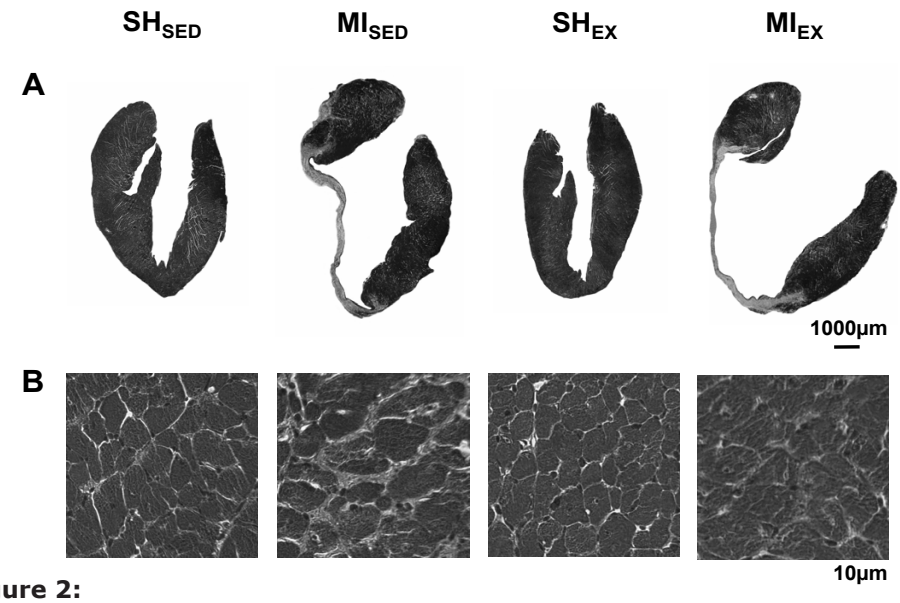

Figure 2:
Masson's trichrome staining showing the green/blue infarct area in longitudinal cross-section of the LV, 8 weeks after permanent LAD ligation. MI includes the anterior wall and apical part of the LV (A). Magnifications of the Masson's trichrome image showing collagen and myocyte CSA in viable myocardium (remote zone in MI mice) (B).

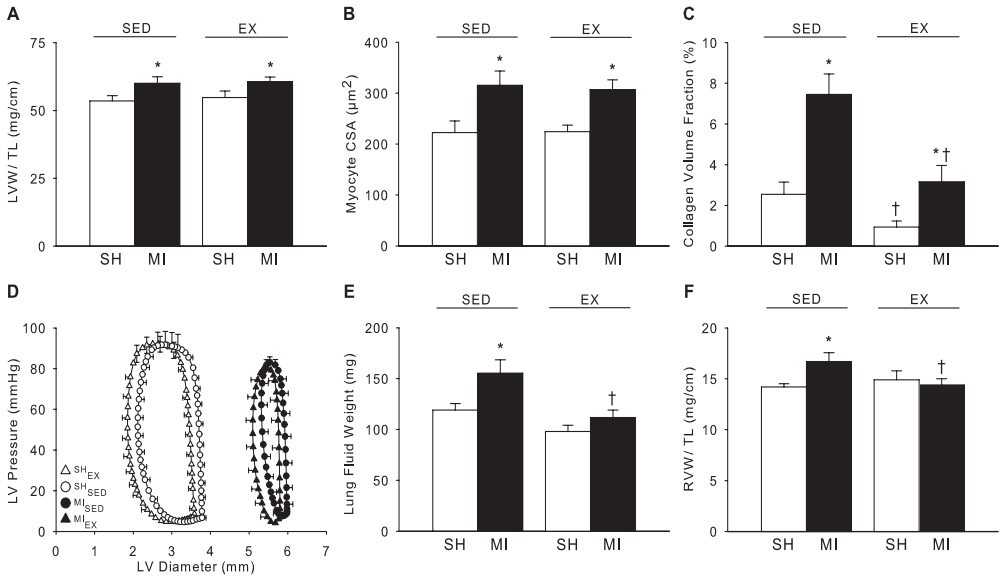

Figure 3:

Effects of MI and exercise on relative LVmass (A), myocyte CSA (B), collagen volume fraction (C), LV pressure-diameter relation (D), lung fluid weight (E), and relative RVmass (F). *P<0.05 versus corresponding SH; †P<0.05 versus corresponding SED.

increased cardiomyocyte-CSA and LV collagen volume fraction within the remote myocardium 8 weeks later (Figure 2 and Figure 3A through 3C). Exercise had no significant effect on cardiomyocyte-CSA and LVW/TL in either Sham or MI mice. However, exercise tended to decrease LV end-diastolic diameter and significantly reduced LV collagen content in both Sham and MI mice (Figure 3).

Global LV function

MI resulted in lower LV systolic pressure, $LVdP/dt_{P30}$, fractional shortening, $LVdP/dt_{min}$ and increased τ, but had no apparent effect on LV end-diastolic pressure (Table). Exercise had minimal effects on LV systolic and diastolic function in Sham mice, but increased both $LVdP/dt_{P30}$ and FS after MI. MI resulted in a marked rightward shift as well as a narrowing of the LV pressure-diameter relation, indicating LV dilation and depressed FS (Figure 3D). Exercise caused a small leftward shift in Sham and MI animals (both P<0.02). Lung-fluid weight and RVW/TL were increased after MI, indicative of pulmonary congestion and RV hypertrophy (Figure 3E and 3F), which were abolished by exercise.

Force development in single permeabilized cardiomyocytes

Passive force was similar in SH_{SED} (3.0±0.4 kN/m^2) and MI_{SED} (2.9±0.3 kN/m^2), but maximal isometric force (F_{max}) was significantly lower in MI_{SED} than in SH_{SED} (Figure 4A). The normalized force-pCa curves also showed a leftward shift in MI animals,

Table 1. LV anatomical and functional data

		Sedentary	Exercise
Anatomical data			
Body weight (g)	Sham	26±1	25±1
	MI	26±1	24±1
LV weight (mg)	Sham	96±4	95±4
	MI	109±4 *	110±4 *
Endocardial infarct length (mm)	MI	3.8±0.3	3.5±0.4
Epicardial infarct length (mm)	MI	4.6±0.3	4.2±0.4
Infarct thickness (mm)	MI	0.11±0.01	0.13±0.02
Infarct area (mm^2)	MI	1.54±0.14	1.59±0.08
Hemodynamic data			
Heart Rate (bpm)	Sham	537±8	555±12
	MI	520±7	528±9
MAP (mmHg)	Sham	70±2	79±5 †
	MI	69±2	71±1 *
LVSP (mmHg)	Sham	93±2	100±4
	MI	83±2 *	82±2 *
LV dP/dt_{P30} (mmHg/s)	Sham	7189±239	7030±267
	MI	5295±207 *	5794±207 * †
Fractional Shortening (%)	Sham	39±2	47±3 †
	MI	8±1 *	12±1 * †
LV dP/dt_{min} (mmHg/s)	Sham	-7879±462	-9329±568
	MI	-5184±285 *	-5203±410 *
Tau (ms)	Sham	7.9±0.5	8.1±1.0
	MI	10.6±0.8 *	10.5±1.1 *
LVEDP (mmHg)	Sham	6.4±0.6	4.3±0.9
	MI	7.9±1.0	5.7±0.8

MAP, mean arterial pressure; LVSP, left ventricular systolic pressure; LVEDP, left ventricular end diastolic pressure. Anatomical and hemodynamic data tested two-tailed; SH_{SED} (n=27), SH_{EX} (n=11), MI_{SED} (n=27) and MI_{EX} (n=14). *P<0.05 vs corresponding Sham; †P<0.05 vs corresponding Sedentary.

indicating greater Ca^{2+}-sensitivity in MI_{SED} than in SH_{SED}, which was accompanied by an decreased steepness (nHill) of the force-pCa curves in MI compared with Sham (2.4±0.1 versus 2.6±0.1 respectively, P<0.05). Treatment with the catalytic subunit of PKA decreased pCa_{50} in both MI_{SED} and SH_{SED}, reflecting a PKA-induced decrease in myofilament Ca^{2+}-sensitivity.

Importantly, after PKA the Ca^{2+}-sensitivity was no longer different between MI_{SED} and SH_{SED} (Figure 4), suggesting that loss of PKA-mediated myofilament protein phosphorylation contributed to the increased myofilament Ca^{2+}-sensitivity

after MI. PKA had no effect on F_{max} in MI_{SED} and SH_{SED} (not shown). Exercise had no effect on passive force of cardiomyocytes from either Sham or MI mice (not shown). Exercise had also no effect on contractile properties of cardiomyocytes from Sham mice (Figure 4). However, exercise restored F_{max}, Ca^{2+}-sensitivity and nHill (2.4±0.1 versus 2.7±0.1 in MI_{SED} and MI_{EX} respectively, $P<0.02$) in MI mice. The effects of PKA on Ca^{2+}-sensitivity were now similar in Sham and MI animals. MHC composition (% α-MHC) was not altered following MI, consistent with the maintained Ktr (Figure 5). Exercise had no effect on either MHC composition or Ktr.

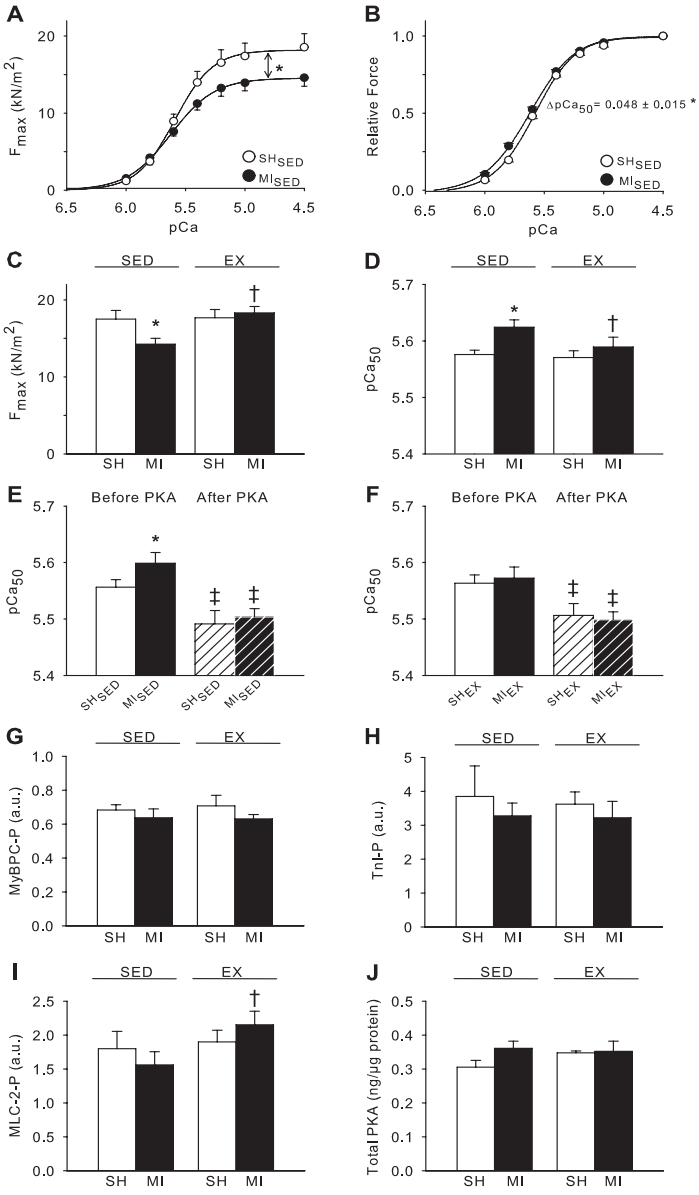

Figure 4:
Absolute (A and C) and normalized (B and D) force-pCa curves and bar charts. The pCa_{50} before and after treatment with exogenous PKA (E and F). The panels (A, B, and E) show the effects of MI in SED mice. The panels (C, D, and F) show the effects of EX in SH and MI. Myofilament protein phosphorylation of MyBP-C (G), TnI (H), MLC-2 (I), and total PKA (J) in LV remote myocardium. *$P<0.05$ versus corresponding SH; †$P<.05$ versus corresponding SED; ‡$P<0.05$ after PKA versus before PKA.

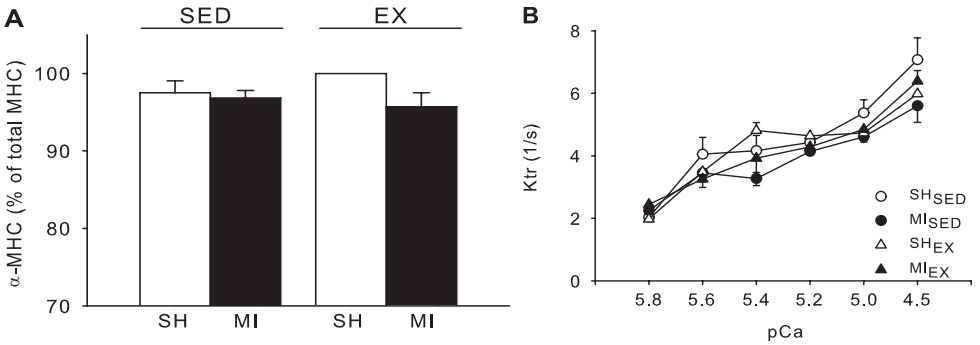

Figure 5:
Effects of MI and exercise on relative α-myosin heavy chain (MHC) levels (A) and rate of force redevelopment (Ktr) at different Ca^{2+}-levels (B). No significant differences were observed.

Myofilament proteins phosphorylation status

There were no significant differences in phosphorylation status of myofilament proteins MyBP-C, troponin I (TnI), myosin light chain (MLC-2) (Figure 4), troponin T (TnT) and desmin (not shown), between SH_{SED} and MI_{SED}. Exercise had no significant effect on the phosphorylation status of MyBP-C, TnI, TnT, and desmin, but increased MLC-2 phosphorylation in MI mice.

β_1-adrenergic signaling

Total PKA levels were not affected by MI and exercise training (Figure 4). Surprisingly, cAMP was also not different in MI_{SED} compared with SH_{SED}. However, exercise after MI significantly increased cAMP levels from 4.5±0.3 pmol/mg protein in MI_{SED} to 6.1±0.8 pmol/mg protein in MI_{EX} (P<0.05).

Western immunoblotting

Protein level of the β_1-adrenergic receptor decreased after MI, but did not change in SH_{EX} mice. The decrease was not accompanied by significant changes in GRK2 and Gαi-3 expressions (Figure 6). Protein levels of SERCA2a decreased in the remodeled myocardium after MI, but were not altered in SH_{EX} mice. PLB levels were maintained in both MI_{SED} and SH_{EX}. PLB phosphorylation at the Ser16 site did not change in both MI and exercise mice, whereas phosphorylation at the Thr17 site was decreased in both groups. Na^+/Ca^{2+}- exchanger levels did not change in MI_{SED} and SH_{EX} mice. Exercise after MI increased β_1-adrenergic receptor levels and Na^+/Ca^{2+}-exchanger levels, but had minimal effects on the expression of GRK2, Gαi-3, SERCA2a, and PLB and PLB phosphorylation at both Ser16 and Thr17 sites (Figure 6).

Contractile properties of isolated intact cardiomyocytes

To further investigate the mechanism by which exercise improved LV function in MI mice, we performed additional experiments in enzymatically isolated intact cardiomyocytes obtained from MI_{SED} and MI_{EX}. Unloaded cell shortening in MI_{EX} was significantly higher compared with MI_{SED} mice (Figure 7). Preliminary cell shortening data of 3 SH_{SED} mice showed values comparable to MI_{EX} (not shown). Basal $[Ca^{2+}]_i$ was lower in MI_{EX} than MI_{SED}, but the $[Ca^{2+}]_i$ transient amplitudes were similar in the 2 groups.

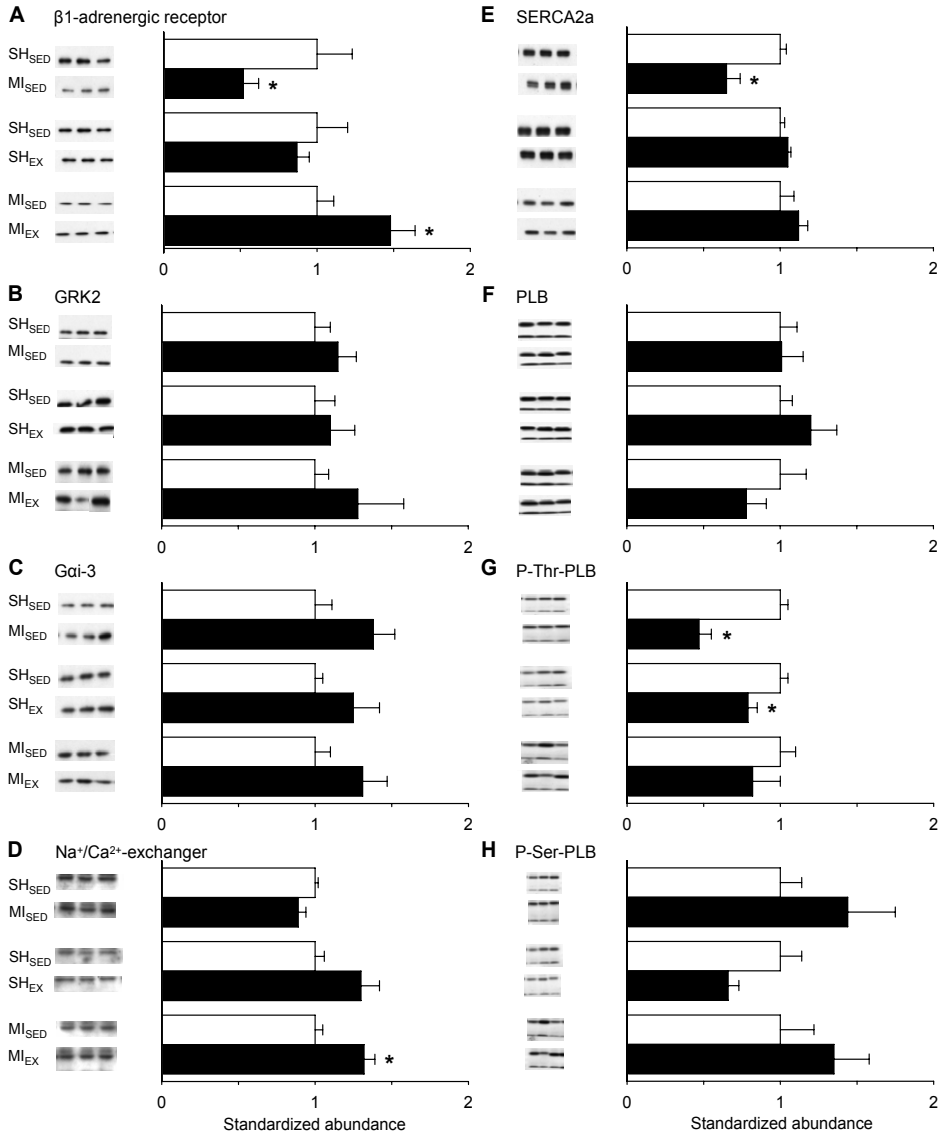

Figure 6:

Immunoblot analyses of β_1-adrenergic signaling and Ca^{2+}-handling proteins of the four groups. Representative Western blots are shown, with summary quantification in bar graphs (n=5 for each bar). Comparisons were made in a pair-wise fashion (normalized within each pair). *P<0.05 versus corresponding white bar.

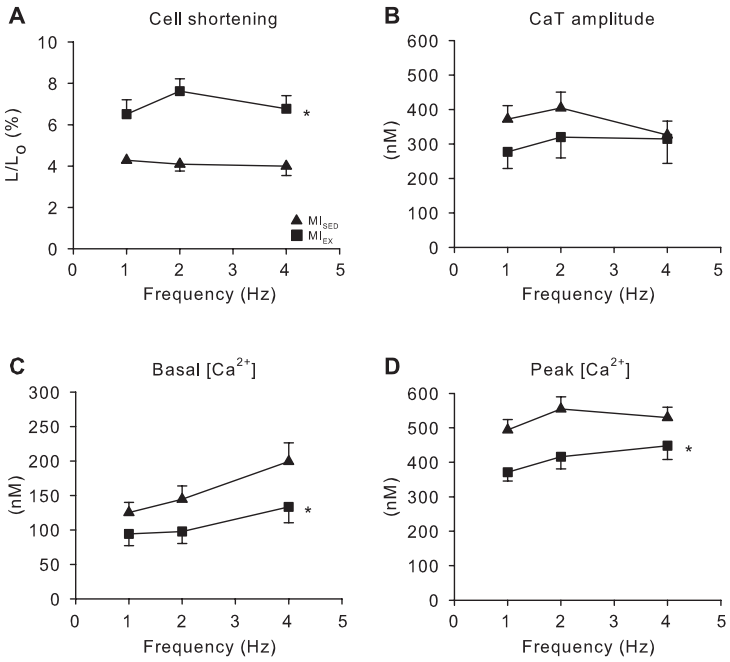

Figure 7:
Single cell shortening during field stimulation at the indicated frequencies (A), $[Ca^{2+}]_i$ transient amplitude (B), basal (C), and peak (D) $[Ca^{2+}]_i$ values during whole cell current clamp recording at the same frequencies. *P<0.05 MI$_{EX}$ versus MI$_{SED}$.

Discussion

The present study investigated the impact of 8 weeks of voluntary exercise training, started early after a large MI, on LV remodeling and dysfunction in mice at the in vivo, cellular and molecular level. The main findings were that: *(i)* exercise had no adverse effect on LV dimensions and hypertrophy, while ameliorating LV dysfunction and backward failure; *(ii)* exercise normalized MI-induced myofilament dysfunction, which likely contributed to the exercise-induced improvement in unloaded shortening of isolated intact cardiomyocytes, as the $[Ca^{2+}]_i$ transient amplitude was not altered by exercise. In addition, basal $[Ca^{2+}]_i$ was reduced by exercise; and *(iii)* exercise likely mediated these effects via increased β_1-adrenoceptor protein and cAMP levels, and Na^+/Ca^{2+}-exchanger protein levels.

Pathophysiology of MI-induced LV dysfunction in mice

In agreement with previous reports,[31,32,37] permanent LAD ligation in mice resulted in LV remodeling, characterized by LV dilation, hypertrophy, and increased collagen deposition in remote noninfarcted myocardium, and resulted in marked LV dysfunction, characterized by decrements in LV pump function (fractional shortening) and decrements in indices of global LV contractility (dP/dt$_{P30}$) and relaxation (dP/dt$_{min}$ and τ), which was associated with LV backward failure reflected in pulmonary edema and RV hypertrophy. The mechanism for LV

dysfunction after MI remains incompletely understood, but has been proposed to be the consequence of alterations in LV geometry with no effect on cardiomyocyte function or β_1-adrenergic responsiveness.[38] Conversely, other investigators reported that alterations in β_1-adrenergic signaling[21] and Ca^{2+}-handling[22,23] of the remote myocardium also contribute to global LV dysfunction. In agreement with the latter notion, we observed downregulation of β_1-adrenergic receptor levels and reductions in SERCA2a after MI, whereas no clear changes were found in protein levels of GRK2, Gαi-3, PLB and Na^+/Ca^{2+}-exchanger. Unexpectedly, cAMP levels were not depressed in MI mice, which is consistent with the observation that cAMP-dependent PKA mediated phosphorylation of PLB-Ser16 was also not decreased after MI, but suggests that the β_1-adrenergic signaling in MI mice is not a simple function of the β_1-receptor density. PLB-Thr17 phosphorylation, which is mediated by CaM-kinase II, was attenuated following MI. Reduced phosphorylation of PLB-Thr17 will inhibit SERCA2a function in the mouse heart particularly at higher heart rates.[39] The latter could, in conjunction with decreased β_1-adrenoceptor and SERCA2a expression, contribute to perturbations in cardiomyocyte Ca^{2+}-handling in MI mice, particularly during increased activity.

In agreement with observations in swine,[2] remodeling of noninfarct myocardium in mice was associated with altered myofilament function, characterized by decreased F_{max} and increased Ca^{2+}-sensitivity of tension development in single permeabilized cardiomyocytes. The small increase in Ca^{2+}-sensitivity after MI was likely the result of the minor reduction of PKA-mediated phosphorylation of TnI and MyBP-C,[2,40] as treatment with exogenous PKA abolished the difference in Ca^{2+}-sensitivity between Sham and MI animals. However, Pro-Q Diamond analysis did not reveal significant decreases in phosphorylation status of myofilament proteins in MI_{SED} compared with SH_{SED}. It is possible that a small decrease in PKA-mediated phosphorylation of TnI and MyBP-C was obscured by increased PKC-mediated phosphorylation of these myofilament proteins as increased PKC activity was observed in rat hearts within 1 to 8 weeks after MI.[41]

The mechanism underlying the MI-induced reduction in F_{max} is less clear. A role for degradation of TnI in reducing F_{max}, as suggested in pigs,[2] is unlikely in the post-MI remodeled mouse heart. First, no degradation products were observed in remodeled myocardium. Second, Narolska *et al* recently demonstrated that exchange of truncated TnI in human cardiomyocytes had no effect on F_{max}.[42] A likely alternative candidate responsible for the reduction in F_{max} is increased PKC-mediated phosphorylation, because the absence of a change in TnI phosphorylation in MI animals is consistent with increased PKC activity. Studies in rodent myocardium

indicated a central role for PKC-mediated phosphorylation of TnI[43] and TnT[44] in decreasing F_{max}. Based on our Pro-Q analysis PKC-mediated TnT phosphorylation can be excluded as possible cause for the reduction in F_{max}, because there were no differences in TnT phosphorylation among the groups. Although speculative, our myofilament force measurements and phosphoprotein data could be interpreted to suggest that the increase in Ca^{2+}-sensitivity and the decrease in F_{max} are because of reduced PKA-mediated and increased PKC-mediated phosphorylation of TnI, respectively.

Surprisingly, we did not observe a shift from α-MHC to β-MHC protein expression, which is a post-MI hypertrophy marker in rats[45] and mice.[29] In contrast, protein levels of another hypertrophy marker ANP were elevated in remote LV of MI_{SED} (5.41±2.39 a.u.) compared with SH_{SED} (0.10±0.09 a.u.; $P<0.016$), correlating well with lung-fluid weight ($R^2<0.55$; $P<0.014$). An explanation for this unexpected lack of MHC-isoform shift is not readily found, but it should be noted that our observations are consistent with the unchanged Ktr after MI.

In conclusion, the present study supports the concept that alterations at the cellular level in remote noninfarcted myocardium contribute to decreased global LV function after MI. Future studies, using catecholamine challenges, are required to determine in greater detail the importance of perturbations in kinase-phosphatase signaling cascades in post-MI remodelled mouse heart.

Mechanism of beneficial effects of exercise training after MI

Rat studies on exercise after MI have reported no changes in parameters of LV function such as LV dP/dt_{max},[23,25] fractional shortening,[23] PV-relation[24] or cardiac output,[46] irrespective of whether exercise was started early[24,25] or late,[25,46] irrespective of a small[24,25] or large[25,46] MI, and irrespective of treadmill[23,46] or swim[24,25] training. Nevertheless, exercise was reported to improve cardiomyocyte function.[21–23,47] Thus, exercise after healed MI (~3 weeks after MI) attenuates β-MHC expression [21] and restores cardiomyocyte Ca^{2+}- handling and Ca^{2+}-responsiveness, and SERCA2a and Na^+/ Ca^{2+}-exchanger levels.[23] Furthermore, exercise was reported to blunt cardiomyocyte hypertrophy, and to restore Ca^{2+}-transients, and SERCA2a and Na^+/Ca^{2+}-exchanger expression[22,47] Thus, beneficial effects of exercise on LV remote myocardium in rats are clearly observed when exercise is initiated late after MI. To date no study has investigated the effects of exercise started early after MI on β_1-adrenergic signaling, Ca^{2+}-handling and myofilament function.

In view of the concern that early exercise may aggravate LV remodeling after a large MI, we investigated the effects of exercise started immediately after MI on LV

remodeling and dysfunction in the mouse. The results indicate that in mice, even after a large MI (comprising ~43% of LV mass), 8 weeks of voluntary exercise does not aggravate LV remodeling, as relative LV mass and cardiomyocyte size as well as infarct geometry were unchanged, whereas exercise decreased collagen content and actually tended to decrease LV end-diastolic diameter. These observations are in agreement with a recent study by Konhilas *et al* [48] who reported that 8 weeks of moderate exercise in mice with hypertrophic cardiomyopathy reversed collagen deposition with little effect on cardiac hypertrophy. Interestingly, exercise also reversed expression of hypertrophy markers and components of apoptosis pathways. In view of the minimal effects of exercise on LV remodeling and the pronounced effects on LV dysfunction, we elected to focus on the effects of exercise training on myofilament function and Ca^{2+}-handling. However, the study by Konhilas *et al* warrants future studies that include the analysis of hypertrophy and survival signaling pathways in the model of post-MI remodeling.

Exercise attenuated LV dysfunction and ameliorated LV backward failure, which was likely because of improved cardiomyocyte function, as shortening of isolated cardiomyocytes was increased by exercise. Ca^{2+}-transient amplitude remained unaltered, consistent with the lack of effect of exercise on SERCA2a and PLB expression. However, basal (diastolic) calcium concentrations were reduced by exercise after MI. In the absence of changes in SERCA2a and PLB protein levels or phosphorylation, a potential explanation for the reduction in basal $[Ca^{2+}]_i$ is the increased expression of Na^+/Ca^{2+}-exchange. However, future studies are needed to determine whether this small increase in Na^+/Ca^{2+}-exchanger protein levels is indeed responsible for the exercise-induced reduction in diastolic Ca^{2+} levels, or whether other mechanisms, including increased sarcolemmal Ca^{2+}-ATPase activity, also contribute.

Importantly, our findings indicate that the improved cell shortening was not because of increased Ca^{2+}-transient amplitude, suggesting that the exercise-induced normalization of myofilament function was principally responsible for the improved isolated myocyte shortening in vitro and global LV fractional shortening in vivo. Exercise restored myofilament Ca^{2+}-sensitivity after MI, which was likely mediated via increased β_1-adrenergic signaling as suggested by the increased β_1-adrenoceptor and cAMP levels and by the normalization of the Ca^{2+}-sensitivity response to PKA. Restoration of β_1-adrenergic signaling also acts to increase MLC-2 phosphorylation via PKA-mediated inhibition of protein phosphatase 1.[49] Indeed, MLC-2 phosphorylation was increased by exercise. It is however unlikely that the exerciseinduced increase in MLC-2 phosphorylation is involved in either the

increased Fmax or reduced Ca^{2+}-sensitivity in MIEX, because phosphorylation of MLC-2 increases myofilament Ca^{2+}-sensitivity without an effect on F_{max}.[50,51] Future studies are required to further delineate the mechanism underlying the exercise-induced normalization of F_{max}.

Clinical implications

The present study indicates that exercise training started early after a large MI is beneficial, resulting in improved LV function and molecular phenotype, without adverse effects on LV remodeling. The beneficial effects appear to be the result of improved β_1-adrenergic signaling and myofilament function. Because some of these cellular adaptations to exercise are also observed following chronic β_1-adrenoceptor blockade,[52,53] future studies should be aimed at investigating whether combined β_1-adrenoceptor blockade and exercise yield added benefit.

References

1. Konstam MA, Udelson JE, Sharpe N. *Prevention and reversal of left ventricular remodeling summation.* J Card Fail 2002;8:S506–S511.
2. van der Velden J, Merkus D, Klarenbeek BR, James AT, Boontje NM, Dekkers DH, Stienen GJM, Lamers JM, Duncker DJ. *Alterations in myofilament function contribute to left ventricular dysfunction in pigs early after myocardial infarction.* Circ Res 2004;95:e85–e95.
3. Gielen S, Schuler G, Hambrecht R. *Exercise training in coronary artery disease and coronary vasomotion.* Circulation 2001;103:E1–E6.
4. Moore RL, Korzick DH. *Cellular adaptations of the myocardium to chronic exercise.* Prog Cardiovasc Dis 1995;37:371–396.
5. Cohen MV. *Coronary and collateral blood flows during exercise and myocardial vascular adaptations to training.* Exerc Sport Sci Rev 1983;11:55–98.
6. Coats AJ. *Exercise training in heart failure.* Curr Control Trials Cardiovasc med 2000;1:155–160.
7. You Fang Z, Marwick TH. *Mechanisms of exercise training in patients with heart failure.* Am Heart J 2003;145:904–911.
8. Adamopoulos S, Coats AJ, Brunotte F, Arnolda L, Meyer T, Thompson CH, Dunn JF, Stratton J, Kemp GJ, Radda GK, *et al*. *Physical training improves skeletal muscle metabolism in patients with chronic heart failure.* J Am Coll Cardiol 1993;21:1101–1106.
9. Kubo N, Ohmura N, Nakada I, Yasu T, Katsuki T, Fujii M, Saito M. *Exercise at ventilatory threshold aggravates left ventricular remodeling in patients with extensive anterior acute myocardial infarction.* Am Heart J 2004;147:113–120.
10. Jugdutt BI, Michorowski BL, Kappagoda CT. *Exercise training after anterior Q wave myocardial infarction: importance of regional left ventricular function and topography.* J Am Coll Cardiol 1988;12:362–372.
11. Giannuzzi P, Tavazzi L, Temporelli PL, Corra U, Imparato A, Gattone M, Giordano A, Sala L, Schweiger C, Malinverni C. *Long-term physical training and left ventricular remodeling after anterior myocardial infarction: results of the Exercise in Anterior Myocardial Infarction (EAMI) trial. EAMI Study Group*. J Am Coll Cardiol 1993;22:1821–1829.
12. Dubach P, Myers J, Dziekan G, Goebbels U, Reinhart W, Vogt P, Ratti R, Muller P, Miettunen R, Buser P. *Effect of exercise training on myocardial remodeling in patients with reduced left ventricular function after myocardial infarction: application of magnetic resonance imaging.* Circulation 1997;95:2060–2067.
13. Otsuka Y, Takaki H, Okano Y, Satoh T, Aihara N, Matsumoto T, Yasumura Y, Morii I, Goto Y. *Exercise training without ventricular remodeling in patients with moderate to severe left ventricular dysfunction early after acute myocardial infarction.* Int J Cardiol 2003;87:237–244.
14. Sullivan MJ, Higginbotham MB, Cobb FR. *Exercise training in patients with severe left ventricular dysfunction. Hemodynamic and metabolic effects.* Circulation 1988;78:506 –515.
15. Koizumi T, Miyazaki A, Komiyama N, Sun K, Nakasato T, Masuda Y, Komuro I. *Improvement of left ventricular dysfunction during exercise by walking in patients with successful percutaneous coronary intervention for acute myocardial infarction.* Circ J 2003;67:233–237.
16. Giannuzzi P, Temporelli PL, Corra U, Gattone M, Giordano A, Tavazzi L. *Attenuation of unfavorable remodeling by exercise training in postinfarction patients with left ventricular dysfunction: results of the Exercise in Left Ventricular Dysfunction (ELVD) trial.* Circulation 1997;96:1790–1797.
17. Ehsani AA, Biello DR, Schultz J, Sobel BE, Holloszy JO. *Improvement of left ventricular contractile function by exercise training in patients with coronary artery disease.* Circulation 1986;74:350–358.
18. Giannuzzi P, Temporelli PL, Corra U, Tavazzi L. *Antiremodeling effect of long-term exercise training in patients with stable chronic heart failure: results of the Exercise in Left Ventricular Dysfunction and Chronic Heart Failure (ELVD-CHF) Trial.* Circulation 2003;108:554–559.
19. Musch TI, Moore RL, Leathers DJ, Bruno A, Zelis R. *Endurance training in rats with chronic heart failure induced by myocardial infarction.* Circulation 1986;74:431– 441.
20. Libonati JR. *Exercise and diastolic function after myocardial infarction.* Med Sci Sports Exerc 2003;35:1471–1476.
21. Orenstein TL, Parker TG, Butany JW, Goodman JM, Dawood F, Wen WH, Wee L, Martino T, McLaughlin PR, Liu PP. *Favorable left ventricular remodeling following large myocardial infarction by exercise training. Effect on ventricular morphology and gene expression.* J Clin Invest 1995;96:858–866.
22. Zhang LQ, Zhang XQ, Musch TI, Moore RL, Cheung JY. *Sprint training restores normal contractility in postinfarction rat myocytes.* J Appl Physiol 2000;89:1099–1105.
23. Wisloff U, Loennechen JP, Currie S, Smith GL, Ellingsen O. *Aerobic exercise reduces cardiomyocyte hypertrophy and increases contractility, Ca^{2+}-sensitivity and SERCA-2 in rat after myocardial infarction.* Cardiovasc Res 2002;54:162–174.
24. Alhaddad IA, Hakim I, Siddiqi F, Lagenback E, Mallavarapu C, Nethala V, Mounce D, Ross PL, Brown EJ, Jr. *Early exercise after experimental myocardial infarction: effect on left ventricular remodeling.* Coron Artery Dis 1998;9:319–327.
25. Gaudron P, Hu K, Schamberger R, Budin M, Walter B, Ertl G. *Effect of endurance training early or late after coronary artery occlusion on left ventricular remodeling, hemodynamics, and survival in rats with chronic transmural myocardial infarction.* Circulation 1994;89:402–412.
26. Kloner RA, Kloner JA. *The effect of early exercise on myocardial infarct scar formation.* Am Heart J 1983;106:1009–1013.
27. Hochman JS, Healy B. *Effect of exercise on acute myocardial infarction in rats.* J Am Coll Cardiol 1986;7:126–132.

28. Oh BH, Ono S, Rockman HA, Ross J, Jr. *Myocardial hypertrophy in the ischemic zone induced by exercise in rats after coronary reperfusion.* Circulation 1993;87:598–607.
29. Flaim SF, Minteer WJ, Clark DP, Zelis R. *Cardiovascular response to acute aquatic and treadmill exercise in the untrained rat.* J Appl Physiol 1979;46:302–308.
30. Bernstein D. *Exercise assessment of transgenic models of human cardiovascular disease.* Physiol Genomics 2003;13:217–226.
31. van Rooij E, Doevendans PA, Crijns HJ, Heeneman S, Lips DJ, van Bilsen M, Williams RS, Olson EN, Bassel-Duby R, Rothermel BA, De Windt LJ. *MCIP1 overexpression suppresses left ventricular remodeling and sustains cardiac function after myocardial infarction.* Circ Res 2004;94:e18–e26.
32. van den Bos EJ, Mees BM, de Waard MC, de Crom R, Duncker DJ. *A novel model of cryoinjury-induced myocardial infarction in the mouse: a comparison with coronary artery ligation.* Am J Physiol Heart Circ Physiol 2005;289: H1291–H1300.
33. Faloona GR, Srere PA. *Escherichia coli citrate synthase. Purification and the effect of potassium on some properties.* Biochemistry 1969;8:4497–4503.
34. van der Velden J, Klein LJ, van der Bijl M, Huybregts MA, Stooker W, Witkop J, Eijsman L, Visser CA, Visser FC, Stienen GJM. *Isometric tension development and its calcium sensitivity in skinned myocyte-sized preparations from different regions of the human heart.* Cardiovasc Res 1999;42:706–719.
35. van der Velden J, Papp Z, Boontje NM, Zaremba R, de Jong JW, Janssen PM, Hasenfuss G, Stienen GJM. *The effect of myosin light chain-2 dephosphorylation on Ca^{2+}-sensitivity of force is enhanced in failing human hearts.* Cardiovasc Res 2003;57:505–514.
36. Antoons G, Mubagwa K, Nevelsteen I, Sipido KR. *Mechanisms underlying the frequency dependence of contraction and $[Ca^{2+}]_i$ transients in mouse ventricular myocytes.* J Physiol 2002;543:889–898.
37. Jones SP, Greer JJ, van Haperen R, Duncker DJ, de Crom R, Lefer DJ. *Endothelial nitric oxide synthase overexpression attenuates congestive heart failure in mice.* Proc Natl Acad Sci U S A 2003;100:4891–4896.
38. Gupta S, Prahash AJ, Anand IS. *Myocyte contractile function is intact in the post-infarct remodeled rat heart despite molecular alterations.* Cardiovasc Res 2000;48:77–88.
39. Zhao W, Uehara Y, Chu G, Song Q, Qian J, Young K, Kranias EG. *Threonine-17 phosphorylation of phospholamban: a key determinant of frequency-dependent increase of cardiac contractility.* J Mol Cell Cardiol 2004;37:607–612.
40. Strang KT, Sweitzer NK, Greaser ML, Moss RL. *Beta-adrenergic receptor stimulation increases unloaded shortening velocity of skinned single ventricular myocytes from rats.* Circ Res 1994;74:542–549.
41. Wang J, Liu X, Sentex E, Takeda N, Dhalla NS. *Increased expression of protein kinase C isoforms in heart failure because of myocardial infarction.* Am J Physiol Heart Circ Physiol 2003;284:H2277–H2287.
42. Narolska NA, Piroddi N, Belus A, Boontje NM, Scellini B, Deppermann S, Zaremba R, Musters RJ, dos Remedios C, Jaquet K, Foster DB, Murphy AM, van Eyk JE, Tesi C, Poggesi C, van der Velden J, Stienen GJM. *Impaired diastolic function after exchange of endogenous troponin I with C-terminal truncated troponin I in human cardiac muscle.* Circ Res 2006;99:1012–1020.
43. Burkart EM, Sumandea MP, Kobayashi T, Nili M, Martin AF, Homsher E, Solaro RJ. *Phosphorylation or glutamic acid substitution at protein kinase C sites on cardiac troponin I differentially depress myofilament tension and shortening velocity.* J Biol Chem 2003;278:11265–11272.
44. Sumandea MP, Pyle WG, Kobayashi T, de Tombe PP, Solaro RJ. *Identification of a functionally critical protein kinase C phosphorylation residue of cardiac troponin T.* J Biol Chem 2003;278:35135–35144.
45. Hashimoto T, Kambara N, Nohara R, Yazawa M, Taguchi S. *Expression of MHC-beta and MCT1 in cardiac muscle after exercise training in myocardial-infarcted rats.* J Appl Physiol 2004;97:843–851.
46. Musch TI, Moore RL, Smaldone PG, Riedy M, Zelis R. *Cardiac adaptations to endurance training in rats with a chronic myocardial infarction.* J Appl Physiol 1989;66:712–719.
47. Zhang LQ, Zhang XQ, Ng YC, Rothblum LI, Musch TI, Moore RL, Cheung JY. *Sprint training normalizes Ca^{2+}-transients and SR function in postinfarction rat myocytes.* J Appl Physiol 2000;89:38-46.
48. Konhilas JP, Watson PA, Maass A, Boucek DM, Horn T, Stauffer BL, Luckey SW, Rosenberg P, Leinwand LA. *Exercise can prevent and reverse the severity of hypertrophic cardiomyopathy.* Circ Res 2006;98:540–548.
49. Braz JC, Gregory K, Pathak A, Zhao W, Sahin B, Klevitsky R, Kimball TF, Lorenz JN, Nairn AC, Liggett SB, Bodi I, Wang S, Schwartz A, Lakatta EG, DePaoli-Roach AA, Robbins J, Hewett TE, Bibb JA, Westfall MV, Kranias EG, Molkentin JD. *PKC-alpha regulates cardiac contractility and propensity toward heart failure.* Nat Med 2004;10:248–254.
50. Morano I. *Effects of different expression and posttranslational modifications of myosin light chains on contractility of skinned human cardiac fibers.* Basic Res Cardiol 1992;87 Suppl1:129–141.
51. Rajashree R, Blunt BC, Hofmann PA. *Modulation of myosin phosphatase targeting subunit and protein phosphatase 1 in the heart.* Am J Physiol Heart Circ Physiol 2005;289:H1736–H1743.
52. Sun YL, Hu SJ, Wang LH, Hu Y, Zhou JY. *Effect of beta-blockers on cardiac function and calcium handling protein in postinfarction heart failure rats.* Chest 2005;128:1812–1821.
53. Min JY, Ding B, Wang JF, Sullivan MF, Morgan JP. *Metoprolol attenuates postischemic depressed myocardial function in papillary muscles isolated from normal and postinfarction rat hearts.* Eur J Pharmacol 2001;422:115–125.

Chapter 3

Beneficial effects of exercise training after myocardial infarction requires full eNOS activity

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Submitted

Abstract

Exercise training attenuates left ventricular (LV) dysfunction after myocardial infarction (MI). It could be speculated that these effects of exercise are mediated by increased endothelial NO synthase (eNOS) activity. In the present study we tested the hypothesis that eNOS plays a critical role in the exercise-induced amelioration of LV dysfunction after MI. MI or sham was induced in $eNOS^{-/-}$, $eNOS^{+/-}$ and $eNOS^{+/+}$ mice. After 8 weeks of voluntary wheel running (~7km/day in all groups) or sedentary housing, global cardiac function was determined in vivo and (immuno)histochemistry was performed to assess cardiomyocyte size, fibrosis, capillary density and apoptosis in remote myocardium. At baseline $eNOS^{-/-}$ mice had higher mean aortic pressure compared to $eNOS^{+/-}$ and $eNOS^{+/+}$ mice, but had normal global cardiac function. MI resulted in marked LV remodeling, including cardiomyocyte hypertrophy and a reduction in capillary density, increased fibrosis and apoptosis, as well as LV systolic and diastolic dysfunction to the same extent in all genotypes. In $eNOS^{+/+}$ MI mice exercise abolished fibrosis and apoptosis in the remote myocardium, attenuated LV systolic dysfunction and ameliorated pulmonary congestion. These beneficial effects were lost in $eNOS^{+/-}$ and $eNOS^{-/-}$ mice, while LV systolic dysfunction and pulmonary congestion in $eNOS^{+/-}$ mice were aggravated by exercise. The beneficial effects of exercise after MI on LV remodeling and dysfunction depend critically on endogenous eNOS. The observation that the lack of one eNOS allele is sufficient to negate all beneficial effects of exercise, strongly suggests that exercise depends on full eNOS availability and activity.

Introduction

Left ventricular (LV) remodeling after myocardial infarction (MI) is a compensatory mechanism that serves to restore LV pump function. This adaptation process involves LV dilation, LV and cardiomyocyte hypertrophy and myocardial fibrosis. Despite the apparent appropriateness of LV remodeling to maintain cardiac pump function early after MI, remodeling is an independent risk factor for the development of congestive heart failure.[1] The mechanism underlying the progression from LV remodeling to overt heart failure remains incompletely understood, but may include loss of cardiomyocytes through apoptosis,[2] reduction in contractile function of the surviving myocardium[3] and/or alterations in extracellular matrix leading to LV dilation.[4] Experimental and clinical studies suggest that the vascular endothelium plays an important role in modulating the progression of ventricular and vascular remodeling in heart failure. For example, an increase in NO bioavailability has been shown to improve many of the processes perturbed in LV remodeling, including angiogenesis,[5] cardiac fibrosis,[6] and hypertrophy.[7] Furthermore, in vivo experiments in eNOS overexpressing[8] and eNOS deficient mice[9] have provided evidence that eNOS protects against LV remodeling and LV dysfunction after MI.

In chapter 2 of this thesis we reported that exercise training (exercise) following MI attenuates LV pump dysfunction in a mouse model.[10] In patients, physical activity has been associated with a reduction in cardiovascular morbidity and mortality.[11-13] The beneficial effects of exercise on cardiac function can be attributed, at least in part, to an exercise-induced upregulation of endogenous endothelial nitric oxide synthase (eNOS) expression and activity,[14,15] but this has not been investigated to date. Consequently, we tested the hypothesis that eNOS plays an obligatory role in the beneficial effects of exercise training on cardiac remodeling and dysfunction after MI. For this purpose we subjected wild type ($eNOS^{+/+}$) and eNOS-deficient mice lacking either one ($eNOS^{+/-}$) or both ($eNOS^{-/-}$) functional eNOS alleles to induction of MI followed by either sedentary housing or voluntary exercise training. Studies in $eNOS^{+/-}$ mice were included as previous studies have shown that $eNOS^{+/-}$ mice have maintained basal levels of eNOS expression but show nevertheless selective loss of exercise-induced increase in eNOS expression.[16]

Materials and Methods

The investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, Revised 1996) and were approved by the Erasmus MC Animal Care Committee.

Animals

Female $eNOS^{+/-}$ and male $eNOS^{-/-}$ eNOS deficient mice in C57Bl/6J background were bred to generate $eNOS^{-/-}$ and $eNOS^{+/-}$ offspring. Wild type C57Bl/6J mice ($eNOS^{+/+}$) were purchased from Harlan. Mice were genotyped by a polymerase chain reaction method using genomic DNA extracted from tail biopsies. A total of 71 $eNOS^{-/-}$, 117 $eNOS^{+/-}$, and 225 $eNOS^{+/+}$ mice of either sex (~12-weeks old) entered the study and were randomly assigned to one of four experimental groups. Sham-operated mice (SH) and mice with MI were housed sedentary (SH_{SED}, MI_{SED}) or subjected to voluntary exercise training by wheel running (SH_{EX}, MI_{EX}) for 8 weeks.

Experimental procedures

Mice were weighed, sedated with 4% isoflurane, intubated and pressure-controlled ventilated with O_2-N_2O (1:2, vol/vol) containing ~2.5% isoflurane for anesthesia. MI was produced by permanent ligation of the left-anterior-descending-coronary-artery (LAD) with a 7-0 silk suture (BBraun, Germany) as described before.[10,17] Sham animals underwent the operation without infarct induction. Eight weeks after entering the study, hemodynamic measurements were performed under anesthesia as described before.[10] In brief, M-mode LV echocardiography (Prosound SSD-4000, ALOKA, Japan) from long and short axis views was performed and LV diameters at end-diastole (LVEDD) and end-systole (LVESD) were measured, and fractional shortening calculated. A 1.4F microtipped pressure transducer catheter (SPR-671, Millar Instruments, USA) was inserted into the LV and pressure-diameter relations were obtained from M-mode images synchronized with LV-pressure by simultaneous ECG recording. All data were recorded, digitized and analyzed with a software program written in Matlab® for determination of heart rate, mean aortic pressure, LV systolic pressure, and LV end-diastolic pressure, the afterload-insensitive contractility parameter $LVdP/dt_{P30}$ (rate of rise of LV pressure at 30 mmHg), and the relaxation parameters $LVdP/dt_{min}$ (the maximum rate of LV pressure decay) and τ (time constant of LV pressure decay).

Tissue analysis

Weights

At the end of each experiment, the weight of the right ventricle (RVW) and LV (LVW) including septum, and tibia length (TL) were determined. The accumulation of lung fluid weight was assessed by measuring the difference between the wet and dry lung weights, which is an indication for pulmonary edema.

Cardiomyocyte hypertrophy and collagen content

The LV was fixed overnight in freshly prepared 4% paraformaldehyde at 4°C. Paraffin-embedded LVs were cut into 4-μm-thin sections and stained with Masson's Trichrome for determination of cardiomyocyte cross-sectional area and collagen content. Four microscopic fields from two sections per animal within the remote non-infarcted LV were photographed and analyzed by a blinded observer.[10]

Capillary density

Endothelial cells were detected using lectin staining. Briefly, paraffin sections were deparaffinized, cleared and hydrated to tris-buffered saline, pH 7.4, using a descending series of ethanol. Sections were treated with trypsin, pH 7.0, for 20min at 37°C, washed and treated with hydrogen peroxide 3% for 10min at room temperature (to inhibit the endogenous peroxide). Sections were incubated overnight at 4°C with peroxidase-conjugated lectin from Bandeira simplicifolia (Sigma). Horseradish peroxidase was activated by incubation for 1-2min with a diaminobenzidine commercial kit (Dako). Sections were washed, counterstained with haematoxillin, dehydrated with graded ethanol solutions, cleared in xylene and mounted. Vessels were detected as brown endothelial cells and capillary density was determined as the number of vessels per mm^2.

Apoptosis

To localize DNA degradation in remote non-infarcted LV, we used the In Situ Cell Death Detection Kit (Roche Diagnostics, Germany), which is a modification of the TUNEL assay (3' terminal deoxynucleotide transferase (TdT)-mediated dUTP nick end labeling). In brief, paraffin sections were deparaffinized, cleared and hydrated to phosphate-buffered saline (PBS), pH 7.4, using a descending series of ethanol. Sections were treated with Proteinase-K for 30min at 37°C, washed in PBS, treated with the TUNEL reaction mixture and incubated in the dark for 1h at 37°C, followed by washes in PBS. Both positive (DNaseI-treated sections) and negative controls (no TDT included in the reaction mixture) were included. Sections were covered with Vectashield including DAPI and directly photographed using fluorescence microscopy. TUNEL positive cells emit red fluorescence and all nuclei were blue. Apoptosis was determined as the number of TUNEL-positive nuclei per 105 nuclei.

Statistical analysis

Survival analysis was performed by the Kaplan-Meier method and between-group difference in survival was tested by the log-rank (Mantel-Cox) test. All other data

were analyzed by three-way (eNOS genotype x EX x MI) ANOVA, followed by post-hoc testing with Student-Newman-Keuls test (STATVIEW) when appropriate. Significance was accepted when $P<0.05$. Data are means ± SEM.

Results

Survival and exercise

MI resulted in 30-50% mortality during the first 2 weeks after MI. Mortality was not significantly different between $eNOS^{+/+}$, $eNOS^{+/-}$ and $eNOS^{-/-}$ MI mice and was not significantly affected by exercise (Figure 1A). MI_{EX} mice ran a shorter distance compared to sham mice, particularly during the first 4 weeks ($P<0.05$ by 2-way ANOVA), while $eNOS^{+/-}$ mice ran a slightly longer distance than $eNOS^{+/+}$ or $eNOS^{-/-}$, particularly in the sham mice ($P<0.05$ by 2-way ANOVA). Importantly, however, there was no significant difference between $eNOS^{+/+}MI_{EX}$, $eNOS^{+/-}MI_{EX}$, and $eNOS^{-/-}$ MI_{EX} mice in the total distance run (Figure 1B and C).

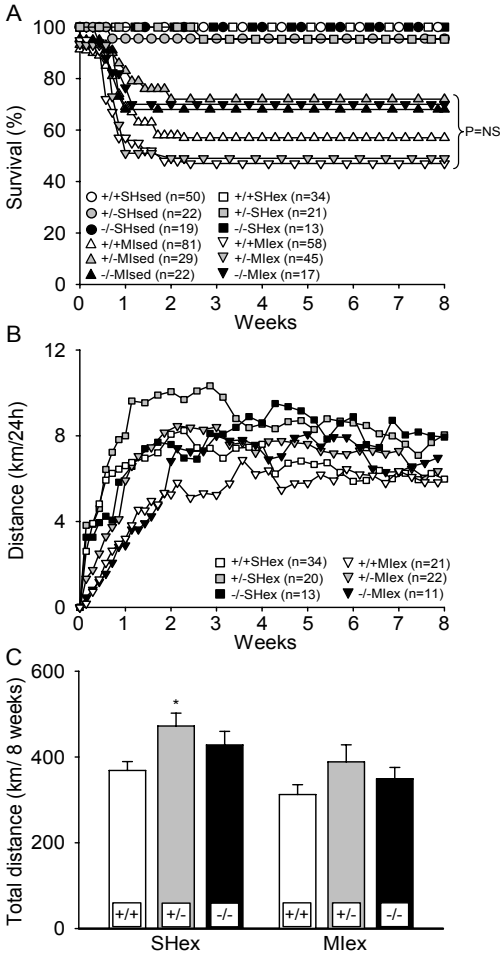

Figure 1:
Kaplan-Meier survival curves for all groups. Time point zero represents the immediate postoperative survival (A). No significant differences were observed within the sham or MI groups. MI significantly reduced survival ($P<0.05$). Daily running distance of mice that survived the 8-week follow-up period (B). Total distance run over 8 weeks (C). *$P<0.05$ versus corresponding $eNOS^{+/+}$

Hemodynamics and LV function

Mean aortic blood pressure was increased in $eNOS^{-/-}SH_{SED}$ (11±5 mmHg; $P<0.05$) but not in $eNOS^{+/-}SH_{SED}$ compared to $eNOS^{+/+}SH_{SED}$, while LV systolic and diastolic function were normal in both $eNOS^{+/-}SH_{SED}$ and $eNOS^{-/-}SH_{SED}$ mice. Exercise had no effect on any of the LV systolic and diastolic function parameters in any of the sham-operated groups, but normalized aortic blood pressure in $eNOS^{-/-}$ mice (Table 1, Figure 2).

MI in sedentary $eNOS^{+/+}$ mice resulted in loss of LV systolic (fractional shortening and $LVdP/dt_{P30}$) and diastolic ($LVdP/dt_{min}$, Tau, and LV end-diastolic pressure) function, and pulmonary congestion reflected in the increased lung fluid weight and RV hypertrophy (Table 1, Figure 2). Similar degrees of MI-induced LV dysfunction and pulmonary congestion were observed in $eNOS^{+/-}MI_{SED}$ and $eNOS^{-/-}MI_{SED}$ mice. Exercise after MI improved fractional shortening and $LVdP/dt_{P30}$, and blunted pulmonary congestion in $eNOS^{+/+}$ mice. In contrast, exercise failed to ameliorate LV dysfunction after MI in $eNOS^{+/-}$ and $eNOS^{-/-}$ mice, and even aggravated pulmonary congestion in $eNOS^{+/-}MI$ mice (Table 1, Figure 2).

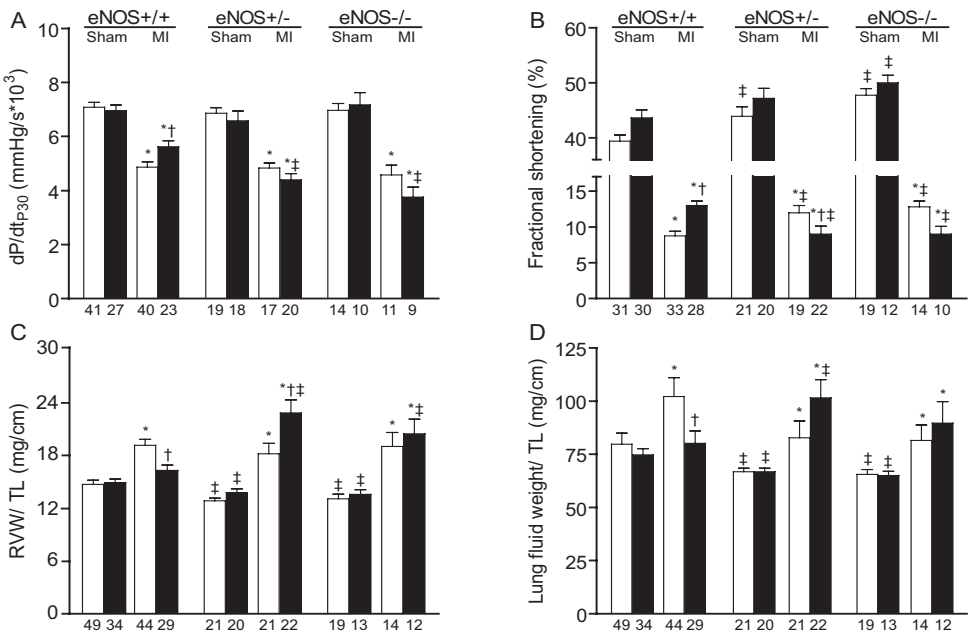

Figure 2:

Effects of MI and exercise in $eNOS^{+/+}$, $eNOS^{+/-}$ and $eNOS^{-/-}$ mice on global LV contractility (dP/dt_{P30}) (A), global LV pump function (fractional shortening) (B), relative RV mass (C) and relative lung fluid weight (D). *$P<0.05$ versus corresponding sham, †$P<0.05$ versus corresponding sedentary, ‡$P<0.05$ versus corresponding $eNOS^{+/+}$, §$P<0.05$ versus corresponding $eNOS^{+/-}$. □Sedentary and ■Exercise. Numbers of animals are indicated below the figures.

Table 1: Anatomical and Functional Data

		$eNOS^{+/+}$		$eNOS^{+/-}$		$eNOS^{-/-}$	
		SED	EX	SED	EX	SED	EX
Animal number	Sham	49	34	21	20	19	13
	MI	44	29	21	22	14	12
Body weight (g)	Sham	26±1	25±1	23±1	23±1‡	21±1‡	20±1‡
	MI	25±1	24±1	24±1	23±1	22±1‡	22±1‡
Tibia length (cm)	Sham	1.82±0.01	1.78±0.02	1.77±0.01‡	1.76±0.01‡	1.74±0.01‡	1.72±0.02‡
	MI	1.80±0.01	1.79±0.01	1.78±0.01	1.76±0.01	1.76±0.01‡	1.74±0.02‡
LV weight (mg)	Sham	97±3	94±2	80±2‡	81±2‡	79±3‡	78±2
	MI	113±2*	112±2*	110±3*	111±3*	101±3*‡	109±5*
LV weight/ BW (mg)	Sham	3.83±0.09	3.82±0.05	3.48±0.09‡	3.62±0.09‡	3.74±0.10	3.82±0.12
	MI	4.52±0.08*	4.73±0.09*†	4.53±0.08*	4.83±0.08*†	4.52±0.08*	4.91±0.15*†
RV weight (mg)	Sham	26±1	25±1	22±1‡	23±1‡	22±1‡	22±1‡
	MI	33±1*	29±1*†	32±2*	39±2*‡	32±3*	34±3*‡
RV weight/ BW (mg)	Sham	1.01±0.03	1.04±0.02	0.95±0.02	1.04±0.04†	1.04±0.03‡§	1.11±0.04
	MI	1.35±0.06*	1.19±0.05*†	1.34±0.09*	1.69±0.11*†‡	1.46±0.13*‡	1.56±0.12*‡
Heart rate (bpm)	Sham	530±10	515±10	490±10	500±15	500±15	495±20
	MI	515±10	525±10	515±20	510±10	480±15	500±10
MAP (mmHg)	Sham	75±2	80±3	81±2	73±3†	86±4‡§	76±4
	MI	71±2	71±2*	74±4*	68±3	75±2	67±6
LVSP (mmHg)	Sham	97±2	99±3	102±2	93±2*‡	104±4	106±3§
	MI	84±2*	85±2*	88±3*	78±3*†	85±5*	79±6*
dP/dt_{min} (mmHg/s)	Sham	-7040±540	-7200±580	-8120±290	-7830±540	-6880±500	-8070±440
	MI	-3590±170*	-4190±270*	-4830±260*	-4180±270*	-4580±450*	-3880±350*
Tau (ms)	Sham	8.7±0.6	8.6±0.7	7.9±0.3	8.4±1.0	9.8±0.9	6.6±0.6†
	MI	12.4±1.0*	13.5±1.1*	11.5±0.6*	10.9±0.5*	11.0±1.0	10.5±0.9*
LVEDP (mmHg)	Sham	6.9±0.6	4.6±0.6	5.6±0.8	4.2±0.6	5.7±1.0	4.5±1.1
	MI	9.8±0.9*	8.0±0.9*	8.5±1.0	8.6±1.1*	8.3±1.3*	9.7±1.9*

LV, left ventricle; BW, body weight; RV, right ventricle; MAP, mean arterial pressure; LVSP, left ventricular systolic pressure; LVEDP, left ventricular end-diastolic pressure. *P<0.05 versus corresponding Sham; †P<0.05 versus corresponding Sedentary; ‡P<0.05 versus corresponding $eNOS^{+/+}$; §P<0.05 versus corresponding $eNOS^{+/-}$.

LV remodeling

At similar age of 10-12 weeks, eNOS$^{+/-}$SH$_{SED}$ and eNOS$^{-/-}$SH$_{SED}$ exhibited a lower body weight and consequently a slightly smaller LV diameter, reflected in a leftward shift of the LV pressure-diameter relation, compared to eNOS$^{+/+}$SH$_{SED}$ (Figure 3). Similarly, absolute LV weight was lower in eNOS$^{+/-}$SH$_{SED}$ and eNOS$^{-/-}$SH$_{SED}$ mice (Table 1), whereas relative LV weight was not different from eNOS$^{+/+}$SH$_{SED}$ mice (Figure 4A). Cardiomyocyte size (Figure 4B) was similar in eNOS$^{+/+}$SH$_{SED}$ and eNOS$^{+/-}$SH$_{SED}$ mice, but was greater in eNOS$^{-/-}$SH$_{SED}$ mice. Exercise had no effect on relative LV weight, LV cardiomyocyte size, or LV diameter in eNOS$^{+/+}$ sham-operated mice. Exercise of eNOS$^{-/-}$ and eNOS$^{+/-}$ sham-operated mice resulted in a small decrease in LV diameter, while a small increase in cardiomyocyte size was observed in eNOS$^{+/-}$ mice.

MI resulted in marked LV dilation (Figure 3) and myocardial hypertrophy (Figure

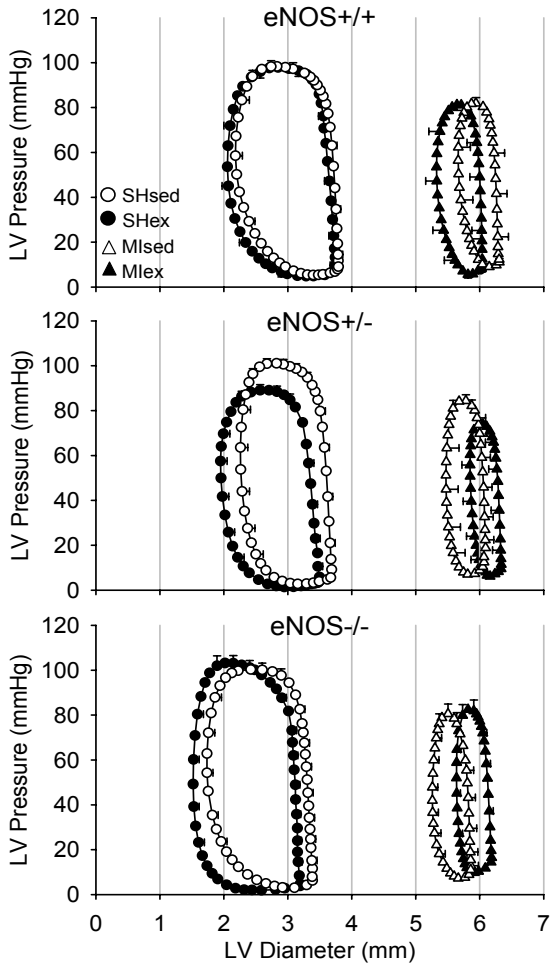

Figure 3:
Effects of MI and exercise on LV pressure-diameter relation in eNOS$^{+/+}$, eNOS$^{+/-}$ and eNOS$^{-/-}$ mice. Number of animals in eNOS$^{+/+}$, eNOS$^{+/-}$ and eNOS$^{-/-}$ mice respectively for SH$_{SED}$ (n=24,18,17), SH$_{EX}$ (n=27,17,11), MI$_{SED}$ (n=29,19,11) and MI$_{EX}$ (n=25,20,10).

4A, B) in $eNOS^{+/+}$, $eNOS^{+/-}$ and $eNOS^{-/-}$ mice. Exercise had no effect on the MI-induced LV myocardial hypertrophy, but blunted LV dilation in $eNOS^{+/+}$ mice. In contrast, exercise aggravated the MI-induced LV dilation in $eNOS^{+/-}$ and $eNOS^{-/-}$ mice.

Collagen content

$eNOS^{+/-}SH_{SED}$ mice had higher basal LV myocardial interstitial collagen content compared to $eNOS^{+/+}$ and $eNOS^{-/-}$ mice (Figure 4C). Exercise reduced LV myocardial interstitial collagen content in eNOS+/+ sham-operated mice. In contrast, exercise in $eNOS^{+/-}$ and $eNOS^{-/-}$ sham-operated mice failed to reduce collagen content.

MI resulted in increased interstitial collagen content of the remote surviving LV myocardium, which was of a similar magnitude in sedentary $eNOS^{+/+}$, $eNOS^{+/-}$ and $eNOS^{-/-}$ mice. Exercise abolished the MI-induced increase in interstitial collagen content in $eNOS^{+/+}$ mice. In contrast, exercise failed to reduce the collagen content in $eNOS^{+/-}$MI and $eNOS^{-/-}$MI mice (Figure 4C, 5).

Capillary density

Capillary density in the LV myocardium was significantly lower in $eNOS^{+/-}SH_{SED}$ and $eNOS^{-/-}SH_{SED}$ mice, compared to $eNOS^{+/+}SH_{SED}$ mice (Figure 4D). Exercise had no significant effect on capillary density in any of the sham-operated groups.

LV remodeling after MI resulted in a decreased capillary density in $eNOS^{+/+}$ sedentary mice, but did cause a further lowering of capillary density in $eNOS^{+/-}$ and $eNOS^{-/-}$ sedentary mice. Exercise after MI did not affect capillary density in $eNOS^{+/+}$, $eNOS^{+/-}$, or $eNOS^{-/-}$ mice (Figure 4D, 5).

Apoptosis

The number of TUNEL positive nuclei in LV myocardium were similar between $eNOS^{+/+}$, $eNOS^{+/-}$ and $eNOS^{-/-}$ sham-operated sedentary mice (Figure 4E). Exercise reduced the number of TUNEL positive nuclei in $eNOS^{+/+}$SH mice. In contrast, exercise had no effect in $eNOS^{+/-}$SH mice, and even increased the number of TUNEL positive nuclei in $eNOS^{-/-}$SH mice.

MI resulted in an increased number of TUNEL positive nuclei in remote surviving myocardium in $eNOS^{+/+}$ sedentary mice, a response that was exaggerated in $eNOS^{+/-}$ and $eNOS^{-/-}$ sedentary mice. Exercise after MI reduced the number of TUNEL positive nuclei to basal levels in $eNOS^{+/+}$ mice (Figure 4E, 5). In contrast, exercise failed to reduce the number of TUNEL positive nuclei in $eNOS^{+/-}$MI and $eNOS^{-/-}$MI mice.

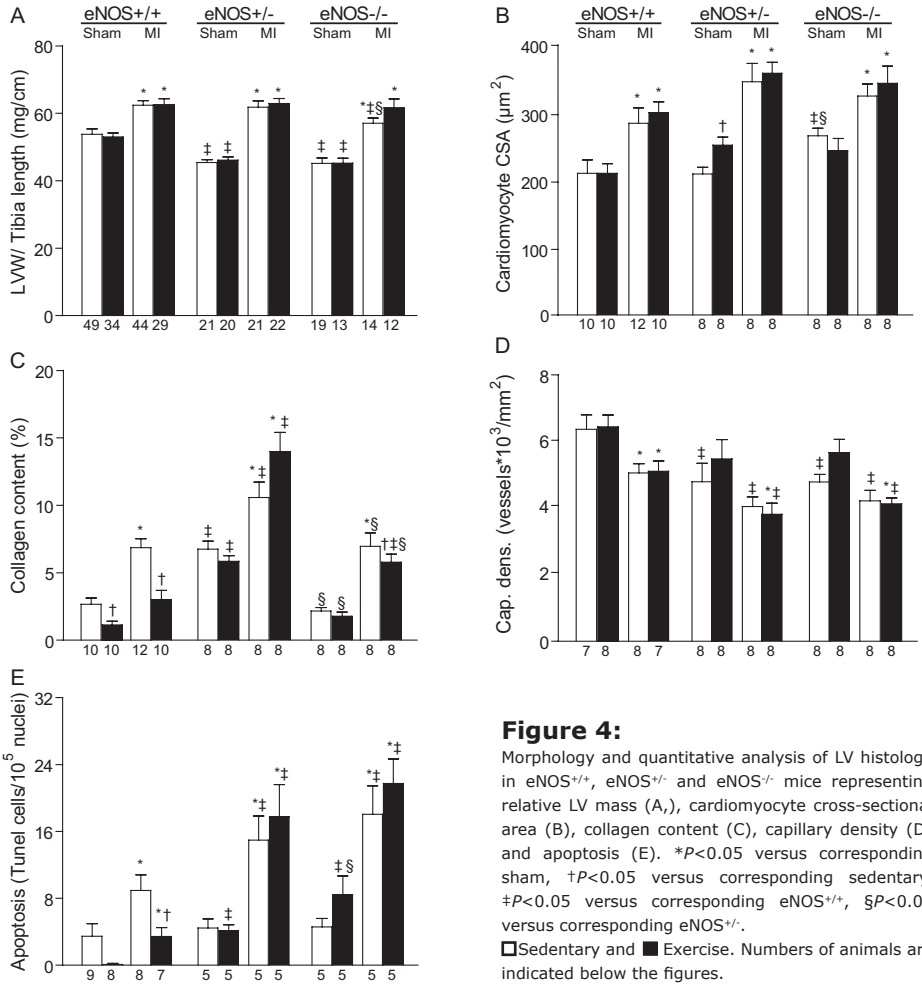

Figure 4:
Morphology and quantitative analysis of LV histology in eNOS$^{+/+}$, eNOS$^{+/-}$ and eNOS$^{-/-}$ mice representing relative LV mass (A,), cardiomyocyte cross-sectional area (B), collagen content (C), capillary density (D) and apoptosis (E). *P<0.05 versus corresponding sham, †P<0.05 versus corresponding sedentary, ‡P<0.05 versus corresponding eNOS$^{+/+}$, §P<0.05 versus corresponding eNOS$^{+/-}$.
□Sedentary and ■ Exercise. Numbers of animals are indicated below the figures.

Discussion

The present study was designed to assess the critical importance of full eNOS availability in the exercise-induced amelioration of LV remodeling and dysfunction after MI in mice. The main findings were: *(i)* MI in eNOS$^{+/+}$ mice resulted in marked LV remodeling, including cardiomyocyte hypertrophy and a reduction in capillary density, increased interstitial collagen content and apoptosis, as well as LV systolic and diastolic dysfunction; *(ii)* The extent of these MI-induced abnormalities were not exaggerated in eNOS$^{+/-}$ or eNOS$^{-/-}$ mice; *(iii)* Exercise abolished interstitial fibrosis and apoptosis in the remote remodeled myocardium, attenuated global LV systolic dysfunction and ameliorated pulmonary congestion in eNOS$^{+/+}$ MI mice; *(iv)* These beneficial effects of exercise after MI were lost in eNOS$^{+/-}$ and eNOS$^{-/-}$ mice, while LV systolic dysfunction and pulmonary congestion in eNOS$^{+/-}$ were even aggravated by exercise. The implications of these findings will be discussed.

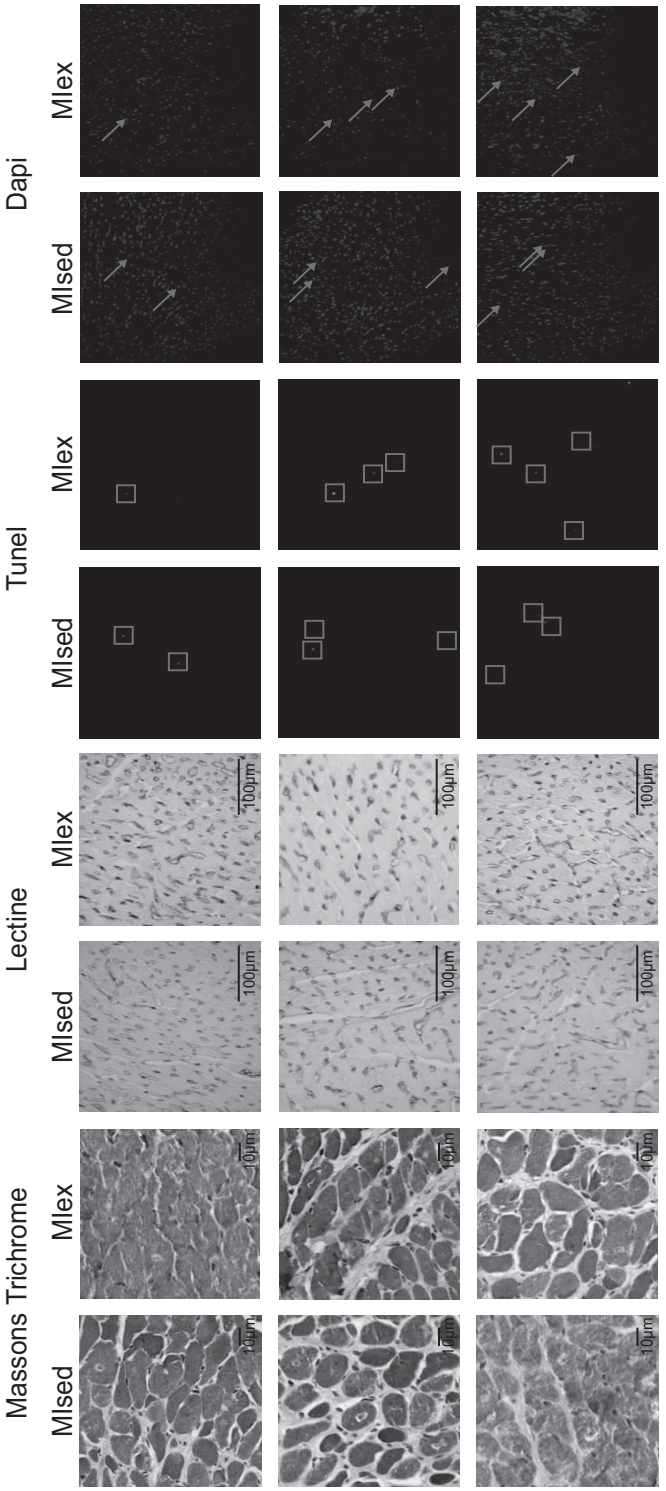

Figure 5:
Representative histological examples from Masson's trichrome, lectin, TUNEL and Dapi staining in MI_{SED} and MI_{EX} mice.

Effects of exercise training after MI in eNOS$^{+/+}$ mice

In agreement with previous observations from our laboratory,[10,17] we found that eNOS$^{+/+}$ mice with a large MI (comprising ~45% of LV mass) exhibited marked LV remodeling, characterized by LV dilation and hypertrophy, increased interstitial collagen deposition and apoptosis and decreased capillary density in remote surviving myocardium. Induction of MI also produced marked LV dysfunction, characterized by decrements in LV pump function (fractional shortening) and indices of global LV contractility (dP/dt_{P30}) and relaxation (dP/dt_{min} and τ), culminating in LV backward failure and hence pulmonary congestion as reflected in pulmonary edema and RV hypertrophy.[10,17]

The effects of exercise on LV remodeling and dysfunction remain incompletely understood, since several studies in humans reported contradictory effects of training on LV remodeling after a MI.[18-27] Careful inspection of these studies suggests that after a small MI, exercise has no detrimental effect[20,26] or even improves[19,22,24] LV geometry and function, independent of whether exercise was started late, i.e. ~1 year,[19,22] or early, i.e. <2 months,[20,24,26] after MI. In contrast, in patients with a large MI, exercise had a beneficial effect on ejection fraction (EF) and LV volumes but only when started late after MI.[19,22] However, when exercise after a large MI is started at a time when LV remodeling is still ongoing (<3-4 months after MI), the majority of studies reported that exercise has either no,[18,20,26,27] or even a detrimental[23,25] effect on LV dimensions and/or EF.

Similar to these clinical studies, studies in rats indicate that exercise started late (>3 weeks) after a moderate to large MI, encompassing 35-50% of LV mass, at a time when infarct healing is complete, does not aggravate,[28,29] or even blunts[30-32] LV dilation[30] and hypertrophy.[28-32] In contrast, when started <1 week after a moderate to large MI,[33-37] exercise resulted in variable outcomes with beneficial,[37] no,[33,35] or detrimental[34,36] effects on LV remodeling. These rodent studies lend further support to the concern that early exercise may have detrimental effects on LV remodeling after a large MI, although interpretation is hampered by the fact that late exercise studies in rats principally employed treadmill running,[22,29,30,32,38] while early exercise studies predominantly used swimming.[31,33-35] This is important because the exercise responses to swimming are markedly different from those to treadmill running.[39,40] In contrast to these studies employing early swim training in rats, we observed (in a previous[10,17] and in the present study) that exercise training through voluntary treadmill running, starting early after a large MI (comprising ~45% of LV mass), blunted LV remodeling and dysfunction, and ameliorated LV backward failure in eNOS$^{+/+}$ mice. A novel observation in the present study is that

exercise also attenuated MI-induced apoptosis, but did not appear to have an effect on capillary density in the remodeling heart. These findings indicate that treadmill running started early after a large MI is beneficial. The lack of a beneficial effect of early exercise in patients with a large MI is difficult to explain but may be related to the presence of risk factors, and hence endothelial dysfunction, in the majority of patients. These findings could be interpreted to suggest that nitric oxide is important to allow a beneficial effect of early exercise after a large MI.

eNOS deficient mice and the response to MI

The cardiovascular phenotype of $eNOS^{-/-}$ mice as observed in the present study is in general agreement with previous studies.[16,41-43] Thus, under basal conditions $eNOS^{-/-}$ exhibited hypertension, but demonstrated normal levels of global LV systolic (fractional shortening, dP/dt_{P30}) and diastolic function (dP/dt_{min}, τ, and LV end-diastolic pressure) under basal conditions. Liu *et al*,[42] also found unperturbed cardiac function, myocardial collagen content and cardiomyocyte cross-sectional area, but in their study a sustained 15-20 mmHg increase in systolic blood pressure was associated with a transient (only up to 6 months of age) increase in relative LV mass in $eNOS^{-/-}$ mice compared to $eNOS^{+/+}$ mice. Furthermore, we found fewer capillaries under basal conditions in $eNOS^{+/-}$ and $eNOS^{-/-}$ compared to $eNOS^{+/+}$ mice, which is in line with observations in neonatal $eNOS^{-/-}$ mice,[43] and supports an important role for nitric oxide in the vasculogenesis/angiogenesis in the heart.[44] We specifically included $eNOS^{+/-}$ mice in the present study, in view of observations by Kojda *et al*[16] that basal levels of eNOS expression and hemodynamics are not different from $eNOS^{+/+}$ mice, but the exercise-induced increase in eNOS expression is selectively abolished.[16,45] Indeed, we observed not only that aortic blood pressure, but also that LV systolic and diastolic function were normal in $eNOS^{+/-}$ mice. In addition, levels of apoptosis and cardiomyocyte cross sectional area were also similar to $eNOS^{+/+}$ mice. However, we did observe that collagen content was higher in $eNOS^{+/-}$ mice and that, similar to the $eNOS^{-/-}$ mice, capillary densities were lower.

Scherrer-Crosbie *et al*[9] reported that 4 weeks after a small MI (resulting in a decrease in fractional shortening from 58±2% to 44±2% in $eNOS^{+/+}$ mice), LV remodeling and dysfunction were aggravated in $eNOS^{-/-}$ compared to $eNOS^{+/+}$ mice. In contrast, we found that the LV remodeling and dysfunction, as well as cardiomyocyte hypertrophy, interstitial fibrosis and increased apoptosis that occurred after a large MI (decreasing fractional shortening from ~40% to ~10%) was similar in $eNOS^{+/+}$, $eNOS^{+/-}$ and $eNOS^{-/-}$ mice. Our observations are in agreement

with the study of Liu *et al*[42] in which LV remodeling and the development of heart failure over a 6 month follow-up period after a large MI (decreasing fractional shortening from ~65% to ~20%) were also similar in $eNOS^{-/-}$ and $eNOS^{+/+}$ mice. Taken together these findings are consistent with the concept that lack of eNOS does not aggravate myocardial remodeling and LV dysfunction after a large MI. Future studies are necessary to investigate the mechanisms responsible for this effect after MI in eNOS deficient mice, and suggest that in more chronic post-MI remodeling an adaptation through compensatory mechanisms (prostacyclin,[46] nNOS,[47] adaptation of remote myocardium) has occurred.

Exercise training after MI in eNOS deficient mice

Several studies suggest an important role of endogenous production of NO in the beneficial effects of exercise.[15] For example, in vivo studies have shown that exercise results in an increased vascular expression of eNOS.[48,49] Furthermore, exercise training in healthy individuals elevated NO bioavailability through a variety of mechanisms including increased NOS enzyme expression and activity.[50] Such adaptations likely contribute to cardiovascular protection by regular physical exercise in individuals with elevated cardiovascular risk or established coronary artery disease. eNOS is known to be present in myocardium of normal mice[51] and $eNOS^{+/-}$ mice,[45] but absent in hearts of $eNOS^{-/-}$ mice.[41] We examined a mouse MI model to investigate the role of eNOS in the exercise-induced benefits on cardiac function and remodeling after MI. Although, exercise after MI in $eNOS^{+/-}$ and $eNOS^{-/-}$ mice did not aggravate LV myocardial hypertrophy, the beneficial effects of exercise on MI-induced LV dilation and LV systolic dysfunction, as observed in $eNOS^{+/+}$ mice, were lost in $eNOS^{+/-}$ and $eNOS^{-/-}$ mice. Furthermore, the exercise-induced amelioration of LV backward failure that was observed in $eNOS^{+/+}$ mice was lost in $eNOS^{-/-}$ mice and even reversed to a worsening of pulmonary congestion in $eNOS^{+/-}$ mice. At the myocardial level, the beneficial effects of exercise in $eNOS^{+/+}$ mice on interstitial fibrosis and apoptosis within non-infarcted remote myocardium was lost in $eNOS^{+/-}$ and $eNOS^{-/-}$ mice. Our findings indicate that eNOS is essential for the exercise-induced improvement of LV geometry (both at the macroscopic and microscopic level) and LV function after MI. Interestingly, $eNOS^{+/-}$ mice which miss only one eNOS allele, appear to respond even slightly worse to exercise after MI than $eNOS^{-/-}$ mice. Kojda *et al*[16] proposed that when two eNOS alleles are present, as in $eNOS^{+/+}$ mice, under basal conditions both are being transcribed at a submaximal rate. Exercise thus increases transcriptional activity of both genes. The absence of one eNOS allele results in the opposite allele functioning at near

peak transcription rate, such that it cannot be further activated by exercise.[45] It could be speculated that in the absence of eNOS, other factors, including prostacyclin[46] or nNOS,[47] may be upregulated or increased in eNOS$^{-/-}$ mice and act to compensate for the absence of eNOS, thereby preventing further worsening of cardiac remodeling and function. If one eNOS allele is still functional and results in normal basal eNOS expression, such compensatory mechanisms may not occur, leaving the heart more vulnerable to the effects of exercise. Future studies are needed to study the influence of potential compensating factors.

Clinical implications and conclusions

When exercise in patients with a large MI is started at a time when LV remodeling is still ongoing (<3-4 months after MI), the majority of studies reported that exercise had either no,[18,20,26] or even a detrimental[23,25] effect on LV dimensions and/or EF. The results of the present study could be interpreted to suggest that endothelial dysfunction in these patients and consequently loss of NO bioavailability may have blunted the beneficial effects of exercise. Future studies in patients and/or animal models of hyperlipidemia and/or metabolic syndrome are required to test the hypothesis that adjunctive therapy aimed improving endothelial function can preserve the beneficial effects of exercise training after a recent large MI.

In conclusion, the beneficial effects of exercise training after a myocardial infarction on LV remodeling and dysfunction depend critically on endogenous eNOS. Furthermore, the observation that the lack of one eNOS allele is sufficient to negate all beneficial effects of exercise, strongly suggests that exercise training depends on full eNOS availability and activity.

References

1. Konstam MA, Udelson JE, Sharpe N. *Prevention and reversal of left ventricular remodeling: summation.* J Card Fail 2002;8:S506-511.
2. Narula J, Haider N, Arbustini E, Chandrashekhar Y. *Mechanisms of disease: apoptosis in heart failure--seeing hope in death.* Nat Clin Pract Cardiovasc Med 2006;3:681-688.
3. van der Velden J, Merkus D, Klarenbeek BR, James AT, Boontje NM, Dekkers DH, *et al*. *Alterations in myofilament function contribute to left ventricular dysfunction in pigs early after myocardial infarction.* Circ Res 2004;95:e85-95.
4. Spinale FG. *Myocardial matrix remodeling and the matrix metalloproteinases: influence on cardiac form and function.* Physiol Rev 2007;87:1285-1342.
5. Massion PB, Balligand JL. *Modulation of cardiac contraction, relaxation and rate by the endothelial nitric oxide synthase (eNOS): lessons from genetically modified mice.* J Physiol 2003;546:63-75.
6. Kim NN, Villegas S, Summerour SR, Villarreal FJ. *Regulation of cardiac fibroblast extracellular matrix production by bradykinin and nitric oxide.* J Mol Cell Cardiol 1999;31:457-466.
7. Ritchie RH, Schiebinger RJ, LaPointe MC, Marsh JD. *Angiotensin II-induced hypertrophy of adult rat cardiomyocytes is blocked by nitric oxide.* Am J Physiol 1998;275:H1370-1374.
8. Jones SP, Greer JJ, van Haperen R, Duncker DJ, de Crom R, Lefer DJ. *Endothelial nitric oxide synthase overexpression attenuates congestive heart failure in mice.* Proc Natl Acad Sci U S A 2003;100:4891-4896.
9. Scherrer-Crosbie M, Ullrich R, Bloch KD, Nakajima H, Nasseri B, Aretz HT, *et al*. *Endothelial nitric oxide synthase limits left ventricular remodeling after myocardial infarction in mice.* Circulation 2001;104:1286-1291.
10. de Waard MC, van der Velden J, Bito V, Ozdemir S, Biesmans L, Boontje NM, *et al*. *Early exercise training normalizes myofilament function and attenuates left ventricular pump dysfunction in mice with a large myocardial infarction.* Circ Res 2007;100:1079-1088.
11. Ekelund LG, Haskell WL, Johnson JL, Whaley FS, Criqui MH, Sheps DS. *Physical fitness as a predictor of cardiovascular mortality in asymptomatic North American men. The Lipid Research Clinics Mortality Follow-up Study.* N Engl J Med 1988;319:1379-1384.
12. Paffenbarger RS, Jr., Hyde RT, Wing AL, Lee IM, Jung DL, Kampert JB. *The association of changes in physical-activity level and other lifestyle characteristics with mortality among men.* N Engl J Med 1993;328:538-545.
13. Blair SN, Kohl HW, 3rd, Barlow CE, Paffenbarger RS, Jr., Gibbons LW, Macera CA. *Changes in physical fitness and all-cause mortality. A prospective study of healthy and unhealthy men.* Jama 1995;273:1093-1098.
14. Sessa WC, Pritchard K, Seyedi N, Wang J, Hintze TH. *Chronic exercise in dogs increases coronary vascular nitric oxide production and endothelial cell nitric oxide synthase gene expression.* Circ Res 1994;74:349-353.
15. Shen W, Zhang X, Zhao G, Wolin MS, Sessa W, Hintze TH. *Nitric oxide production and NO synthase gene expression contribute to vascular regulation during exercise.* Med Sci Sports Exerc 1995;27:1125-1134.
16. Kojda G, Cheng YC, Burchfield J, Harrison DG. *Dysfunctional regulation of endothelial nitric oxide synthase (eNOS) expression in response to exercise in mice lacking one eNOS gene.* Circulation 2001;103:2839-2844.
17. van den Bos EJ, Mees BM, de Waard MC, de Crom R, Duncker DJ. *A novel model of cryoinjury-induced myocardial infarction in the mouse: a comparison with coronary artery ligation.* Am J Physiol Heart Circ Physiol 2005;289:H1291-1300.
18. Dubach P, Myers J, Dziekan G, Goebbels U, Reinhart W, Vogt P, *et al*. *Effect of exercise training on myocardial remodeling in patients with reduced left ventricular function after myocardial infarction: application of magnetic resonance imaging.* Circulation 1997;95:2060-2067.
19. Ehsani AA, Biello DR, Schultz J, Sobel BE, Holloszy JO. *Improvement of left ventricular contractile function by exercise training in patients with coronary artery disease.* Circulation 1986;74:350-358.
20. Giannuzzi P, Tavazzi L, Temporelli PL, Corra U, Imparato A, Gattone M, *et al*. *Long-term physical training and left ventricular remodeling after anterior myocardial infarction: results of the Exercise in Anterior Myocardial Infarction (EAMI) trial. EAMI Study Group.* J Am Coll Cardiol 1993;22:1821-1829.
21. Giannuzzi P, Temporelli PL, Corra U, Gattone M, Giordano A, Tavazzi L. *Attenuation of unfavorable remodeling by exercise training in postinfarction patients with left ventricular dysfunction: results of the Exercise in Left Ventricular Dysfunction (ELVD) trial.* Circulation 1997;96:1790-1797.
22. Giannuzzi P, Temporelli PL, Corra U, Tavazzi L. *Antiremodeling effect of long-term exercise training in patients with stable chronic heart failure: results of the Exercise in Left Ventricular Dysfunction and Chronic Heart Failure (ELVD-CHF) Trial.* Circulation 2003;108:554-559.
23. Jugdutt BI, Michorowski BL, Kappagoda CT. *Exercise training after anterior Q wave myocardial infarction: importance of regional left ventricular function and topography.* J Am Coll Cardiol 1988;12:362-372.
24. Koizumi T, Miyazaki A, Komiyama N, Sun K, Nakasato T, Masuda Y, *et al*. *Improvement of left ventricular dysfunction during exercise by walking in patients with successful percutaneous coronary intervention for acute myocardial infarction.* Circ J 2003;67:233-237.
25. Kubo N, Ohmura N, Nakada I, Yasu T, Katsuki T, Fujii M, *et al*. *Exercise at ventilatory threshold aggravates left ventricular remodeling in patients with extensive anterior acute myocardial infarction.* Am Heart J 2004;147:113-120.
26. Otsuka Y, Takaki H, Okano Y, Satoh T, Aihara N, Matsumoto T, *et al*. *Exercise training without ventricular remodeling in patients with moderate to severe left ventricular dysfunction early after acute myocardial infarction.* Int J Cardiol 2003;87:237-244.
27. Sullivan MJ, Higginbotham MB, Cobb FR. *Exercise training in patients with severe left ventricular dysfunction. Hemodynamic and metabolic effects.* Circulation 1988;78:506-515.
28. Libonati JR. *Exercise and diastolic function after myocardial infarction.* Med Sci Sports Exerc 2003;35:1471-1476.

29. Musch TI, Moore RL, Leathers DJ, Bruno A, Zelis R. *Endurance training in rats with chronic heart failure induced by myocardial infarction.* Circulation 1986;74:431-441.
30. Orenstein TL, Parker TG, Butany JW, Goodman JM, Dawood F, Wen WH, *et al*. *Favorable left ventricular remodeling following large myocardial infarction by exercise training. Effect on ventricular morphology and gene expression.* J Clin Invest 1995;96:858-866.
31. Wisloff U, Loennechen JP, Currie S, Smith GL, Ellingsen O. *Aerobic exercise reduces cardiomyocyte hypertrophy and increases contractility, Ca^{2+} sensitivity and SERCA-2 in rat after myocardial infarction.* Cardiovasc Res 2002;54:162-174.
32. Zhang LQ, Zhang XQ, Musch TI, Moore RL, Cheung JY. *Sprint training restores normal contractility in postinfarction rat myocytes.* J Appl Physiol 2000;89:1099-1105.
33. Alhaddad IA, Hakim I, Siddiqi F, Lagenback E, Mallavarapu C, Nethala V, *et al*. *Early exercise after experimental myocardial infarction: effect on left ventricular remodeling.* Coron Artery Dis 1998;9:319-327.
34. Gaudron P, Hu K, Schamberger R, Budin M, Walter B, Ertl G. *Effect of endurance training early or late after coronary artery occlusion on left ventricular remodeling, hemodynamics, and survival in rats with chronic transmural myocardial infarction.* Circulation 1994;89:402-412.
35. Hochman JS, Healy B. *Effect of exercise on acute myocardial infarction in rats.* J Am Coll Cardiol 1986;7:126-132.
36. Kloner RA, Kloner JA. *The effect of early exercise on myocardial infarct scar formation.* Am Heart J 1983;106:1009-1013.
37. Oh BH, Ono S, Rockman HA, Ross J, Jr. *Myocardial hypertrophy in the ischemic zone induced by exercise in rats after coronary reperfusion.* Circulation 1993;87:598-607.
38. Flaim SF, Minteer WJ, Clark DP, Zelis R. *Cardiovascular response to acute aquatic and treadmill exercise in the untrained rat.* J Appl Physiol 1979;46:302-308.
39. Bernstein D. *Exercise assessment of transgenic models of human cardiovascular disease.* Physiol Genomics 2003;13:217-226.
40. van Rooij E, Doevendans PA, Crijns HJ, Heeneman S, Lips DJ, van Bilsen M, *et al*. *MCIP1 overexpression suppresses left ventricular remodeling and sustains cardiac function after myocardial infarction.* Circ Res 2004;94:e18-26.
41. Huang PL, Huang Z, Mashimo H, Bloch KD, Moskowitz MA, Bevan JA, *et al*. *Hypertension in mice lacking the gene for endothelial nitric oxide synthase.* Nature 1995;377:239-242.
42. Liu YH, Xu J, Yang XP, Yang F, Shesely E, Carretero OA. *Effect of ACE inhibitors and angiotensin II type 1 receptor antagonists on endothelial NO synthase knockout mice with heart failure.* Hypertension 2002;39:375-381.
43. Zhao X, Lu X, Feng Q. *Deficiency in endothelial nitric oxide synthase impairs myocardial angiogenesis.* Am J Physiol Heart Circ Physiol 2002;283:H2371-2378.
44. Murohara T, Asahara T. *Nitric oxide and angiogenesis in cardiovascular disease.* Antioxid Redox Signal 2002;4:825-831.
45. Kojda G, Laursen JB, Ramasamy S, Kent JD, Kurz S, Burchfield J, *et al*. *Protein expression, vascular reactivity and soluble guanylate cyclase activity in mice lacking the endothelial cell nitric oxide synthase: contributions of NOS isoforms to blood pressure and heart rate control.* Cardiovasc Res 1999;42:206-213.
46. Godecke A, Decking UK, Ding Z, Hirchenhain J, Bidmon HJ, Godecke S, *et al*. *Coronary hemodynamics in endothelial NO synthase knockout mice.* Circ Res 1998;82:186-194.
47. Meng W, Ma J, Ayata C, Hara H, Huang PL, Fishman MC, *et al*. *ACh dilates pial arterioles in endothelial and neuronal NOS knockout mice by NO-dependent mechanisms.* Am J Physiol 1996;271:H1145-1150.
48. Woodman CR, Muller JM, Laughlin MH, Price EM. *Induction of nitric oxide synthase mRNA in coronary resistance arteries isolated from exercise-trained pigs.* Am J Physiol 1997;273:H2575-2579.
49. Delp MD, Laughlin MH. *Time course of enhanced endothelium-mediated dilation in aorta of trained rats.* Med Sci Sports Exerc 1997;29:1454-1461.
50. Maroun MJ, Mehta S, Turcotte R, Cosio MG, Hussain SN. *Effects of physical conditioning on endogenous nitric oxide output during exercise.* J Appl Physiol 1995;79:1219-1225.
51. Gyurko R, Kuhlencordt P, Fishman MC, Huang PL. *Modulation of mouse cardiac function in vivo by eNOS and ANP.* Am J Physiol Heart Circ Physiol 2000;278:H971-981.

Chapter 4

Reduction of blood pressure, plasma cholesterol and atherosclerosis by elevated endothelial nitric oxide

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Abstract

In the vascular system, nitric oxide is generated by endothelial NO synthase (eNOS). NO has pleiotropic effects, most of which are believed to be atheroprotective. Therefore, it has been argued that patients suffering from cardiovascular disease could benefit from an increase in eNOS activity. However, increased NO production can cause oxidative damage, cell toxicity, and apoptosis and hence could be atherogenic rather than beneficial. To study the in vivo effects of increased eNOS activity, we created transgenic mice overexpressing human eNOS. Aortic blood pressure was ~20 mmHg lower in the transgenic mice compared with control mice because of lower systemic vascular resistance. The effects of eNOS overexpression on diet-induced atherosclerosis were studied in apolipoprotein E-deficient mice. Elevation of eNOS activity decreased blood pressure (~20 mmHg) and plasma levels of cholesterol (~17%), resulting in a reduction in atherosclerotic lesions by 40%. We conclude that an increase in eNOS activity is beneficial and provides protection against atherosclerosis.

Introduction

Endothelial nitric oxide synthase (eNOS) plays an important role in the regulation of vascular tone, vascular biology, and hemostasis. For example, NO produced by eNOS causes vasodilation. Thus, eNOS knockout mice are hypertensive,[1] whereas eNOS transgenic mice have hypotension.[2] In addition, NO reduces the activation and aggregation of platelets,[3,4] attenuates adhesion of leukocytes to the endothelium,[5–7] reduces the permeability of the endothelium, and inhibits proliferation and migration of vascular smooth muscle cells.[8] Impaired activity of eNOS is associated with endothelial cell dysfunction.[9] For these reasons eNOS has been proposed to modulate atherosclerotic disease.[10–12] Indeed, impairment or deficiency of eNOS gives rise to accelerated atherosclerosis in animal models,[10,12–14] indicating that physiological levels of eNOS are anti-atherogenic. This suggests that patients at increased risk of atherosclerotic vascular disease could possibly benefit from an increase of eNOS activity by pharmacological means or (local) gene therapy. However, eNOS-derived NO also has detrimental effects,[15] such as the generation of superoxides,[16] making it difficult to predict whether increased eNOS activity is beneficial or harmful.[17] To determine whether increased eNOS activity may be beneficial, we created transgenic mice that express the human eNOS gene. These mice were crossbred to apolipoprotein E-deficient (apoE0) mice in order to evaluate the effects of a constitutive increase in eNOS activity on the development of atherosclerosis.

Materials and Methods

Mice

A DNA fragment containing the human eNOS gene was isolated from a homemade human genomic cosmid library[18] using eNOS cDNA (kindly donated by Dr. S. Janssens, Leuven, Belgium; Ref. 19) as a probe. In addition, the DNA fragment contained ~6 kb of 5`-natural flanking sequence, including the native eNOS promoter, and ~3 kb of 3`-sequence to the gene. Vector sequences were removed by restriction endonucleases. A solution of 1–2 µg/ml DNA was used for microinjection of fertilized oocytes from FVB donor mice and transplanted into the oviducts of pseudopregnant B10 x CBA mice. Founder mice and offspring were genotyped by PCR on DNA isolated from tail biopsies. Primers used were sense, 5`-GTCCTGCAGACCGTGCAGC-3`, and antisense, 5`-GGCTGTTGGTGTCTGAGCCG-3`. Mice were backcrossed to C57Bl/6J for at least five generations (~96% C57Bl/6J). All eNOS transgenic mice were hemizygous. ApoE0 mice were obtained from The Jackson Laboratory, Bar Harbor, ME. Male mice were used in all experiments.

All animal experiments were performed in compliance with institutional (Erasmus Medical Center, Rotterdam, The Netherlands) and national (Ministry of Health, Welfare, and Sport, The Hague, The Netherlands) guidelines.

Western blotting and immunohistochemistry

Aortas were collected and homogenized in 50 mM Tris-HCl, pH 7.4, containing 1 mM EDTA, 0.25 M sucrose, and 20 mM CHAPS. Western blotting was performed as described previously.[20] 25 µg of protein (BCA protein assay kit; Pierce) was applied to each lane. Anti-eNOS was obtained from Santa Cruz Biotechnology Inc., Santa Cruz, CA. This antibody was also used for immunohistochemistry experiments, which were performed according to Bakker *et al*.[21]

Hemodynamic measurements

Baseline blood pressure measurements

Mice were weighed, anesthetized with ketamine (100 mg/kg intraperiteoneal) and xylazine (20 mg/kg intraperitoneal), intubated, and ventilated with a mixture of O_2 and N_2 (1/2 v/v) with a pressure-controlled ventilator (Servo ventilator 900C, Siemens-Elema, Sweden). The ventilation rate was set at 100 strokes/min with a peak inspiration pressure of 18 cm H_2O and a positive end expiration pressure of 6 cm H_2O. After intubation the mice were placed on a heating pad to maintain body temperature at 37°C, and a polyethylene catheter (PE10) was inserted into the right carotid artery and advanced into the aortic arch for the measurement of aortic pressure. In the first part of the study we used 12 eNOS-Tg2 and 5 eNOS-Tg3 mice for screening of eNOS expression and compared them to 33 wild type mice. Ten minutes after a second intraperitoneal bolus of anesthetics (100 mg/kg ketamine and 20 mg/kg xylazine), baseline blood pressure recordings were obtained.

Effect of L-NAME on systemic vascular resistance

Subsequently, we chose the eNOS-Tg2 line to determine whether the lower aortic blood pressure was the result of a NO-mediated decrease in systemic vascular resistance. For this purpose, in 17 eNOS-Tg2 mice and 17 wild type mice a polyethylene catheter (PE 10) was inserted into the right carotid artery and advanced into the aortic arch for the measurement of aortic pressure, while another PE 10 catheter was introduced into the right external jugular vein and advanced into the superior caval vein for infusion of L-NAME. After thoracotomy through the second right intercostal space, the ascending aorta was exposed and a transit-time flow probe (ID 1.5 mm; T206; Transonics Systems Inc.) was placed around the

aorta for measuring aorta flow. Ten minutes after a second intraperitoneal bolus of 100 mg/kg ketamine and 20 mg/kg xylazine, baseline recordings were obtained. A continuous 10-min intravenous infusion of L-NAME (100 mg/kg) was started; 10 min after completion of the infusion, measurements were repeated.

Effects of dietary suppletion of L-arginine on baseline hemodynamics

In the eNOS-Tg2 line we studied whether L-arginine deficiency contributed to the modest effects of eNOS overexpression on systemic vascular resistance. For this purpose, in six eNOS-Tg2 male mice and seven wild type male mice, L-arginine was supplemented in their drinking water (2.5% w/v). One week later, animals were instrumented as described above, and hemodynamic measurements were performed under baseline conditions.

Data analysis

Hemodynamic data were recorded and digitized using an online 4-channel data acquisition program (ATCODAS, Dataq Instruments, Akron, OH), for later analysis with a program written in MatLab (Mathworks Inc, Natick, MA). Fifteen consecutive beats were selected for determination of heart rate, aortic pressure, and aorta blood flow.

eNOS activity assay

Aortas were collected and homogenized in 50 mM Tris-HCl, pH 7.4, containing 1 mM EDTA, 0.25 M sucrose, and 20 mM CHAPS. eNOS activity assays were performed by measuring L-arginine to L-citrulline conversion using a nitric oxide synthase assay kit (Calbiochem, La Jolla, CA; catalogue no. 482700) according to the manufacturer's instructions. Protein content was measured by the BCA protein assay kit (Pierce).

Lipid measurements

Blood was collected via orbital puncture after an overnight fasting period. Plasma was frozen freshly or subjected to ultracentrifugation in a Beckman 42.2 Ti rotor (42,000 rpm, 3 h, 12 °C) at d >1.063 g/ml. Tubes were sliced, and two fractions were collected: very low density lipoprotein (VLDL) + low density lipoprotein (LDL), d >1.063 g/ml; and high density lipoprotein (HDL), d >1.063 g/ml. Cholesterol was measured with the F-chol kit (Roche Molecular Biochemicals) after hydrolysis of cholesteryl esters with cholesterol esterase from Candida cylindracea (Roche Molecular Biochemicals).

Atherosclerosis

Atherosclerosis experiments were performed in age- and sexmatched mice. Male mice of 8 weeks were fed a Western type diet containing 15% (w/w) cacoa butter and 0.25% (w/w) cholesterol (diet W, Hope Farms, Woerden, The Netherlands) for 6 weeks, which leads to appreciable atherosclerosis.[22–24] Animals were anesthetized using isoflurane, and in situ fixation was performed via the left ventricle of the heart using phosphate-buffered formalin (4%, v/v). A Sony digital camera was used to obtain images of sections of the aortic root. These were analyzed by Scion Image processing and analyzing software (available from www.scioncorp.com). Atherosclerosis was quantified in five sections per mouse with 80 μm intervals using the method of Paigen *et al.*[25]

Data analysis

Analysis of data was performed using two- or one-way analysis of variance followed by the Scheffé test, as appropriate. Statistical significance was accepted when $P<0.05$ (two-tailed). Data are presented as mean±S.E.

Results and Discussion

For the generation of eNOS transgenic mice, we used a DNA fragment that comprised the complete human eNOS genomic sequence, including all exons and introns as well its natural flanking sequences. Therefore, our mice are different from those described by Ohashi *et al,*[2] which overexpressed bovine eNOS cDNA, driven by the murine preproendothelin-1 promoter. Our approach was chosen to preserve the natural regulation of the gene and to prevent ectopic expression. For example, the endothelium enhancer element that is located 4.9 kilobases upstream from the transcription start site of the eNOS gene[26] is included in this construct. By using this construct we mimic the human situation in terms of regulation and tissue distribution of eNOS as much as possible.

Two independent lines with appreciable overexpression of the transgene, as measured by RT-PCR (not shown), were selected and arbitrarily designated eNOStg2 and eNOStg3. Production of human eNOS protein was demonstrated by Western blotting of aorta homogenates (Figure 1A). Subsequently, eNOS activity in aortas from control (wild type) and eNOS overexpressing mice was measured, using the L-arginine to L-citrulline conversion assay.[27] In aorta, the level of active eNOS enzyme was 10-fold increased in eNOStg2 mice and 7.5-fold in eNOStg3 mice when compared with wild type animals (Figure 1B). Expression of human eNOS was also investigated by immunohistochemistry. There was no human eNOS

staining of aortas of wild type mice, whereas the endothelial layer of the aorta was clearly stained in both human eNOS transgenic lines (Figure 1C).

The expression pattern of the human eNOS transgene was investigated by immunohistochemistry in heart (Figure 2A), liver (Figure 2B), kidney (Figure 2C), adrenal (Figure 2D), and testis (Figure 2E). Sections from wild type controls show virtually no immunostaining (not shown). The lining of the larger vessels is clearly stained (Figure 2, A, D, and E). Staining of capillaries is visible in the heart between the cardiomyocytes. Immunoreactivity is observed in the sinusoids in the liver and in the kidney in the peritubular capillaries as well as in the capillaries from the glomeruli. In the adrenal, the cortical capillaries as well as medullary capillary sinusoids and veins are stained. In the testis, only blood vessels between the seminiferous tubules are stained. No appreciable immunoreactivity is perceptible in the parenchyma cells of any of these organs. Similar results were found with sections from eNOStg3 mice (not shown).

Our results show that the genomic sequences included in the DNA fragment we used are sufficient for expression in endothelial cells. Although eNOS activity is tightly regulated at the post-translational level,[28,29] there is also extensive regulation at the transcriptional level.[30,31] The present study shows that our construct results in high level expression of human eNOS that is not prevented by a feedback mechanism.

To study the effect of increased eNOS activity on blood pressure and vascular tone, we performed hemodynamic studies.[32] Heart rates were similar for wild type controls and eNOS transgenic lines, although the eNOS transgenic lines each exhibited a 20–25 mmHg lower mean aortic blood pressure compared with littermate controls (Figure 3A). Subsequent hemodynamic studies were performed in eNOStg2, the transgenic mouse line with the highest expression. These experiments showed that the lower aortic blood pressure was the result of a 30% lower systemic vascular resistance, because mean aortic blood flow and heart rate were similar in both groups (Figure 3B). Subsequent infusion of the NOS inhibitor, L-NAME, increased systemic vascular resistance and abolished the difference between control and eNOStg2 mice. We therefore conclude that the lower basal systemic vascular resistance in eNOStg2 mice is the result of increased NO production (Figure 3B). This is corroborated by the significantly larger increase in blood pressure in response to L-NAME in the transgenic mice. Suppletion of L-arginine had no effect on the already lower mean aorticblood pressure and heart rate in the transgenic mice (Figure 3C). We therefore also conclude that the blood pressure-lowering effect of eNOS overexpression was not limited by a shortage of

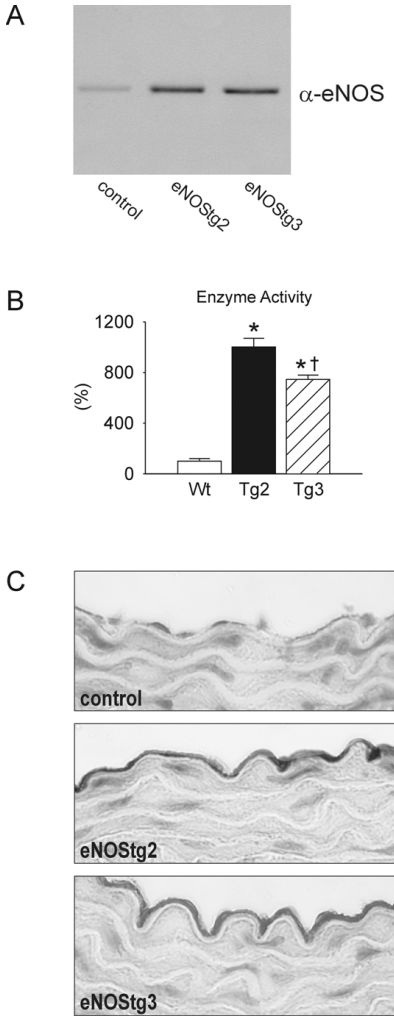

Figure 1: Expression of eNOS in transgenic mice

A, Western blot analysis of aortas from control (wild type littermates), eNOStg2, or -3 mice. 25 µg of homogenate was applied per lane. The blot was probed with antihuman eNOS antibody. A single protein band of the expected molecular size (~130 kDa) was detected. B, eNOS activity was measured in aortas from control (Wt), eNOStg2 (Tg2), or -3 (Tg3) mice by the L-arginine to L-citrulline conversion assay using a nitric oxide synthase assay kit (Calbiochem). Activity is expressed as percentage of the activity in controls, which was 1.56±0.31 pmol/µg/min. Each value represents the mean ± S.E. of three animals. One of three experiments is shown. **P*<0.05 versus controls; †*P*<0.05 versus eNOStg2 mice (analysis of variance followed by the Scheffé test). C, immunohistochemistry on aortas from control, eNOStg2, or -3 mice. Aortas were collected after in situ fixation. Paraffin sections were incubated with antihuman eNOS antibody and a peroxidase-conjugated secondary antibody. Original magnification, x630.

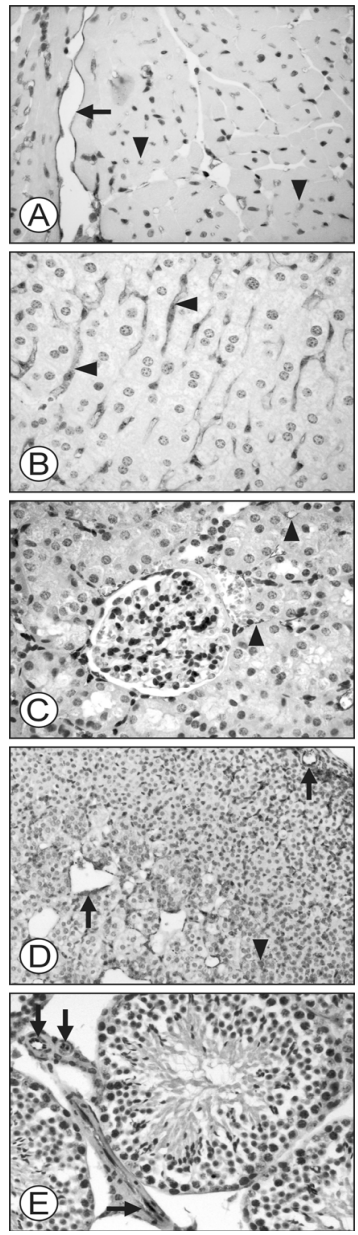

Figure 2: Expression pattern of human eNOS in transgenic mice

Organs from eNOStg2 mice were collected after in situ fixation. Paraffin sections were incubated with antihuman eNOS antibody and a peroxidase-conjugated secondary antibody. A, heart. B, liver. C, kidney. D, adrenal. E, testis tissue. Arrowheads indicate representative immunoreactive capillaries; arrows indicate larger blood vessels. Original magnification, ×400.

substrate.

The present study shows that the lower blood pressure associated with eNOS overexpression (as reported in Ref. 2) is the result of a lower systemic vascular resistance. Thus eNOS activity was not only increased in the larger blood vessels but also in the microcirculation. Although eNOStg2 and -3 mice showed a slight variation in eNOS activity (Figure 1B), the degree to which blood pressure was lowered was not different. This suggests that another rate-limiting factor or one or more compensatory mechanisms prevented a further decrease in blood pressure in the eNOStg2 mice.

To study whether increased expression of eNOS protects the mice against the development of diet-induced atherosclerosis, eNOS transgenic mice were cross-bred to apoE0 mice, which represent a well known mouse model for studying atherosclerosis.[33,34] The animals were fed a Western type diet for 6 weeks. As shown in Figure 4A, overexpression of eNOS also caused a decrease in systemic blood pressure under these conditions, although heart rates were similar. Plasma cholesterol levels were measured before the start of the atherogenic diet (i.e. on normal chow diet) and at the end of the experiment (Table I).

Both eNOS transgenic lines showed a decrease in plasma cholesterol of ~15% when compared with apoE0 mice when fed a normal chow diet. As expected, the Western diet resulted in a dramatic increase (~3-fold) in plasma cholesterol levels, whereas the total cholesterol concentration remained ~16% lower in the eNOS transgenic animals when compared with apoE0 controls. This difference was because of variations in VLDL and LDL, which contain the bulk of the plasma cholesterol under these conditions: HDL-cholesterol concentration in plasma was 0.4 mM and did not differ between the groups (Table I). These findings indicate that elevated eNOS activity results in a slightly more favorable (i.e. less atherogenic) lipoprotein profile, because VLDL and LDL contain the atherogenic portion of plasma cholesterol,[35] whereas HDL is protective against the development of atherosclerosis.[36,37]

To study the effect of eNOS overexpression on atherosclerosis, the atherosclerotic lesion areas in the aortic roots were measured. Figure 4B shows representative examples of histological sections. Compared with apoE0 mice, we observed a decrease in atherosclerosis in both lines of eNOS transgenic mice studied (Figure 4C).

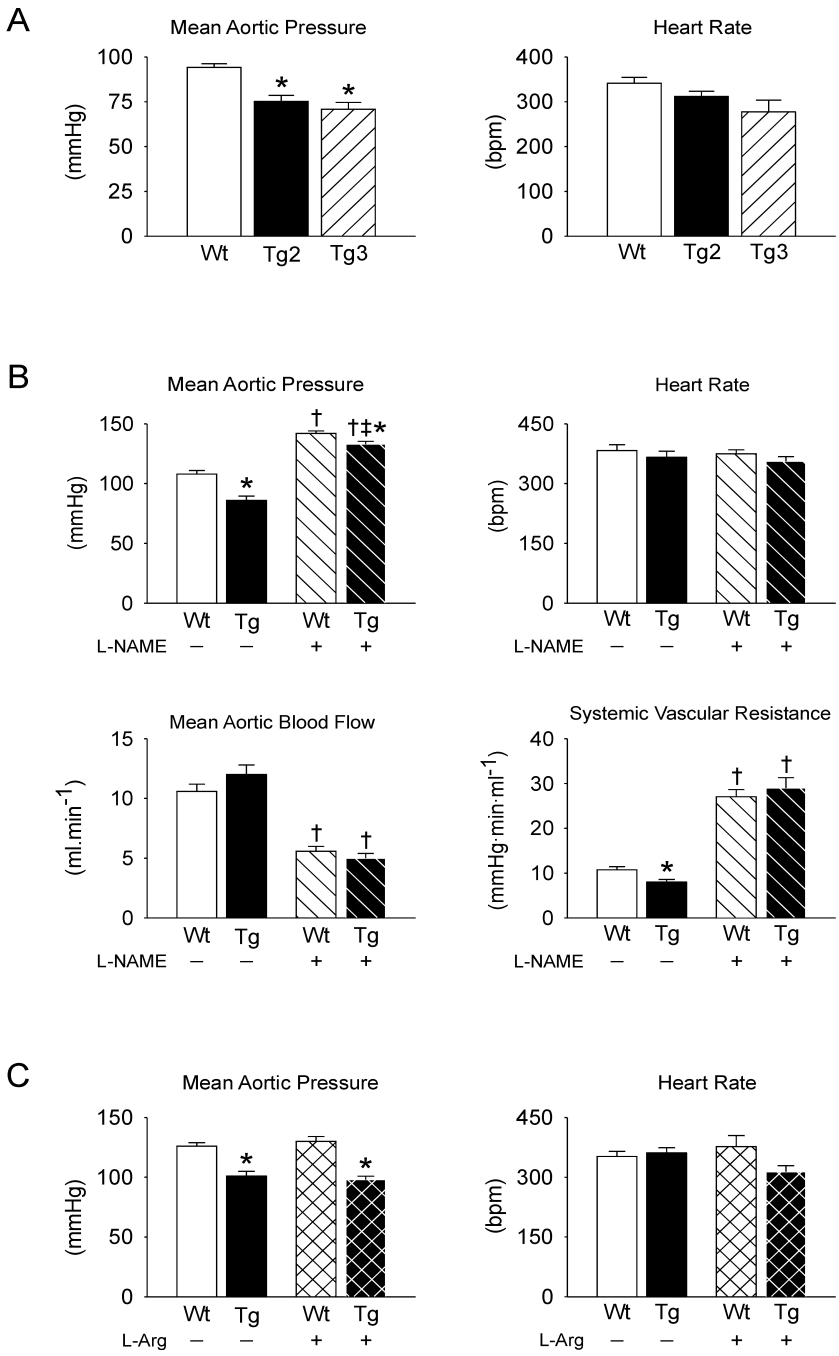

Figure 3: Hemodynamic measurements

A, mean aortic pressure and heart rate were measured in anesthetized control (wild type littermates, Wt), eNOStg2 (Tg2), or -3 (Tg3) mice. B, mean aortic pressure, heart rate, mean aortic blood flow, and systemic vascular resistance were analyzed in Wt or eNOStg2 mice before and following infusion of L-NAME. C, mean aortic pressure and heart rate were measured in anesthetized Wt or Tg2 mice following 1 week of drinking water with or without L-arginine supplementation (L-Arg, 2.5% w/v). Each value represents the mean ± S.E. of five animals. *P<0.05 versus Wt. †P<0.05 versus baseline (before infusion of L-NAME). ‡P<0.05 versus Wt after infusion of L-NAME (analysis of variance followed by the Scheffé test).

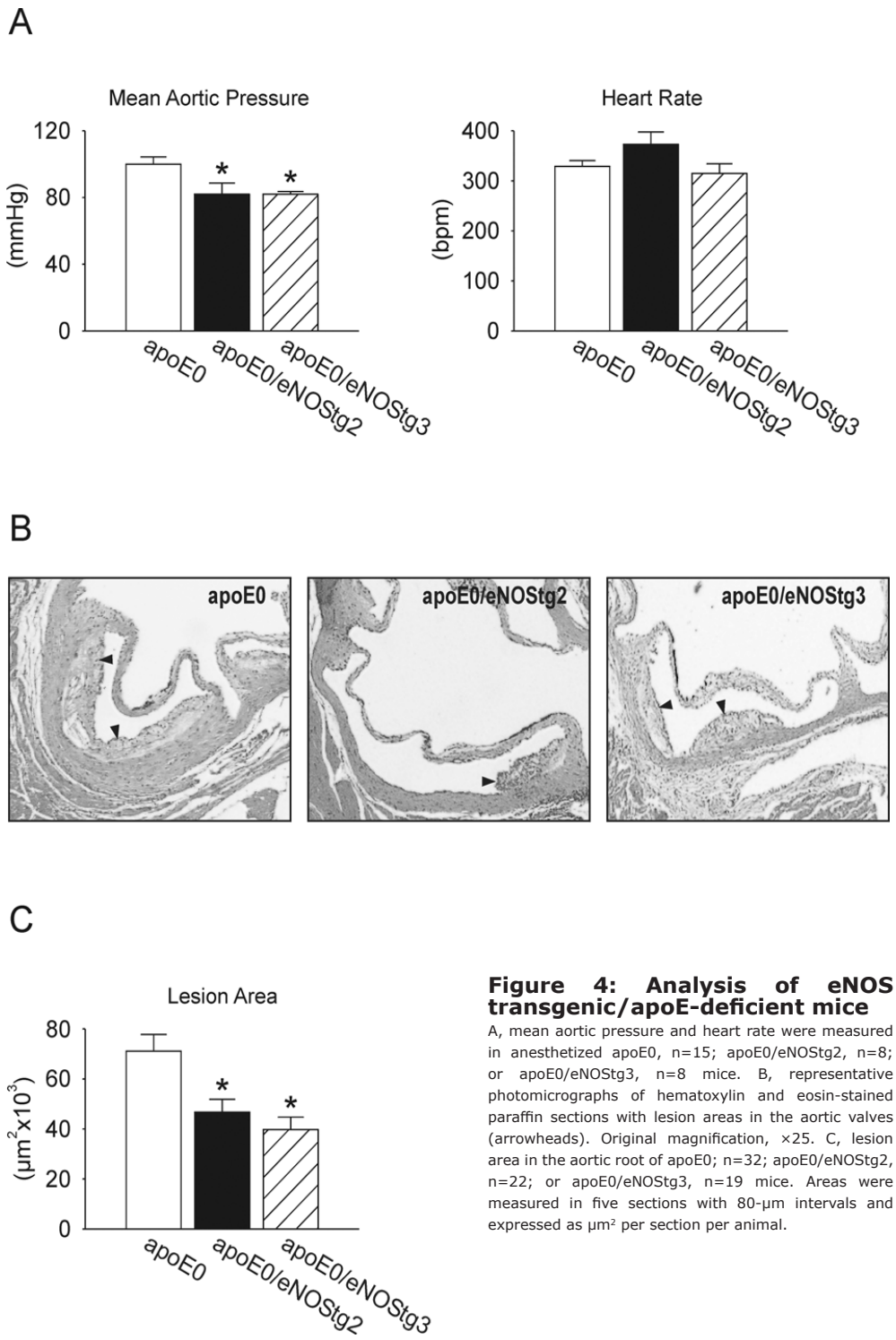

Figure 4: Analysis of eNOS transgenic/apoE-deficient mice

A, mean aortic pressure and heart rate were measured in anesthetized apoE0, n=15; apoE0/eNOStg2, n=8; or apoE0/eNOStg3, n=8 mice. B, representative photomicrographs of hematoxylin and eosin-stained paraffin sections with lesion areas in the aortic valves (arrowheads). Original magnification, ×25. C, lesion area in the aortic root of apoE0; n=32; apoE0/eNOStg2, n=22; or apoE0/eNOStg3, n=19 mice. Areas were measured in five sections with 80-μm intervals and expressed as μm^2 per section per animal.

Table I: Plasma lipid and lipoprotein analysis

Total cholesterol concentrations in plasma from apoE-deficient control (apoE0), apoE0/eNOStg2, or apoE0/eNOStg3 mice after feeding a normal chow diet or an atherogenic diet. Cholesterol concentrations were determined by enzymatic methods. VLDL + LDL and HDL fractions were isolated by ultracentrifugation. TC, total cholesterol (mM); VLDL + LDL, cholesterol in VLDL and LDL (mM); HDL, cholesterol in HDL (mM).

				Atherogenic diet		
	n	Chow TC	TC	VLDL + LDL	HDL
apoE0	32	10.9 ± 0.3	28.4 ± 0.9	27.3 ± 0.8	0.4 ± 0.02
apoE0/eNOStg2	22	9.2 ± 0.4[a]	23.3 ± 1.1[a]	21.9 ± 1.1[b]	0.4 ± 0.02
apoE0/eNOStg3	19	9.0 ± 0.2[b]	24.4 ± 1.1[c]	24.3 ± 1.0[c]	0.4 ± 0.02

[a] $P<0.01$, [b] $P<0.001$, [c] $P<0.05$

Several studies have described a relation between plasma cholesterol and eNOS activity.[38–40] However, these investigations exclusively focused on the effects of changes in plasma cholesterol levels on eNOS activity. Hypercholesterolemia is associated with decreased eNOS activity, probably via an interaction of oxidized LDL with caveolae, the plasma membrane domains in which eNOS resides.[41] Cholesterol synthesis inhibitors (statins) have been reported to increase eNOS activity in addition to their cholesterol lowering effects, but these actions appear to results be independent.[38] In the present study we observed that the level of eNOS expression affects the level of plasma cholesterol: plasma levels of cholesterol were about 15% lower in eNOStg/apoE0 mice as compared with apoE0 controls. A similar difference in plasma cholesterol levels was found after feeding the mice a Western type diet for 6 weeks, indicating that eNOS overexpression alleviates dietinduced hypercholesterolemia. This effect is not caused by ectopic expression in organs involved in lipid metabolism, e.g. the liver, because the expression pattern is restricted to endothelial cells in all organs that were examined (Figure 2). Recently it was shown that hypercholesterolemia in mice results in CD36-mediated cholesterol depletion of caveolae, followed by translocation of eNOS from caveolae and subsequent inactivation of the enzyme.[42] Thus, eNOS activity is directly related to the cholesterol content of caveolae. Possibly, the moderate decrease in plasma cholesterol that we observed in our eNOS transgenic mice is caused by a recruitment of plasma cholesterol by the endothelial cells in order to handle the increased level of eNOS protein. This small decrease in plasma cholesterol likely contributed, at least in part, to the observed lower susceptibility to diet-induced atherosclerosis. Although it has been proposed that elevation of eNOS activity would attenuate atherosclerosis,[11] serious doubts have also been expressed as to whether an increase in eNOS activity in vivo would have beneficial effects, because high levels of NO (e.g. as produced by inducible NO synthase) have been implicated in cell toxicity and apoptosis.[15,43] During the preparation

of our report, Ozaki *et al*[44] reported the unexpected observation that relatively modest overexpression of eNOS resulted in increased atherosclerosis. Based on our findings, we conclude that this observation cannot be generalized, taking into account the following considerations. First, because enzyme activity is ~1.5-fold increased, the level of eNOS overexpression in the mice used by Ozaki *et al* is relatively low when compared with the much higher NO production levels by activated inducible NO synthase. A 1.5-fold increase is also rather low in terms of possible drug or gene therapy applications. Second, the observed increase in atherosclerosis is explained by measurements indicating that the overexpressed eNOS enzyme is dysfunctional in the mouse model used by Ozaki *et al*. This finding is not unexpected, given the moderate level of overexpression in their mice, because it has been previously reported that endogenous eNOS is indeed dysfunctional in terms of NO production in apoE0 mice fed a Western type diet[45] The results from the Ozaki *et al* study are probably (at least in part) explained by the construct used, which consists of cDNA (often leading to low expression levels) and a heterologous promoter. In contrast, our results demonstrate that overexpression of human eNOS in endothelial cells indeed results in decreased atherosclerosis, most likely via lowering blood pressure and plasma cholesterol. Our study therefore suggests that elevation of eNOS activity could be beneficial for patients at risk of developing atherosclerotic disease.

References

1. Huang PL, Huang Z, Mashimo H, Bloch KD, Moskowitz MA, Bevan JA, and Fishman MC. *Hypertension in mice lacking the gene for endothelial nitric oxide synthase.* Nature 1995;377:239–242.
2. Ohashi Y, Kawashima S, Hirata K, Yamashita T, Ishida T, Inoue N, Sakoda T, Kurihara H, Yazaki Y, and Yokoyama M. *Hypotension and reduced nitric oxide-elicited vasorelaxation in transgenic mice overexpressing endothelial nitric oxide synthase.* J Clin Invest 1998;102:2061–2071.
3. Riddell DR, and Owen JS. *Nitric oxide and platelet aggregation.* Vitam Horm 1999;57:25–48.
4. Loscalzo J. *Nitric oxide insufficiency, platelet activation, and arterial thrombosis.* Circ Res 2001;88:756–762.
5. Lefer AM. *Nitric oxide: nature's naturally occurring leukocyte inhibitor.* Circulation 1997;95:553–554.
6. Qian H, Neplioueva V, Shetty GA, Channon KM, and George SE. *Nitric oxide synthase gene therapy rapidly reduces adhesion molecule expression and inflammatory cell infiltration in carotid arteries of cholesterol-fed rabbits.* Circulation 1999;99:2979–2982.
7. Niebauer J, Dulak J, Chan JR, Tsao PS, and Cooke JP. *Gene transfer of nitric oxide synthase: effects on endothelial biology.* J Am Coll Cardiol 1999;34:1201–1207.
8. Li H, and Forstermann U. *Nitric oxide in the pathogenesis of vascular disease.* J Pathol 2000;190:244–254.
9. Harrison DG. *Cellular and molecular mechanisms of endothelial cell dysfunction.* J Clin Invest 1997;100:2153–2157.
10. Barton M, Haudenschild CC, d'Uscio LV, Shaw S, Munter K, and Luscher TF. *Endothelin ETA receptor blockade restores NO-mediated endothelial function and inhibits atherosclerosis in apolipoprotein E-deficient mice.* Proc Natl Acad Sci U S A 1998;95:14367–14372.
11. Drummond GR, and Harrison DG. *eNOS-overexpressing mice: too much NO makes the blood pressure low.* J Clin Invest 1998;102:2033–2034.
12. Kano H, Hayashi T, Sumi D, Esaki T, Asai Y, Thakur NK, Jayachandran M, and Iguchi A. A *HMG-CoA reductase inhibitor improved regression of atherosclerosis in the rabbit aorta without affecting serum lipid levels: possible relevance of up-regulation of endothelial NO synthase mRNA.* Biochem Biophys Res Commun 1999;259:414–419.
13. Knowles JW, Reddick RL, Jennette JC, Shesely EG, Smithies O, and Maeda N. J Clin Invest 2000;105:451–458.
14. Kuhlencordt PJ, Gyurko R, Han F, Scherrer-Crosbie M, Aretz TH, Hajjar R, Picard MH, and Huang PL. *Accelerated atherosclerosis, aortic aneurysm formation, and ischemic heart disease in apolipoprotein E/endothelial nitric oxide synthase double-knockout mice.* Circulation 2001;104:448–454.
15. Wever RM, Luscher TF, Cosentino F, and Rabelink TJ. *Atherosclerosis and the two faces of endothelial nitric oxide synthase.* Circulation 1998;97:108–112.
16. Xia Y, Tsai AL, Berka V, and Zweier JL. *Superoxide generation from endothelial nitric-oxide synthase. A Ca2+/calmodulin-dependent and tetrahydrobiopterin regulatory process.* J Biol Chem 1998;273:25804–25808.
17. O'Donnell VB, and Freeman BA. *Interactions between nitric oxide and lipid oxidation pathways: implications for vascular disease.* Circ Res 2001;88:12–21.
18. van Haperen R, van Tol A, Vermeulen P, Jauhiainen M, van Gent T, van den Berg P, Ehnholm S, Grosveld F, van der Kamp A, and de Crom R. *Human plasma phospholipid transfer protein increases the antiatherogenic potential of high density lipoproteins in transgenic mice.* Arterioscler Thromb Vasc Biol 2000;20:1082–1088.
19. Janssens SP, Shimouchi A, Quertermous T, Bloch DB, and Bloch KD. *Cloning and expression of a cDNA encoding human endothelium-derived relaxing factor/nitric oxide synthase.* J Biol Chem 1992;267:14519–14522.
20. de Crom R, van Haperen R, Janssens R, Visser P, Willemsen R, Grosveld F, and van der Kamp A. *Gp96/GRP94 is a putative high density lipoprotein-binding protein in liver.* Biochim Biophys Acta 1999;1437:378–392.
21. Bakker CE, de Diego Otero Y, Bontekoe C, Raghoe P, Luteijn T, Hoogeveen AT, Oostra BA, and Willemsen R. *Immunocytochemical and biochemical characterization of FMRP, FXR1P, and FXR2P in the mouse.* Exp Cell Res 2000;258:162–170.
22. Nakashima Y, Plump AS, Raines EW, Breslow JL, and Ross R. *ApoE-deficient mice develop lesions of all phases of atherosclerosis throughout the arterial tree.* Arterioscler Thromb 1994;14:133–140.
23. Murayama T, Yokode M, Kataoka H, Imabayashi T, Yoshida H, Sano H, Nishikawa S, and Kita T. *Intraperitoneal administration of anti-c-fms monoclonal antibody prevents initial events of atherogenesis but does not reduce the size of advanced lesions in apolipoprotein E-deficient mice.* Circulation 1999;99:1740–1746.
24. Sjoland H, Eitzman DT, Gordon D, Westrick R, Nabel EG, and Ginsburg D. Arterioscler Thromb Vasc Biol 2000;20:846–852.
25. Paigen B, Morrow A, Holmes PA, Mitchell D, and Williams RA. *Quantitative assessment of atherosclerotic lesions in mice.* Atherosclerosis 1987;68:231–240.
26. Laumonnier Y, Nadaud S, Agrapart M, and Soubrier F. J Biol Chem 2000;275:40732–40741.
27. Garcia-Cardena G, Fan R, Stern DF, Liu J, and Sessa WC. *Endothelial nitric oxide synthase is regulated by tyrosine phosphorylation and interacts with caveolin-1.* J Biol Chem 1996;271:27237–27240.
28. Marletta MA. *Another activation switch for endothelial nitric oxide synthase: why does it have to be so complicated?* Trends Biochem Sci 2001;26:519–521.
29. Fulton D, Gratton JP, and Sessa WC. *Post-translational control of endothelial nitric oxide synthase: why isn't calcium/calmodulin enough?* J Pharmacol Exp Ther 2001;299:818–824.
30. Forstermann U, Boissel JP, and Kleinert H. *Expressional control of the 'constitutive' isoforms of nitric oxide synthase (NOS I and NOS III).* Faseb J 1998;12:773–790.
31. Govers, R., and Rabelink, T. J. *Cellular regulation of endothelial nitric oxide synthase.* Am J Physiol Renal Physiol

2001;280:F193–206.
32. Kamphoven JH, Stubenitsky R, Reuser AJ, Van Der Ploeg AT, Verdouw PD, and Duncker DJ. *Cardiac remodeling and contractile function in acid alpha-glucosidase knockout mice.* Physiol Genomics 2001;5:171–179.
33. Breslow JL. *Mouse models of atherosclerosis.* Science 1996;272:685–688.
34. Daugherty A. *Mouse models of atherosclerosis.* Am J Med Sci 2002;323:3–10.
35. Brown MS, and Goldstein JL. *A receptor-mediated pathway for cholesterol homeostasis.* Science 1986;232:34–47.
36. Gordon DJ, and Rifkind BM. *High-density lipoprotein--the clinical implications of recent studies.* N Engl J Med 1989;321:1311–1316.
37. Libby P. *Managing the risk of atherosclerosis: the role of high-density lipoprotein.* Am J Cardiol 2001;88:3N-8N.
38. Sessa WC. *Can modulation of endothelial nitric oxide synthase explain the vasculoprotective actions of statins?* Trends Mol Med 2001;7:189–191.
39. Feron O, Dessy C, Moniotte S, Desager JP, and Balligand JL. *Hypercholesterolemia decreases nitric oxide production by promoting the interaction of caveolin and endothelial nitric oxide synthase.* J Clin Invest 1999;103:897–905.
40. Feron O, Dessy C, Desager JP, and Balligand JL. *Hydroxy-methylglutaryl-coenzyme A reductase inhibition promotes endothelial nitric oxide synthase activation through a decrease in caveolin abundance.* Circulation 2001;103:113–118.
41. Everson WV, and Smart EJ. *Influence of caveolin, cholesterol, and lipoproteins on nitric oxide synthase: implications for vascular disease.* Trends Cardiovasc Med 2001;11:246–250.
42. Kincer JF, Uittenbogaard A, Dressman J, Guerin TM, Febbraio M, Guo L, and Smart EJ. *Hypercholesterolemia promotes a CD36-dependent and endothelial nitric-oxide synthase-mediated vascular dysfunction.* J Biol Chem 2002;277:23525–23533.
43. Hoit BD. *Two faces of nitric oxide: lessons learned from the NOS2 knockout.* Circ Res 2001;89:289–291.
44. Ozaki M, Kawashima S, Yamashita T, Hirase T, Namiki M, Inoue N, Hirata K, Yasui H, Sakurai H, Yoshida Y, Masada M, and Yokoyama M. *Overexpression of endothelial nitric oxide synthase accelerates atherosclerotic lesion formation in apoE-deficient mice.* J Clin Invest 2002;110:331–340.
45. d'Uscio LV, Baker TA, Mantilla CB, Smith L, Weiler D, Sieck G, and Katusic ZS. *Mechanism of endothelial dysfunction in apolipoprotein E-deficient mice.* Arterioscler Thromb Vasc Biol 2001;21:1017–102.

Chapter 5

Detrimental effect of combined exercise and eNOS-overexpression on cardiac remodeling and dysfunction after myocardial infarction

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Abstract

It has been reported that exercise following myocardial infarction (MI) attenuates left ventricular (LV) pump dysfunction by normalization of myofilament function. This benefit could be attributed to an exercise-induced up regulation of endothelial NO synthase (eNOS) expression and activity. Consequently, we first tested the hypothesis that the effects of exercise after MI can be mimicked by elevated eNOS-expression, using transgenic overexpressing mice for human eNOS (eNOSTg). Both exercise and eNOSTg attenuated LV remodeling and dysfunction after MI in mice, and improved cardiomyocyte maximal force development (F_{max}). However, only exercise training restored myofilament Ca^{2+}-sensitivity and SERCA2a protein levels, and improved LVdP/dt at 30 mmHg. Conversely, only eNOSTg improved survival. In view of these partly complementary actions we subsequently tested the hypothesis that combining exercise and eNOSTg would provide additional protection against LV remodeling and dysfunction after MI. Unexpectedly, the combination of exercise and eNOSTg abolished the beneficial effects on LV remodeling and dysfunction of either treatment alone. The latter was likely due to perturbations in the Ca^{2+}-household, as myofilament Fmax actually increased despite marked reductions in phosphorylation status of several myofilament proteins, whereas the exercise-induced increases in SERCA2a protein levels were absent in eNOSTg mice. Anti-oxidant treatment with N-acetylcysteine prevented these detrimental effects on LV function, while partly restoring phosphorylation status of myofilament proteins and further enhancing myofilament F_{max}. In conclusion, the combination of exercise and elevated eNOS-expression abolished the cardioprotective effects of either treatment alone after MI, which was the result of increased oxidative stress secondary to eNOS 'uncoupling'.

Introduction

Left ventricular (LV) remodeling after myocardial infarction (MI) is a compensatory mechanism that serves to restore LV pump function. Despite the apparent appropriateness of LV remodeling to maintain cardiac pump function early after MI, remodeling is an independent risk factor for the development of congestive heart failure.[1] The mechanism underlying the progression from LV remodeling to overt heart failure remains incompletely understood. In chapter 2 of this thesis we reported that exercise training (exercise) following MI attenuates LV pump dysfunction by normalization of myofilament function in mice.[2] The beneficial effects of exercise on cardiac function can be attributed, at least in part, to an exercise-induced up regulation of endothelial nitric oxide synthase (eNOS) expression and activity,[3] which is the major isoform of NOS that is expressed in coronary vascular endothelium and cardiac myocytes.[4] For example, an increase in NO bioavailability has been shown to improve many of the processes perturbed in LV remodeling, including angiogenesis,[5] cardiac fibrosis,[6] and hypertrophy.[7] Indeed, Jones *et al*,[8] reported that elevated expression of the human eNOS gene in the vascular endothelium in transgenic mice (eNOSTg) not only improved survival, but also blunted LV dysfunction and pulmonary congestion after MI. These beneficial effects were ascribed to a reduction in afterload, secondary to a lower peripheral vascular resistance,[9] thereby facilitating LV pump function, whereas global LV contractility was unchanged. In contrast, exercise improved global LV contractility with no beneficial effect on survival. These findings suggest that the beneficial effects of exercise and eNOSTg are only in part overlapping and thus partly complementary.

In view of the complementary mechanisms by which exercise and eNOSTg appear to exert beneficial effects we tested the hypothesis that combined treatment with exercise and eNOSTg exerts an added benefit, and investigated the underlying mechanisms. Contrary to our hypothesis, the results unexpectedly indicated that exercise and eNOSTg abolished the beneficial effects of either treatment alone on global cardiac function after MI. Since a shortage of substrate (L-arginine) and/or cofactors (tetrahydrobiopterin; BH4) of eNOS can result in 'uncoupling' of NOS thereby generating reactive oxygen species such as superoxide rather than NO,[10] we subsequently tested the hypothesis that the detrimental effects of combined exercise training and eNOSTg are the result of increased oxidative stress due to eNOS 'uncoupling'.

Materials and Methods

Experiments complied with The Guide for Care and Use of Laboratory Animals of

the National Institutes of Health (NIH Publication No. 85-23, Revised 1996) and were approved by the Erasmus MC Animal Care Committee.

Animals

A total of 159 eNOSTg mice and 223 Wild type (Wt) littermates in C57Bl/6J background of either sex (~12-weeks old) entered the study and were randomly assigned to one of eight experimental groups. In this study we presented our historical wild type data with additional new time-matched data for the wild type groups. Sham-operated mice (SH) and mice with MI were housed sedentary (SH_{SED}, MI_{SED}) or subjected to voluntary exercise training by wheel running (SH_{EX}, MI_{EX}) for 8-weeks. Treadmills were custom built to allow electronic measurement of the distance run by the mice. Voluntary EX was the training of choice due to its ability to achieve uniform controlled EX in mice and to minimize stress factors, which are present during forced running and particularly during swimming.[11] C57Bl/6J mice were used because they are known to perform excellent in voluntary wheel running.[11] Generation of eNOSTg mice has been previously described.[12,13] Briefly, a DNA fragment containing the human eNOS gene, ~6kb of 5'-natural flanking sequence, including the native eNOS promoter, and ~3kb of 3'-sequence to the gene was used for microinjection of fertilized oocytes. Transgenic offspring was backcrossed to C57Bl/6J for >10 generations. In three additional groups of eNOSTg MI_{EX} mice, the anti-oxidant N-acetylcysteine (NAC, n=11; Sigma), L-arginine (Sigma) without (n=10) or with (n=5) BH4 (Schircks Laboratories) was supplemented either in drinking water (1% NAC, 2.5% L-arginine) or administered by gavage (BH4: 80 mg/kg administered 3 times/week), over the entire 8-week follow-up period.

Experimental procedures

Mice were weighed, sedated with 4% isoflurane, intubated and pressure-controlled ventilated with O_2-N_2O (1:2, vol/vol) containing ~2.5% isoflurane for anesthesia. MI was produced by permanent ligation of the left-anterior-descending-coronary-artery (LAD) with a 7-0 silk suture (BBraun, Aesculap AG&CO. KG, Germany) as described before.[2,14] Sham animals underwent the operation without infarct induction. Eight-weeks after entering the study, hemodynamic measurements were performed under anesthesia as described before.[2] In brief, short and long axis view M-mode LV echocardiography (ALOKA, Prosound SSD-4000; Japan) was performed and LV diameters at end-diastole (LVEDD) and end-systole (LVESD) were measured, and fractional shortening calculated. A 1.4F microtipped pressure

transducer catheter (SPR-671, Millar Instruments, Houston, TX) was inserted into the LV and pressure-diameter relations were obtained from M-mode images synchronized with LV-pressure by simultaneous ECG recording. At the conclusion of each experiment, right (RVW) and left (LVW) ventricular weight, tibial-length (TL) and lung fluid weight were determined. Masson's trichrome-staining was used for analysis of LV collagen volume fraction in non-infarcted LV samples as described before.[2] Briefly, four fields were randomly selected in two sections of eight mice per group and photographed using an Olympus BH 20 microscope (Olympus Corporation, Japan) at a magnification of x400. Within each field, segments representing connective and muscle tissue were identified and manually traced with a digitizing pad and computer image analysis software (Clemex Vision PE 3.5) to calculate the traced area. Collagen volume fraction was calculated in each field as the sum of all connective tissue areas divided by the sum of all connective tissue and muscle areas and averaged for each animal per group.

Force measurements in single permeabilized cardiomyocytes

In the non-infarcted remote area of the myocardium isometric force was measured in single permeabilized cardiomyocytes of 5 mice per group at different [Ca^{2+}] and a sarcomere length of 2.2 μm as described before.[2,15] In brief, mechanically isolated myocytes were permeabilized in relaxing solution containing 0.5% Triton X-100 (5 minutes) a treatment which also removes soluble and membrane-bound kinases and phosphatases, which may alter the phosphorylation status of myofibrillar proteins. To remove Triton, cells were washed twice in relaxing solution. Subsequently, a single myocyte was attached between a force transducer and a piezoelectric motor. After obtaining a complete force-pCa series, myocytes were incubated in relaxing solution containing the exogenous catalytic subunit of protein-kinase A (PKA) and a second force-pCa series was obtained.

Myofilament protein phosphorylation status

To determine phosphorylation status of the myofilament proteins myosin binding protein-C (MyBP-C), troponin T (TnT), troponin I (TnI), myosin light chain 2 (MLC-2), desmin and tropomyosin, LV samples (n=6 per group) were separated on gradient gels and stained with Pro-Q Diamond phosphoprotein gel stain in conjunction with SYPRO Ruby staining of the gels as described before.[2] The phosphorylation signals for myofilament proteins were normalized to the intensities of the SYPRO Ruby stained MyBP-C bands and analyzed using the luminescent image analyzer las-3000 and Aida image analyzer.

Western blotting

Frozen LV tissue samples (n=5 per group) were homogenized and protein concentrations were determined as described before.[2] Blots were pre-incubated in Odyssey blocking buffer (LI-COR Biosciences) and incubated with diluted primary antibodies in blocking buffer containing 0.1% Tween-20. As described before, primary antibodies against phospholamban (PLB), SERCA2A, β_1-adrenergic receptor, and the phospho-serine (P-Ser16) or phospho-threonine (P-Thr17) containing sequence of PLB were used.[2] IRDye 800CW or IRDye 680 conjugated goat anti-rabbit or goat anti-mouse secondary antibody (LI-COR Biosciences) were used. For SERCA2A, phosholamban and phospho-phospholamban detection a rat cardiac membrane preparation was used as positive control. For β_1-adrenergic receptor detection a rat brain extract was used as positive control. Fluorescent signals were detected and quantified using the Odyssey Infrared Imaging System (LI-COR Biosciences).

Dihydroethidium fluorescence

Superoxide anion generation in non-infarcted remote myocardium was determined using dihydroethidium (DHE) fluorescence.[16] Of four mice per group the hearts were excised and LV tissue samples were washed in ice-cold saline, embedded in Tissue-Tek, frozen in liquid nitrogen cooled isopentane, and stored at –80°C. Tissue sections of 5 µm were cut using a cryostat, and stained with 10 µM DHE at 37°C for 30 min. Fluorescent images were obtained with a 585-nm long-pass filter. Generation of superoxide was demonstrated by red fluorescent labeling. Images were analyzed on a microscopy image analysis system (Clemex Technologies, Canada) to quantify the amount of fluorescence.

Statistics

Data were analyzed using two-way ANOVA, followed by post-hoc testing with Student-Newman-Keuls. Survival was analyzed by Kaplan-Meier method and log-rank (Mantel-Cox) test. Significance was accepted when $P<0.05$. Data are means ± SEM.

Results

Survival and exercise

Wt-MI_{SED} mice demonstrated 60% survival compared to Wt-SH_{SED} mice (Figure 1A), which significantly improved to 78% in eNOSTg-MI_{SED} mice. In contrast, exercise had no significant effect on survival in Wt-MI mice, and even tended

to increase mortality in eNOSTg-MI mice (P=0.063). Both Wt-MI_{EX} and eNOSTg-MI_{EX} mice initially ran shorter distances per day compared to their corresponding sham groups (Figure 1B), but neither MI nor eNOSTg significantly influenced total running distance over the 8-week period (Figure 1C).

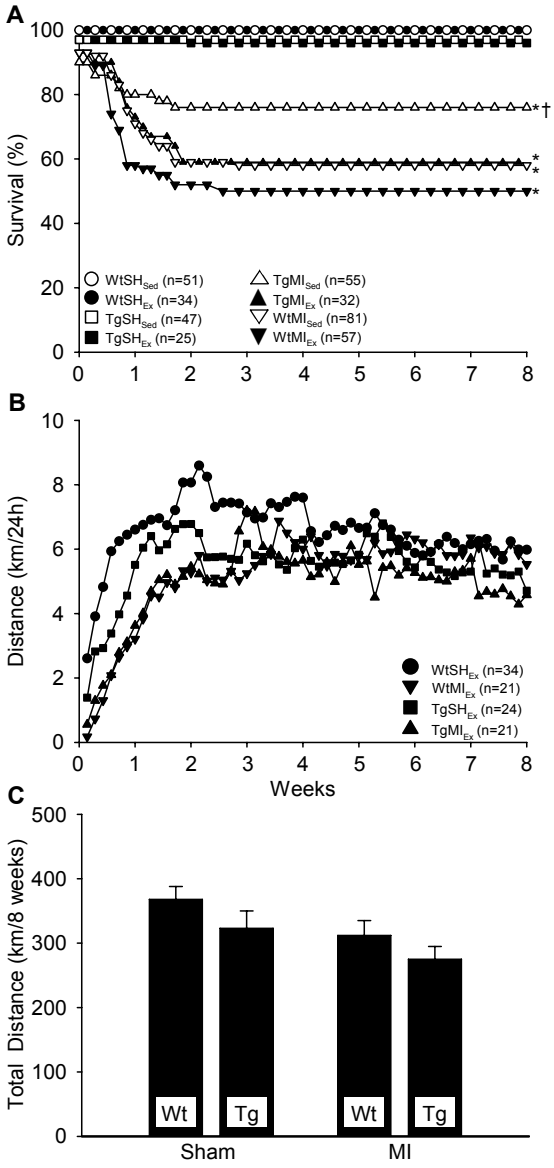

Figure 1:
Kaplan-Meier survival curves for all groups. Time-point zero represents the immediate postoperative survival of 100% in WtSH, 97% in TgSH, 93% in WtMI and 90% in TgMI mice (A). *P<0.05 versus corresponding Sham, †P<0.05 versus corresponding Wt. Daily running distance of mice that survived the entire 8-week follow-up period (B). Total running distance within 8-weeks (C). No significant differences were observed.

LV remodeling and global LV function

eNOSTg-SH_{SED} mice exhibited slightly lower mean aortic pressure (MAP) and LV systolic pressure (LVSP) levels, but had normal global LV function compared to Wt-SH_{SED} mice (Table 1). eNOSTg-SH mice exhibited a slightly larger LV diameter, reflected in the small rightward shift of the LV pressure-diameter relations, compared

to corresponding Wt-SH groups (Figure 2), but LV-, RV- and lung fluid weights were virtually identical (Table 1, Figure 3). Exercise did not affect LV geometry and weight, nor RV and lung fluid weight in either Wt-SH or eNOSTg-SH mice, although it reduced LV interstitial collagen content only in Wt-SH mice. Similarly, exercise had minimal effects on LV systolic and diastolic function in either Wt-SH or eNOSTg-SH mice, although levels of LV fractional shortening and LVdP/dtmin were slightly lower in eNOSTg-SH_{EX} compared to Wt-SH_{EX} mice (Table 1).

Table 1: LV Anatomical and Functional Data

		Sham		MI	
		Sed	Ex	Sed	Ex
Animal numbers	Wt	41	27	40	23
	eNOSTg	43	21	30	18
Body weight (g)	Wt	27±1	25±1	25±1	24±1
	eNOSTg	26±1	24±1	25±1	25±1
LV weight (mg)	Wt	97±3	94±2	113±2*	112±3*
	eNOSTg	98±3	98±4	117±4*	123±4*†
RV weight (mg)	Wt	26±1	25±1	33±1*	29±1*‡
	eNOSTg	27±1	26±1	32±1*	35±2*†
Heart rate (bpm)	Wt	526±6	515±11	514±7	523±7
	eNOSTg	531±5	529±6	529±9	496±10*†
MAP (mmHg)	Wt	75±2	80±3	71±2	71±2*
	eNOSTg	69±2†	66±3†	61±2*†	63±2†
LVSP (mmHg)	Wt	97±2	99±3	84±2*	85±2*
	eNOSTg	88±2†	83±2†	75±2*†	74±2*†
LV dP/dt_{P30} (mmHg/s)	Wt	7070±180	6960±210	4870±200*	5620±220*‡
	eNOSTg	6920±220	6280±340	4380±210*†	4280±240*†
Fractional shortening (%)	Wt	39±1	44±1	8±1*	12±1*‡
	eNOSTg	38±1	36±2†	12±1*†	9±1*†‡
LV dP/dt_{min} (mmHg/s)	Wt	-7520±370	-7860±440	-4650±240*	-4840±310*
	eNOSTg	-6780±280	-5560±400†‡	-4040±240*†	-3920±250*†
Tau (ms)	Wt	8.7±0.6	8.6±0.7	12.4±1.0*	13.5±1.1*
	eNOSTg	9.0±0.7	10.6±1.2	11.8±1.0*	12.3±1.2
LVEDP (mmHg)	Wt	6.9±0.6	4.6±0.6‡	9.8±0.9*	8.0±0.9*
	eNOSTg	5.6±0.6	6.6±1.1	8.5±1.1*	8.2±1.4

Wt,wild type; eNOSTg,eNOS transgenic; LV,left ventricle; RV,right ventricle; MAP,mean arterial pressure; LVSP,left ventricular systolic pressure; LVEDP,left ventricular end diastolic pressure. *P<0.05 vs corresponding sham; †P<0.05 vs corresponding Wt; ‡P<0.05 vs corresponding Sedentary.

eNOSTg had no effect on infarct size measured 24 hours after ligation (39±2% of the total LV area in 6 eNOSTg-MI_{SED} mice versus 43±3% in 8 Wt-MI_{SED}). Also, there was no effect of elevated eNOS-expression or exercise on infarct size eight weeks after LAD ligation as the infarct area was 18±1%, 20±1%, 19±1% and 18±1% of the LV total area in Wt-MI_{SED}, Wt-MI_{EX}, eNOSTg-MI_{SED} and eNOSTg-MI_{EX}, respectively. MI resulted in marked LV dilation (Figure 2), and LV hypertrophy, pulmonary congestion (increase in lung fluid weight), RV hypertrophy, and increased collagen content of the remote surviving LV myocardium in Wt-MI_{SED} mice (Figure 3), which were associated with depressed systolic and diastolic LV function (Table 1). LV remodeling and dysfunction was also observed in eNOSTg-MI_{SED} mice. However, in comparison to Wt-MI_{SED} mice, LV diameter and collagen

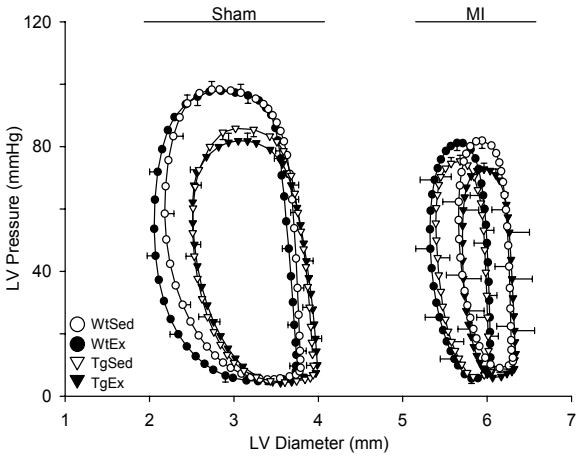

Figure 2:
Effects of MI and exercise on LV pressure-diameter relation in Wt and eNOSTg mice.

content remained significantly lower, while LV fractional shortening remained significantly higher, and pulmonary congestion was prevented in eNOSTg-MI_{SED} mice. Interestingly, LV dP/dt_{P30}, an index of global LV contractility, was slightly lower than in Wt-MI_{SED}, suggesting that the improved LV fractional shortening was the result of the lower aortic blood pressure in eNOSTg-MI_{SED} mice, rather than the result of improved LV contractility. Similar to eNOSTg, exercise had no effect on the MI-induced LV hypertrophy, but blunted LV dilation, interstitial fibrosis, pulmonary congestion and RV hypertrophy, and improved fractional shortening. Unlike eNOSTg, exercise had no effect on aortic blood pressure but improved $LVdP/dt_{P30}$. Despite these complementary actions, we unexpectedly observed that exercise of eNOSTg-MI mice aggravated LV remodeling, interstitial fibrosis, LV dysfunction, pulmonary congestion and RV hypertrophy (Table 1, Figure 2 and 3).

Force development in single permeabilized cardiomyocytes

Passive force was significantly lower in eNOSTg-SH_{SED} (2.0±0.3 kN/m^2) compared to Wt-SH_{SED} (3.0±0.4 kN/m^2) mice, but maximal isometric force development (F_{max}) and myofilament Ca^{2+}-sensitivity (pCa_{50}) were similar in both groups (Figure 3). Exercise had no effect on F_{max} or Ca^{2+}-sensitivity in Wt-SH mice, but increased F_{max} in eNOSTg-SH mice. Treatment with the catalytic subunit of PKA decreased Ca^{2+}-sensitivity to a similar extent in all four Sham groups (ΔpCa_{50}=0.060±0.003), indicating preserved PKA signaling in eNOSTg-SH_{SED}, Wt-SH_{EX}, and eNOSTg-SH_{EX} mice.

In Wt_{SED} mice, MI produced a reduction in F_{max} and an increase in Ca^{2+}-sensitivity. The latter was corrected by the addition of the catalytic subunit of PKA, indicating that the increase in Ca^{2+}-sensitivity resulted from reduced PKA signaling. eNOSTg in MI_{SED} was associated with a marked increase in F_{max}, but did not significantly

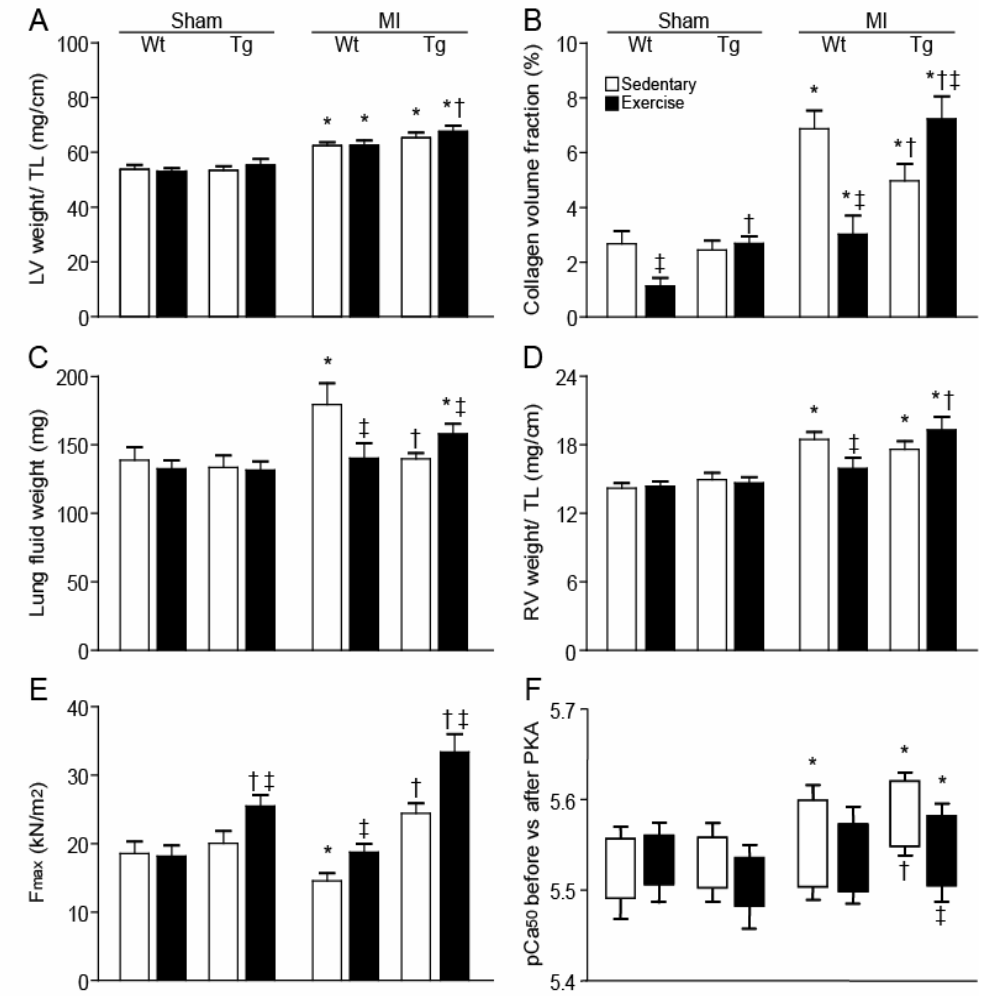

Figure 3:
Effects of MI and exercise in Wt and eNOSTg mice on relative LV mass (A), collagen volume fraction (B), lung fluid weight (C), relative RV mass (D), maximal force development (E), and Ca^{2+}-sensitivity before (top means) and after (bottom means) incubation with PKA (F). *P<0.05 versus corresponding Sham, †P<0.05 versus corresponding Wt, ‡P<0.05 versus corresponding Sedentary.

affect Ca^{2+}-sensitivity (P=0.27 versus Wt-MI_{SED}). Conversely, exercise in Wt-MI mice restored both F_{max} and Ca^{2+}-sensitivity to Sham levels. The combination of exercise and eNOSTg in MI mice increased F_{max} even further, while Ca^{2+}-sensitivity decreased to levels observed in Wt-MI_{EX} mice. PKA decreased Ca^{2+}-sensitivity in sedentary and exercise trained eNOSTg MI mice. However, even after treatment with PKA Ca^{2+}-sensitivity remained higher in eNOSTg-MI_{SED} compared to Wt-MI_{SED} mice. These findings suggest that, in contrast to Wt mice, loss of PKA-mediated protein phosphorylation does not contribute to the increased Ca^{2+}-sensitivity in eNOSTg mice after MI.

Myofilament protein phosphorylation status

There were no differences between Wt-SH_{SED}, Wt-SH_{EX}, eNOSTg-SH_{SED}, and eNOSTg-SH_{EX} mice with respect to phosphorylation status of several myofilament proteins (Table 2). Also, MI did not produce alterations in the phosphorylation status of myofilament proteins in either Wt_{SED} or $eNOSTg_{SED}$ mice. Exercise increased MLC-2 phosphorylation in Wt-MI mice. However, the combination of exercise and eNOSTg caused marked decrements in phosphorylation status of MyBP-C, TnI, desmin, MLC-2 and tropomyosin after MI.

Table 2: Myofilament-protein Phosphorylation Data

		Sham		MI	
		Sed	Ex	Sed	Ex
Animal numbers	Wt	6	5	7	7
	eNOSTg	7	7	5	5
MyBP-C (a.u.)	Wt	0.68±0.03	0.71±0.06	0.64±0.05	0.63±0.03
	eNOSTg	0.74±0.03	0.71±0.04	0.72±0.05	0.53±0.03*†‡
TnI (a.u.)	Wt	3.85±0.90	3.63±0.36	3.28±0.38	3.22±0.48
	eNOSTg	3.61±0.41	3.53±0.28	3.62±0.42	1.62±0.20*†‡
TnT (a.u.)	Wt	1.97±0.35	1.91±0.17	1.66±0.15	1.78±0.25
	eNOSTg	1.91±0.35	1.82±0.22	1.97±0.36	1.33±0.05
Desmin (a.u.)	Wt	1.15±0.19	1.19±0.09	0.94±0.11	1.22±0.14
	eNOSTg	1.01±0.14	1.10±0.09	1.11±0.18	0.63±0.14*†‡
MLC-2 (a.u.)	Wt	1.80±0.26	1.90±0.17	1.56±0.19	2.15±0.20‡
	eNOSTg	1.77±0.38	1.64±0.13	1.81±0.27	0.91±0.06*†‡
Tropomyosin (a.u.)	Wt	0.65±0.10	0.51±0.01	0.54±0.06	0.58±0.07
	eNOSTg	0.45±0.06	0.43±0.06	0.49±0.07	0.30±0.01†

Wt,wild type; eNOSTg,eNOS transgenic; MyBPC,myosin binding protein C; TnI,troponin I; TnT,troponin T; MLC,myosin light chain. *P<0.05 vs corresponding Sham; †P<0.05 vs corresponding Wt; ‡P<0.05 vs corresponding Sedentary.

Western blotting

Protein levels of β_1-adrenoceptors and SERCA2a were significantly lower in eNOSTg-SH compared with Wt-SH mice, while PLB and phosphorylation at either its Ser16 or Thr17 site were not significantly different (Figure 4). Exercise training had minimal effects on β_1-adrenoceptors, SERCA2a, PLB, SERCA2a/PLB ratio and PLB phosphorylation at the Thr17 site in both Wt-SH and eNOSTg-SH mice, but produced a small increase in PLB phosphorylation at the Ser16 site in Wt-SH.

MI resulted in a significant decrease in protein levels of β_1-adrenoceptors and SERCA2a, with no effect on PLB so that the SERCA2a/PLB ratio was lower in the remote surviving myocardium of Wt-MI_{SED} compared to Wt-SH_{SED} mice (Figure 4). Furthermore, PLB phosphorylation at the Ser16 increased, while phosphorylation at the Thr17 site decreased. In $eNOSTg_{SED}$ mice, MI did not produce further alterations in β_1-adrenoceptors and SERCA2a levels, and had no effect on PLB or its phosphorylation status. Exercise in Wt-MI mice normalized levels of β_1-

adrenoceptors and SERCA2a, with no effect on PLB or its phosphorylation. In contrast, exercise in eNOSTg-MI mice had no beneficial effect on calcium regulatory protein levels.

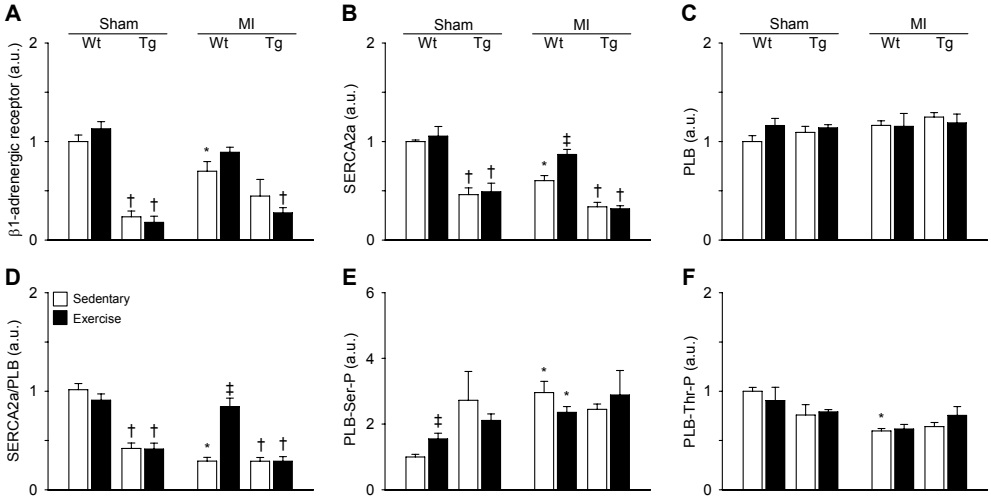

Figure 4:

Semi-quantitative Western blot analysis of Ca^{2+}-handling proteins and β_1-adrenergic receptor normalized to Wt-SH_{SED}. Western blot signals were quantified as described in Methods. *P<0.05 versus corresponding Sham, †P<0.05 versus corresponding Wt, ‡P<0.05 versus corresponding Sedentary.

Dihydroethidium fluorescence

DHE reacts with superoxide anions to form ethidium bromide, which, in turn, intercalates with DNA to provide nuclear fluorescence as a marker for superoxide anion generation. As shown in Figure 5 DHE fluorescence was enhanced in non-infarcted remote myocardium after MI compared with Sham mice. Exercise normalized the MI-induced increase in DHE fluorescence. The combination of exercise and eNOSTg after MI even further increased the DHE fluorescence, which was abolished by NAC treatment.

Effect of anti-oxidant, L-arginine and BH4 treatment

Adjunctive treatment of eNOSTg-MI_{EX} mice with the anti-oxidant NAC improved survival from 60% in eNOSTg-MI_{EX} without NAC to 85% (not shown; P<0.05), reversed LV remodeling, interstitial fibrosis, global LV dysfunction, pulmonary congestion and RV hypertrophy (Figure 6). In contrast, NAC treatment had no significant effect on LV weight and function in eNOSTg-SH_{EX} or Wt-MI_{SED} mice (data not shown). At the myofilament level, treatment of eNOSTg-MI_{EX} mice with NAC produced further increases in F_{max}, with no effect on myofilament Ca^{2+}-sensitivity, and partially reversed phosphorylation of myofilament protein MLC-2, tropomyosin and desmin. NAC had no effect on MyBP-C and TnI phosphorylation in eNOSTg-MI_{EX} mice.

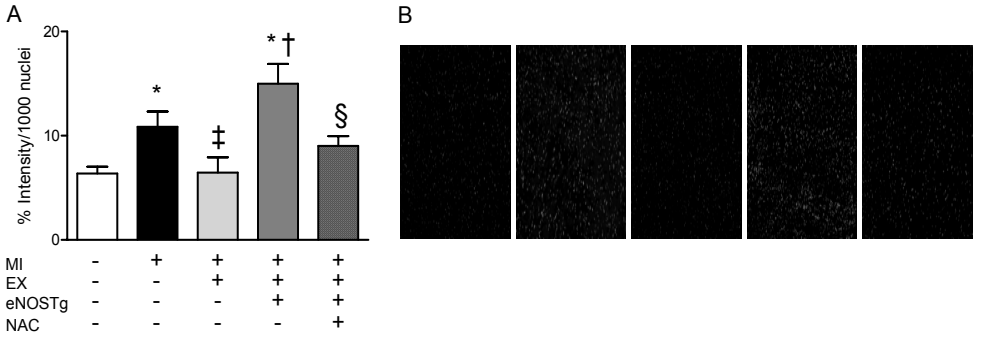

Figure 5:
Average intensity of dihydroethidium fluorescence per 1000 nuclei in non-infarcted remote myocardium (A), representative examples of dihydroethidium fluorescence (x200). Panels are in the same order from left to right as Figure 5A (B). **P*<0.05 versus Sham, †*P*<0.05 versus corresponding Wt, ‡*P*<0.05 versus corresponding Sedentary, §*P*<0.05 versus eNOSTg MI-mice without treatment with NAC.

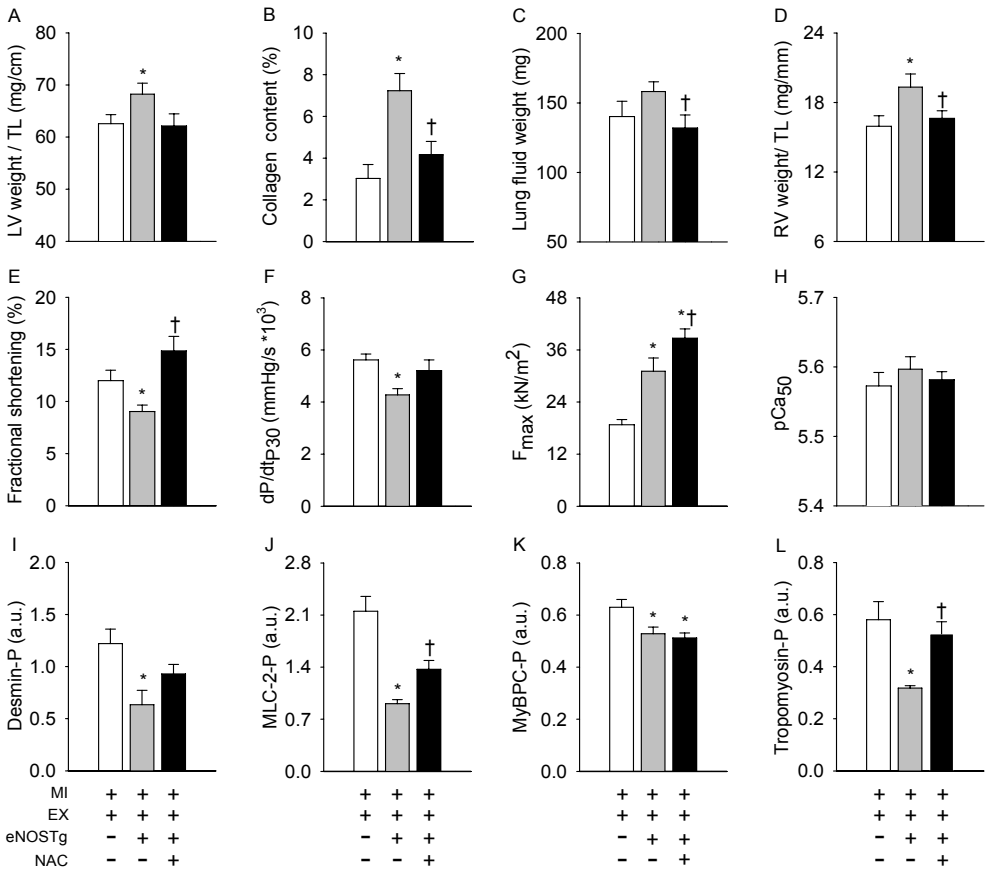

Figure 6:
Effects of anti-oxidant treatment in eNOSTg mice on relative LV mass (A), collagen content (B), lung fluid weight (C), relative RV weight (D), fractional shortening (E), dP/dt_{P30}, maximal force development (G), Ca^{2+}-sensitivity (H), phosphorylation of desmin (I), myosin light chain-2 (J), myosin binding protein-C (K) and tropomyosin (L). ***P*=0.056 versus Wt-MI_{EX}, **P*<0.05 versus Wt-MI_{EX}, †*P*<0.05 versus eNOSTg-MI_{EX}.

To investigate whether the increased oxidative stress resulted from a shortage of eNOS substrate and/or cofactor BH4, we investigated whether L-arginine supplementation alone or together with BH4 ameliorated the detrimental effects of combining exercise and eNOS-overexpression after MI (Table 3). Treatment of eNOSTg-MI_{EX} mice with L-arginine had no significant effects on relative LV mass, LV end-diastolic-diameter, lung fluid weight, relative RV mass, fractional shortening and dP/dt_{P30}, suggesting that substrate deficiency was not the cause of the increased oxidative stress. In contrast, when eNOSTg-MI_{EX} mice were treated with BH4 in addition to L-arginine, LV remodeling, dysfunction and backward failure were ameliorated. These observations suggest that deficiency of BH4 in these mice results in eNOS 'uncoupling' and thereby increased oxidative stress.

Table 3: Data from L-arginine and BH_4 Treatment

	Wt-MI_{EX}	eNOSTg-MI_{EX}	eNOSTg-MI_{EX}	eNOSTg-MI_{EX}
		No treatment	L-arginine	L-arginine + BH_4
LV weight/TL (mg/cm)	63±2	68±2*	70±3*	66±4
LV end-diastolic-diameter (mm)	5.8±0.2	6.4±0.3*	6.3±0.3*	5.5±0.2†
Lung fluid weight (mg)	140±11	158±7	152±11	115±4*†‡
RV weight/TL (mg/cm)	16±1	19±1*	20±1*	17±1
Fractional shortening (%)	12±1	9±1*	10±1*	15±2†‡
LV dP/dt_{P30} (mmHg/s)	5620±220	4280±240*	4550±370*	5210±270†

Wt,wild type; eNOSTg,eNOS transgenic; LV,left ventricle; TL,tibia length; RV,right ventricle.
*P<0.05 versus Wt-MI_{EX}; †P<0.05 versus eNOSTg-MI_{EX}; ‡P<0.05 versus eNOSTg-MI_{EX} + L-arginine.

Discussion

The present study investigated the impact of elevated eNOS-expression and exercise training on LV remodeling and dysfunction after MI in mice at the global left ventricular and cardiomyocyte level, either as single or combined therapy. The main findings were: *(i)* In mice with MI, single therapy of either exercise or elevated eNOS-expression improved global LV fractional shortening and cardiomyocyte force development, reduced collagen content and attenuated pulmonary congestion, whereas only eNOSTg improved survival; *(ii)* The combination of elevated eNOS-expression and exercise abolished the beneficial effects of either treatment alone after MI; *(iii)* These beneficial effects were restored by co-treatment with either the anti-oxidant N-acetylcysteine or the combination of L-arginine and BH4 supplementation. The implications of these findings will be discussed.

MI-induced LV dysfunction in mice

In agreement with previous observations, we observed that permanent LAD ligation in mice had resulted in significant LV remodeling eight weeks later, characterized by LV dilation, hypertrophy, and increased collagen deposition in remote surviving myocardium. LAD ligation also produced marked LV dysfunction, characterized by decrements in LV pump function (fractional shortening) and indices of global LV contractility (dP/dt_{P30}) and relaxation (dP/dt_{min} and τ), resulting in LV backward failure reflected in pulmonary edema and RV hypertrophy.[2,14,17] In agreement with observations in swine[15] and mice[2] we found that remodeling of non-infarcted myocardium was associated with altered myofilament function, characterized by decreased F_{max} and increased Ca^{2+}-sensitivity of tension development in single permeabilized cardiomyocytes.

Exercise training after MI

In agreement with a recent study from our laboratory,[2] we observed that in mice with a large MI (comprising ~43% of LV mass), exercise does not aggravate LV remodeling, as relative LV mass and infarct geometry were unchanged, whereas exercise reduced collagen content and tended to decrease LV end-diastolic diameter. Furthermore, exercise attenuated LV dysfunction and ameliorated LV backward failure. These beneficial effects probably result from an improved cardiomyocyte function, in particular an increased maximum force development (F_{max}), rather than improved calcium transients.

eNOS overexpression after MI

NO can modulate many of the processes involved in cardiac remodeling. For example, long term administration of nitrates (NO-donor compounds) limited LV remodeling in patients with a recent MI.[18] Conversely, eNOS deficient mice demonstrated exaggerated LV dilation and hypertrophy, and lower fractional shortening and dP/dt_{max}, four weeks after MI, suggesting that eNOS derived NO attenuates LV dysfunction and remodeling.[19] In the present study we investigated the effects of elevated eNOS-expression on global LV function and isolated cardiomyocyte function after MI. In agreement with a study by Jones *et al*,[8] we found that in mice overexpressing eNOS by 10 to 12-fold improved survival, global LV pump function, LV dilation and pulmonary edema after MI, and additionally observed that eNOSTg blunted interstitial fibrosis. Conversely, eNOSTg did not attenuate cardiac hypertrophy or improve global cardiac contractility and relaxation parameters. Interestingly, all of these improvements by elevated eNOS-expression occurred

without significant differences in baseline ventricular morphology or function in normal hearts. The only difference that was noted under basal conditions was that protein levels of β_1-adrenergic receptor and SERCA2a were markedly lower. These findings mirror the increase in β_1-adrenoceptor mRNA levels in eNOS$^{-/-}$ mice,[20] and the increase in SERCA2a protein expression in iNOS$^{-/-}$ mice.[21] Interestingly, S-nitrosylation of SERCA2a has been shown to increase its activity.[22] Thus, it could be speculated that an increased excitation-contraction coupling, arising from the elevated cardiomyocyte NO levels and subsequent protein S-nitrosylation, acted to maintain normal cardiac contractile function, in the face of lower protein expression levels of β_1-adrenergic receptor and SERCA2a.[23]

The mechanism underlying the improved survival and cardiac function in eNOSTg mice after MI remains unclear. Based on the lack of increase in indices of contractility or relaxation, it has been suggested that the beneficial effects of eNOSTg on the post-MI heart is principally due to an afterload reduction,[8] which occurs secondary to the lower systemic vascular resistance and hence lower aortic and LV systolic pressure.[9] To further investigate the effects of eNOSTg on cardiomyocyte function, we investigated myofilament function, myofilament protein phosphorylation status and performed Western blotting. Interestingly, we noted that similar to exercise training, elevated eNOS-expression increased maximal force development (F_{max}) of isolated permeabilized cardiomyocytes but that, in contrast to exercise, elevated eNOS-expression did not correct the high myofilament Ca^{2+}-sensitivity in MI, had no effect on MLC-2 protein phosphorylation, and on β_1-adrenergic receptor and SERCA2a protein levels. These findings suggest that in addition to the afterload reduction, an improvement in myofilament F_{max} could have contributed to eNOSTg-induced improvement in LV pump function after MI. The mechanism by which eNOSTg increased F_{max} remains to be determined, but it is of interest to note that Sun *et al* have recently shown that S-nitrosylation of myofilament proteins, including the myofilament protein alpha-myosin heavy chain, myosin light chain kinase 1 and myomesin, may be involved in the cardioprotection by ischemic preconditioning against ischemia-reperfusion induced sarcomeric damage in mice.[22,24] It remains to be investigated whether S-nitrosylation of myofilaments is altered and contributes to the enhanced myofilament contractility in our model.

Combination of exercise and eNOS overexpression: role of reactive oxygen species

The complementary actions of exercise and eNOSTg led us to hypothesize that the combination of exercise and eNOSTg could have an added benefit on LV remodeling

and function after MI. However, we unexpectedly observed that combining the two treatments abolished the beneficial effects of either modality alone. Thus, LV remodeling, LV pump dysfunction, LV backward failure and interstitial fibrosis were all aggravated compared to either treatment alone. The adverse effects occurred despite an increase in myofilament F_{max} and a maintained pCa_{50} suggesting that, despite the marked decreases in phosphorylation of myofilament proteins, including MyBP-C, TnI, desmin, MLC-2 and tropomyosin, overt myofilament dysfunction was not involved. The observations in the present study suggest that perturbations in β_1-adrenergic signaling and Ca^{2+}-household were involved in the loss of beneficial effect of exercise on LV dysfunction in eNOSTg-MI mice, which is supported by the lack of effect of exercise on β_1-adrenoceptors and particularly SERCA2a expression in eNOSTg-MI mice. In support of this notion, Ichinose and colleagues recently demonstrated in a model of sepsis induced cardiac dysfunction that cardiomyocyte-specific elevated eNOS-expression preserved Ca^{2+}-transients and myocardial contractile function.[25]

The adverse effects of combined exercise and eNOSTg on LV remodeling and function were reversed by the anti-oxidant NAC, demonstrating a role for reactive oxygen species (ROS) in the adverse effects. Interestingly, phosphorylation of myofilaments was partly restored and F_{max} further increased, suggesting that ROS-induced myofilament protein de-phosphorylation had blunted the increase in F_{max} produced by combined exercise and eNOSTg. Although the mechanism by which ROS induced de-phosphorylation of myofilament proteins remains to be determined, it is of interest to note that S-nitrosylation is an important inhibitor of phosphatase activity, including protein-phosphatase-1 (PP-1),[26,27] so that increased oxidative stress could have resulted in increased phosphatase activity. Accordingly, heart failure is associated with increased PKC protein content and activity,[28] and activated PP-1, as a result of which MLC-2 phosphorylation is decreased.[29] This effect is enhanced by increased ROS via activation of PKC.[30] Only recently, Vahebi *et al* showed that chronic activation of p38α MAPK in a transgenic mouse model reduced myofilament F_{max} in association with decreased tropomyosin phosphorylation.[31] As ROS has been shown to enhance p38α-MAPK signaling,[32] blocking of ROS with the anti-oxidant NAC and the concomitant enhancement of F_{max} might be explained, at least in part, by the restored tropomyosin phosphorylation. Besides these effects on myofilament function, ROS as well as NO can to directly influence function of the sarcoplasmic reticular ryanodine receptor, i.e the Ca^{2+}-release channel,[33] and SERCA2a.[22,34] These observation in conjunction with the unperturbed myofilament function in the present study, suggest that the increased oxidative stress affected

Ca^{2+}-household.

We hypothesized that increased ROS production was due to 'uncoupling' of eNOS, which may have resulted from shortage of substrate and co-factors,[35-37] as a result of elevated eNOS-expression in conjunction with exercise training.[38] Indeed, the combination of BH4 and L-arginine supplementation attenuated LV remodeling, dysfunction and backward failure in eNOSTg-MI_{EX} mice. Taking together our observations suggest that diminished levels of BH4 resulted in eNOS 'uncoupling' and thereby in elevated oxidative stress.

Clinical implications

The present study indicates that exercise and elevated eNOS-expression as single therapies attenuated LV remodeling, interstitial fibrosis and pulmonary congestion and improved global LV fractional shortening and cardiomyocyte force development, whereas only eNOS-overexpression improved survival in mice with a large MI. The combination of exercise and eNOS-overexpression abolished the beneficial effects of either treatment alone. This detrimental interaction was principally the result of increased oxidative stress, secondary to 'uncoupling' of eNOS. Several investigators have proposed a role for eNOS in patients with cardiovascular disease.[39,40] The results of the present study indicate that eNOS gene therapy in combination with physical exercise (as part of lifestyle changes) requires careful food supplementation of L-arginine and BH4 to avoid potential eNOS 'uncoupling' and increased oxidative stress.

References

1. Konstam MA, Udelson JE, Sharpe N. *Prevention and reversal of left ventricular remodeling: summation.* J Card Fail 2002;8:S506-511.
2. de Waard MC, van der Velden J, Bito V, Ozdemir S, Biesmans L, Boontje NM, *et al. Early exercise training normalizes myofilament function and attenuates left ventricular pump dysfunction in mice with a large myocardial infarction.* Circ Res 2007;100:1079-1088.
3. Sessa WC, Pritchard K, Seyedi N, Wang J, Hintze TH. *Chronic exercise in dogs increases coronary vascular nitric oxide production and endothelial cell nitric oxide synthase gene expression.* Circ Res 1994;74:349-353.
4. Massion PB, Balligand JL. *Modulation of cardiac contraction, relaxation and rate by the endothelial nitric oxide synthase (eNOS): lessons from genetically modified mice.* J Physiol 2003;546:63-75.
5. Murohara T, Asahara T, Silver M, Bauters C, Masuda H, Kalka C, *et al. Nitric oxide synthase modulates angiogenesis in response to tissue ischemia.* J Clin Invest 1998;101:2567-2578.
6. Kim NN, Villegas S, Summerour SR, Villarreal FJ. *Regulation of cardiac fibroblast extracellular matrix production by bradykinin and nitric oxide.* J Mol Cell Cardiol 1999;31:457-466.
7. Ritchie RH, Schiebinger RJ, LaPointe MC, Marsh JD. *Angiotensin II-induced hypertrophy of adult rat cardiomyocytes is blocked by nitric oxide.* Am J Physiol 1998;275:H1370-1374.
8. Jones SP, Greer JJ, van Haperen R, Duncker DJ, de Crom R, Lefer DJ. *Endothelial nitric oxide synthase overexpression attenuates congestive heart failure in mice.* Proc Natl Acad Sci U S A 2003;100:4891-4896.
9. van Deel ED, Merkus D, van Haperen R, de Waard MC, de Crom R, Duncker DJ. *Vasomotor control in mice overexpressing human endothelial nitric oxide synthase.* Am J Physiol Heart Circ Physiol 2007;293:H1144-1153.
10. Pou S, Pou WS, Bredt DS, Snyder SH, Rosen GM. *Generation of superoxide by purified brain nitric oxide synthase.* J Biol Chem 1992;267:24173-24176.
11. Bernstein D. *Exercise assessment of transgenic models of human cardiovascular disease.* Physiol Genomics 2003;13:217-226.
12. van Haperen R, de Waard M, van Deel E, Mees B, Kutryk M, van Aken T, *et al. Reduction of blood pressure, plasma cholesterol, and atherosclerosis by elevated endothelial nitric oxide.* J Biol Chem 2002;277:48803-48807.
13. van Haperen R, Cheng C, Mees BM, van Deel E, de Waard M, van Damme LC, *et al. Functional expression of endothelial nitric oxide synthase fused to green fluorescent protein in transgenic mice.* Am J Pathol 2003;163:1677-1686.
14. van den Bos EJ, Mees BM, de Waard MC, de Crom R, Duncker DJ. *A novel model of cryoinjury-induced myocardial infarction in the mouse: a comparison with coronary artery ligation.* Am J Physiol Heart Circ Physiol 2005;289:H1291-1300.
15. van der Velden J, Merkus D, Klarenbeek BR, James AT, Boontje NM, Dekkers DH, *et al. Alterations in myofilament function contribute to left ventricular dysfunction in pigs early after myocardial infarction.* Circ Res 2004;95:e85-95.
16. Prutz WA. *Inhibition of DNA-ethidium bromide intercalation due to free radical attack upon DNA. I. Comparison of the effects of various radicals.* Radiat Environ Biophys 1984;23:1-6.
17. van Rooij E, Doevendans PA, Crijns HJ, Heeneman S, Lips DJ, van Bilsen M, *et al. MCIP1 overexpression suppresses left ventricular remodeling and sustains cardiac function after myocardial infarction.* Circ Res 2004;94:e18-26.
18. *GISSI-3: effects of lisinopril and transdermal glyceryl trinitrate singly and together on 6-week mortality and ventricular function after acute myocardial infarction.* Gruppo Italiano per lo Studio della Sopravvivenza nell'infarto Miocardico. Lancet 1994;343:1115-1122.
19. Scherrer-Crosbie M, Ullrich R, Bloch KD, Nakajima H, Nasseri B, Aretz HT, *et al. Endothelial nitric oxide synthase limits left ventricular remodeling after myocardial infarction in mice.* Circulation 2001;104:1286-1291.
20. Barouch LA, Harrison RW, Skaf MW, Rosas GO, Cappola TP, Kobeissi ZA, *et al. Nitric oxide regulates the heart by spatial confinement of nitric oxide synthase isoforms.* Nature 2002;416:337-339.
21. Khan SA, Hare JM. *The role of nitric oxide in the physiological regulation of Ca^{2+} cycling.* Curr Opin Drug Discov Devel 2003;6:658-666.
22. Sun J, Morgan M, Shen RF, Steenbergen C, Murphy E. *Preconditioning results in S-nitrosylation of proteins involved in regulation of mitochondrial energetics and calcium transport.* Circ Res 2007;101:1155-1163.
23. Hare JM. *Nitric oxide and excitation-contraction coupling.* J Mol Cell Cardiol 2003;35:719-729.
24. Sun J, Steenbergen C, Murphy E. *S-nitrosylation: NO-related redox signaling to protect against oxidative stress.* Antioxid Redox Signal 2006;8:1693-1705.
25. Ichinose F, Buys ES, Neilan TG, Furutani EM, Morgan JG, Jassal DS, *et al. Cardiomyocyte-specific overexpression of nitric oxide synthase 3 prevents myocardial dysfunction in murine models of septic shock.* Circ Res 2007;100:130-139.
26. Berkowitz DE. *Myocyte nitroso-redox imbalance in sepsis: NO simple answer.* Circ Res 2007;100:1-4.
27. Yu CX, Li S, Whorton AR. *Redox regulation of PTEN by S-nitrosothiols.* Mol Pharmacol 2005;68:847-854.
28. Wang J, Liu X, Sentex E, Takeda N, Dhalla NS. *Increased expression of protein kinase C isoforms in heart failure due to myocardial infarction.* Am J Physiol Heart Circ Physiol 2003;284:H2277-2287.
29. Braz JC, Gregory K, Pathak A, Zhao W, Sahin B, Klevitsky R, *et al. PKC-alpha regulates cardiac contractility and propensity toward heart failure.* Nat Med 2004;10:248-254.
30. Gopalakrishna R, Jaken S. *Protein kinase C signaling and oxidative stress.* Free Radic Biol Med 2000;28:1349-1361.
31. Vahebi S, Ota A, Li M, Warren CM, de Tombe PP, Wang Y, *et al. p38-MAPK induced dephosphorylation of alpha-tropomyosin is associated with depression of myocardial sarcomeric tension and ATPase activity.* Circ Res 2007;100:408-415.
32. Sugden PH, Clerk A. *Oxidative stress and growth-regulating intracellular signaling pathways in cardiac myocytes.* Antioxid Redox Signal 2006;8:2111-2124.
33. Xu L, Eu JP, Meissner G, Stamler JS. *Activation of the cardiac calcium release channel (ryanodine receptor) by poly-S-nitrosylation.* Science 1998;279:234-237.

34. Viner RI, Williams TD, Schoneich C. *Nitric oxide-dependent modification of the sarcoplasmic reticulum Ca-ATPase: localization of cysteine target sites.* Free Radic Biol Med 2000;29:489-496.
35. Wever RM, van Dam T, van Rijn HJ, de Groot F, Rabelink TJ. *Tetrahydrobiopterin regulates superoxide and nitric oxide generation by recombinant endothelial nitric oxide synthase.* Biochem Biophys Res Commun 1997;237:340-344.
36. Landmesser U, Dikalov S, Price SR, McCann L, Fukai T, Holland SM, *et al. Oxidation of tetrahydrobiopterin leads to uncoupling of endothelial cell nitric oxide synthase in hypertension.* J Clin Invest 2003;111:1201-1209.
37. Cosentino F, Luscher TF. *Tetrahydrobiopterin and endothelial nitric oxide synthase activity.* Cardiovasc Res 1999;43:274-278.
38. Hambrecht R, Wolf A, Gielen S, Linke A, Hofer J, Erbs S, *et al. Effect of exercise on coronary endothelial function in patients with coronary artery disease.* N Engl J Med 2000;342:454-460.
39. Cooney R, Hynes SO, Sharif F, Howard L, O'Brien T. *Effect of gene delivery of NOS isoforms on intimal hyperplasia and endothelial regeneration after balloon injury.* Gene Ther 2007;14:396-404.
40. Kupatt C, Hinkel R, von Bruhl ML, Pohl T, Horstkotte J, Raake P, *et al. Endothelial nitric oxide synthase overexpression provides a functionally relevant angiogenic switch in hibernating pig myocardium.* J Am Coll Cardiol 2007;49:1575-1584.

Chapter 6

Cardiomyocyte-restricted over-expression of C-type natriuretic peptide prevents cardiac hypertrophy induced by myocardial infarction in mice

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Abstract

Infused C-type natriuretic peptide (CNP) was recently found to play a cardioprotective role in preventing myocardial ischaemia/reperfusion (I/R) injury and improving cardiac remodelling after myocardial infarction (MI) in rats. Our study aimed to investigate the effect of cardiomyocyte-specific CNP over-expression on I/R injury and MI in transgenic mice. We generated transgenic (TG) mice over-expressing CNP in cardiomyocytes. Elevated CNP expression on RNA and protein levels was demonstrated by RNase-protection assay and radioimmunoassay. Male TG mice and age-matched wild-type (WT) littermates were subjected to 1-hour global myocardial ischaemia and 23 h of reperfusion or permanent ligation of the coronary artery for 3 weeks. Infarct size did not differ between the WT and TG groups in mice subjected to I/R. In mice that underwent permanent ligation of coronary arteries, both left and right ventricular hypertrophy were prevented by CNP over-expression 3 weeks post-MI. Histological analysis revealed less necrosis, muscular degeneration and inflammation in infarcted TG mice. Impairment of cardiac function was less pronounced in transgenic animals than in the wild-type controls. Over-expression of CNP in cardiomyocytes does not affect I/R-induced infarct size but prevents cardiac hypertrophy induced by MI. Therefore, CNP may represent a potent therapeutic target for the treatment of patients with cardiac hypertrophy induced by myocardial infarction or other aetiology.

Introduction

Ischaemic heart disease is the most common cause of death in many countries around the world. Temporary or permanently stopped blood supply to the cardiomyocytes results in ischaemia/reperfusion (I/R) injury or myocardial infarction (MI). After MI, left ventricular (LV) remodelling occurs, in which the myocardium changes shape, size, and function in response to increased mechanical and neurohumoral stress. These adaptations include scar maturation and cardiac hypertrophy of remote myocardium to compensate for myocardial loss and increased wall stress[1,2] Despite the apparent appropriateness of this remodelling, it constitutes an independent risk factor for the progression from LV dysfunction to overt congestive heart failure.

C-type natriuretic peptide (CNP) belongs to the natriuretic peptide family, which consists of three structurally related peptides: atrial natriuretic peptide (ANP), brain natriureticpeptide (BNP), and CNP.[3] In contrast to ANP and BNP, which are produced mainly in the cardiac atria and ventricles, CNP is mainly released in an autocrine/paracrine fashion from endothelial cells as an endothelium-derived vasodilator.[4,5] The biological actions of natriuretic peptides are modulated by three different membrane-bound receptor subtypes, natriuretic peptide receptors A, B, and C (NPRA, NPRB and NPRC).[6] NPRA and NPRB are guanylyl cyclase (GC)-coupled receptors, which can convert guanosine triphosphate (GTP) to guanosine 3',5'-cyclic monophosphate (cGMP). NPRC is a single transmembrane receptor with a short 37-amino-acid intracellular tail that lacks GC activity but contains a pertussin toxin-sensitive Gi binding domain.[7] ANP and BNP are ligands of NPRA, while CNP preferentially binds to NPRB. NPRC has similar affinity to all three natriuretic peptides and is commonly considered a clearance receptor.[8,9]

Since Wei and colleagues first confirmed the presence of CNP within the myocardium by immunohistochemistry and radioimmunoassay,[10] accumulating evidence suggests that CNP exhibits important autocrine and paracrine functions within the heart and coronary circulation.[11] Hobbs *et al* demonstrated that, in addition to interacting with its receptor NPRB, endothelium-derived CNP is involved in the regulation of rat coronary circulation via the activation of NPRC.[12] They further showed that this newly defined CNP/NPRC pathway represents a protective mechanism against I/R injury in isolated perfused hearts, since infusion of CNP, either prior to or following ischaemic insult, resulted in a 30–50% reduction in the infarct size.[12] Furthermore, in vivo administration of CNP has been shown to improve cardiac function and attenuate cardiac remodelling after myocardial infarction in rats.[13] However, it is still not certain whether the cardiac CNP effects are

dependent on the endothelium or cardiomyocytes. Thus, we generated transgenic mice selectively over-expressing CNP in cardiomyocytes. This in-vivo model was used to investigate the effect of cardiomyocyte-specific CNP overexpression on I/R injury and MI.

Materials and Methods

Generation of transgenic mice

All animal experiments were done according to the guidelines of the Federal Law on the Use of Experimental Animals in Germany or guidelines provided by the animal committee of The Netherlands and were approved by the local authorities. cDNA encoding rat CNP (RNCNP) was cloned by RT-PCR, then ligated into an expression vector containing the α-myosin heavy chain (αMHC) gene promoter that directs transgene over-expression to cardiomyocytes[14] and the bovine growth hormone (BGH) polyadenylation signal. After linearizing the generated recombinant plasmid (pMHCRNCNP) by Bgl II/Bln I, a 2750-bp fragment was microinjected into the pronuclei of fertilized oocytes isolated from FVB/N mice according to the standard protocol. Chromosomal integration of the CNP transgene was proven by Southern blot analysis as described previously.[15] Ten µg of genomic DNA digested by EcoR I were used to screen for potential founder lines.

Genotyping of transgenic mice

Primers in the αMHC promoter region (MHC51: 5′-CAT CTG TCT CTA CTC TCT CTG CC-3′) and the CNP gene (RNCNP3: 5′-CCG CCT GGA GTC TTG TCA CC-3′) were used to perform PCR to detect the presence of the CNP transgene (35 cycles of denaturation at 94°C for 30 s, annealing at 59°C for 30 s, and extension at 72°C for 50 s with a hot-start at 94°C for 3 min, and a final extension at 72°C for 10 min). The PCR reactions were carried out in a total volume of 45 µl containing 1.25 U of BioTherm™ DNA polymerase, 2 mmol/L of $MgCl_2$, 0.26 mmol/L of dNTP (Rapidozym GmbH, Berlin, Germany), and 2 nmol/L of each primer (BioTeZ GmbH, Berlin, Germany).

RNase protection assay (RPA)

Total RNA was isolated from heart, lung, kidney, testis and brain using the TRIzol reagent (Invitrogen GmbH, Karlsruhe, Germany) with subsequent chloroform-isopropanol extraction according to the manufacturer's instructions. RNCNP, mouse BNP and collagen type III mRNA expression were identified by RPA using the Ambion RPA II kit (Ambion (Europe) Ltd., Huntingdon, U.K.) as described

elsewhere.[16] In brief, T7- or SP6-RNA polymerase transcribed radioactive antisense probes complementary to target mRNA (RNCNP: 405 bp; BNP 290 bp; collagen type III: 476 bp). RNA complementary to 127 nucleotides of rL32 mRNA was used as a positive control. Ten to 15 µg of each RNA sample was hybridized with approximately 40,000 cpm of RNCNP-, BNP- or collagen type III- and 40,000 cpm of rL32-radiolabeled antisense probe in the same reaction. The hybridized fragments protected from RNase A+T1 digestion were separated by electrophoresis on a denaturing gel (5% [w/v] polyacrylamide, 8M urea) and analyzed using a FUJIX BAS 2000 Phospho-Imager system (Raytest GmbH, Straubenhardt, Germany). Quantitative analyses were performed by measuring the intensity of the target bands normalized by the intensity of rL32.

Bioassay for CNP

After weighing the hearts, lungs and kidneys from both genotypes seven volumes of ice-cold 0.5 N acetic acid containing 0.01% Triton X-100 were added and homogenized by a T8 homogenizer (IKA GmbH, Staufen, Germany). The homogenates were boiled for 10 min, and then centrifuged at 15,000 rpm for 40 min at 4°C. The protein concentration of the supernatants was determined with the BCA protein assay kit (Perbio Science GmbH, Bonn, Germany). The supernatants or plasma were extracted using Sep-Pak C18 cartridges, and the eluates were lyophilized and dissolved in an assay buffer of a commercial radioimmunoassay kit for CNP (Immundiagnostik AG, Bensheim, Germany) as previously described.[17] CNP concentration was normalized by total protein concentration and expressed as pg/mg for organs and pg/ml for plasma.

cGMP measurement

Levels of cGMP in the ventricles and plasma were measured using a commercially available low-pH cGMP immunoassay kit (R&D Systems, Minneapolis, MN, USA). The frozen tissue was weighed and homogenized in 10 volumes of 0.1 N HCl. One hundred µl of supernatant were used to measure the cGMP concentration following the nonacetylationprocedure. cGMP concentration was expressed as pmol/mg for organs and pmol/ml for plasma. The sensitivity of the assay was approximately 0.6 pmol/ml.

Animal experimental protocols

Blood pressure measurement

Mice were sedated with 4% isoflurane, intubated and artificially ventilated with a

mixture of 70% room air and 30% oxygen including 2% isoflurane for anesthesia. A PE10 cannula was inserted into the left carotid artery to monitor systolic blood pressure (SBP), mean arterial pressure (MAP), diastolic blood pressure (DBP) and heart rate (HR) for 15 min using BMON software (TSE GmbH, Bad Homburg, Germany).

Cardiac surgery

Before starting instrumentation, the mice received 0.05 mg/kg analgesic (buprenorphine hydrochloride) subcutaneously, which was repeated once 12 h after surgery. Surgical techniques were employed according to Tarnavski *et al*[18] with some minor revisions. Briefly, mice were anaesthetized and artificially ventilated with a mixture of oxygen and N_2O [1:2 (vol/vol)] using a rodent ventilator, to which 2–2.5% isoflurane was added for anaesthesia.[19] The ventilation rate was set at 80 strokes/min, a peak inspiration pressure of 18 cm H_2O and a positive end expiration pressure of 4 cm H_2O. Mice were placed on a heating pad to maintain body temperature at 37°C. Regional I/R was produced by 1-hour occlusion of the left anterior descending coronary artery (LAD) with a sterile 7.0 silk suture followed by 23 h of reperfusion. Mice were then sacrificed and myocardial infarct size was measured.

In another group of mice, MI was produced by permanent ligation of the LAD. Echocardiographic and haemodynamic measurements were performed 3 weeks after MI, after which RV, LV and wet lung weights were obtained. One half of the LV was snap frozen in liquid nitrogen, and the other half was fixed in 4% paraformaldehyde for histological analysis.

Echocardiographic and haemodynamic analysis

Three weeks after MI, echocardiographic and haemodynamic measurements were performed as previously described.[19] M-mode echocardiograms of the LV with simultaneous electrocardiograph (ECG) (ProSound SSD- 4000, Aloka, Tokyo, Japan) were obtained using a 13-MHz probe. LV diameters were measured at end diastole (LVEDD) and end systole (LVESD), and fractional shortening was calculated as FS=(LVEDD−LVESD)/LVEDD×100%. After echocardiography, a polyethylene catheter (PE-10) was inserted into the left carotid artery and advanced into the aortic arch to measure mean aortic blood pressure (MAP). A 1.4-F-microtipped pressure transducer catheter (Millar Instruments, Houston, TX, USA) was inserted into the LV lumen via the right carotid artery to measure LV pressure. Subsequently, baseline recordings were obtained for MAP, HR and LV systolic pressure. In addition,

we measured the contractility parameter LV dP/dt_{max}, the afterload independent LV dP/dt_{P30} (positive LV dP/dt at LV pressure of 30 mmHg) and the relaxation parameters Tau (τ) and LV dP/dt_{min} [19]

Measurement of infarct size

After reperfusion, the ligature around the LAD was retied and 1 ml of 1% Evan's blue dye was injected into the jugular vein to delineate the area at risk (AAR). The heart was quickly excised, frozen for a few minutes at −20°C, and then immediately sliced with a scalpel into 1-mm-thick sections perpendicular to the long axis of the heart. Slices were incubated individually in 2% triphenyltetrazolium chloride (TTC) (Fluka, Buchs, Switzerland) in Sörensen buffer (pH 7.4) at 37°C for 5 min. Thereafter, the RV was removed, and each slice of LV was weighed and photographed on both sides. Evan's blue stained area, Evan's blue negative area (AAR), TTC stained area, and TTC stain negative area (infarct area) were digitally measured using SigmaScan (SPSS, Chicago, IL, USA). The infarct size was calculated according to the method of Kurrelmeyer *et al*,[20] and expressed as a percentage of infarct area (IA) over total AAR.

Histological analysis

Paraffin-embedded LV`s were sectioned along the short axis into 5-μm-thick slices. After staining with hematoxylineosin (H.-E.), muscular degeneration and inflammation (mononuclear inflammatory infiltrate) in the infarct area were evaluated by one pathologist who was blinded to the genotypes. Ladewig staining was performed to assess the degree of fibrosis in the infarcted area and the stains graded with 0× + to 4× +.

Data and statistical analysis

All data were expressed as mean±SEM. Differences between groups were determined using two-way ANOVA followed by post-hoc testing using Student's t test. Delta values were calculated by subtracting the mean of LV/tibia or RV/tibia in sham-operated mice from each sample in MI group and the difference in change between wild-type (WT) and transgenic (TG) lines was compared by an unpaired t-test. A value of $P<0.05$ was considered statistically significant.

Results

Generation and basic characterization of CNP transgenic mice

We generated transgenic mice over-expressing rat CNP. The transgene was driven

by the rat αMHC to ensure specific transgene over-expression in cardiomyocytes. The transgene integration in the chromosomal DNA was proven by Southern blot. Five independent founders 2, 3, 8, 9, and 12 were detected in the first litter (Figure 1A). Transgenic line 1 was identified from a second litter (data not shown). PCR confirmed the transgenic offspring of founders crossed with wild-type FVB/N mice. All lines were fertile and did not differ in body weight compared to the wild-type controls. To prove CNP transgene mRNA expression, RPA was used to investigate the ventricles of 3- to 5-month-old males of the transgenic lines 1, 2, 9, and 12 (data not shown). Since quantitative analysis showed that CNP mRNA expression was the highest in line 1 among the four lines, this line was selected for the experiments.

To investigate the ontogenetic regulation of CNP transgene expression, cardiac CNP mRNA of transgenic mice was determined at different time points. CNP transgene overexpression was detected at all investigated time points with no significant variation in aging (data not shown). To exclude ectopic expression of the

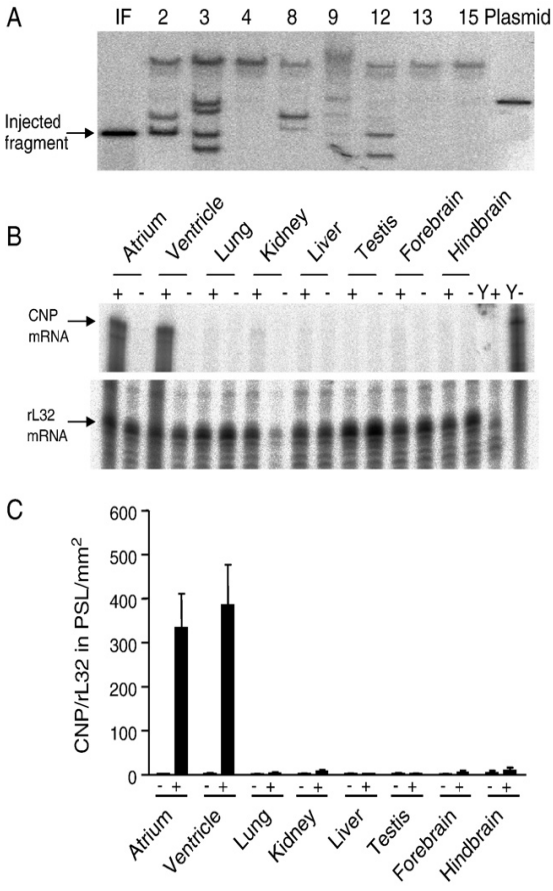

Figure 1. Generation and identification of transgenic mice over-expressing CNP mRNA.

(A) Chromosomal integration of the transgene was detected by Southern blot using EcoR I-digested genomic DNA isolated from tail biopsies of possible founders. Transgenic lines 2, 3, 8, 9, 12 were identified from this litter. Different length and number of bands in founder mice result from random integration of the transgenic cDNA in to the chromosomal DNA. IF: injected fragment, which was released through digestion by Bgl IIand Bln I. Plasmid: recombinant pMHCRNCNP plasmid linearized by EcoRI. (B) Representative RNase-protection assay showing CNP mRNA expression in different investigated organs of 3-month-old CNP1-transgenic(+) and wild-type (−) mice. Y+: yeast RNA with RNase treatment; Y−: yeast RNA without RNase treatment (C) Quantification of CNP transgene expression in different organs of CNP1 transgenic (+) and control (−) mice after autoradiographic signal analysis. Data are shown as multiples after normalization to rL32 mRNA levels, n=3.

transgene, CNP mRNA in the atrium, ventricle, lung, kidney, liver, testis, forebrain, and hindbrain was detected in 3-month-old male TG mice and their WT littermates using RPA (Figure 1B). As expected, high transgene CNP expression was detected in the atrium andventricle, whereas none of the other investigated organs showed detectable transgene expression (Figure 1C).

Peptide concentrations were measured in cardiac tissue, lung, kidney and plasma to confirm that CNP mRNA overexpression also led to more CNP generation. CNP levels were significantly increased in atria (WT: 0.15±0.02 versus TG: 0.30±0.07 pg/mg; $P<0.05$) and ventricles (LV: WT: 0.01± 0.01 versus TG: 0.74±0.12 pg/mg; RV: WT: 0.01±0.01 versus TG: 0.92±0.14 pg/mg; $P<0.001$) of transgenic mice. No change was found in the other organs or plasma (Figure 2A).

Due to its interaction with the NPRB receptor, which couples to guanylyl cyclase, CNP stimulates the generation of second messenger cGMP, an important mediator of effects initiated by natriuretic peptides. cGMP levels in ventricles and plasma were measured to test the hypothesis that CNP over-expression results in higher cGMP concentration. While the cGMP level was significantly increased in ventricles of transgenic mice (WT: 2.5±0.2 versus TG: 3.5± 0.2 pmol/mg; $P<0.05$), it did not differ in plasma in either of the two lines (Figure 2B). CNP-transgenic mice showed no change in cardiac weight as shown by the ratios of LVand RV to body weight (data not shown) or to tibia length (Table 1). Furthermore, CNP over-expression in cardiomyocytes did not modify blood pressure and heart rate compared to their age-matched wild-type littermates (data not shown).

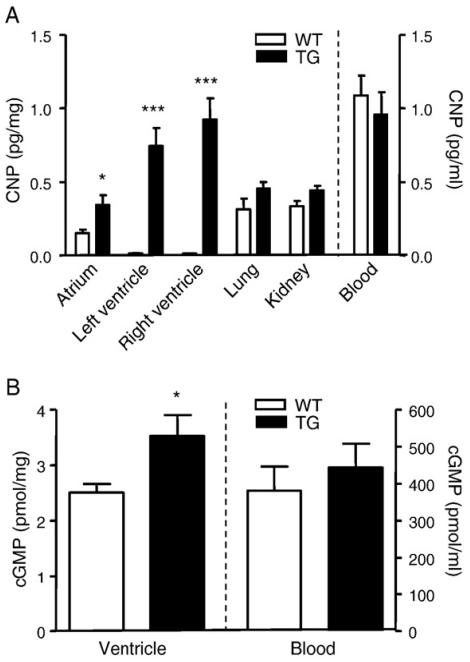

Figure 2. Characterization of CNP peptide expression and cGMP accumulation in transgenic mice.
(A) Quantification of CNP peptide concentration from atrium, ventricle, lung, kidney, and plasma of CNP1-transgenic (TG) and wild-type (WT) mice, analyzed by radioimmunoassay. *$P<0.05$, ***$P<0.001$ versus WT, n=6–8. (B) cGMP concentrations in the ventricles and plasma of CNP-transgenic (TG) and wild-type (WT) mice. *$P<0.05$ versus WT, n=7.

Effect of CNP on I/R injury

To assess impact of CNP over-expression on I/R injury, infarct size was measured after 1 h of regional myocardial ischaemia and 23 h of reperfusion. AAR/LV was similar in both groups (WT: 45±2% versus TG: 43±3%, n=9, per group). No significant difference was observed in myocardial infarct size as defined as the percentage of IA/AAR (WT: 68±8% versus TG: 69±7%, n=9) compared to wild-type controls. Consequently, the infarct size in relation to the left ventricle was also unaltered (WT: 31±4% versus TG: 30±3%, n=9).

Table 1: Characteristics of wild-type (WT) and transgenic (TG) mice 3 weeks after induction of myocardial infarction or sham operation

		WT		TG	
LVW/tibia length (mg/cm)	Sham	46 ± 2		47 ± 2	
	MI	55 ± 1	**	49 ± 1	†
RVW/tibia length (mg/cm)	Sham	13.9 ± 0.7		14.9 ± 0.8	
	MI	16.3 ± 0.8	*	13.5 ± 0.5	†
Lung wet weight (mg)	Sham	158 ± 7		169 ± 10	
	MI	198 ± 11	*	189 ± 10	
MAP (mm Hg)	Sham	77 ± 7		75 ± 5	
	MI	59 ± 4	*	68 ± 4	
LVSP (mm Hg)	Sham	93 ± 7		96 ± 5	
	MI	73 ± 3	*	79 ± 3	**
LVEDP (mm Hg)	Sham	3.8 ± 1.1		6.9 ± 1.0	
	MI	8.9 ± 1.9	*	9.3 ± 2.4	
HR (bpm)	Sham	487 ± 18		465 ± 19	
	MI	456 ± 24		487 ± 25	
LV EDD (mm)	Sham	4.2 ± 0.3		4.3 ± 0.3	
	MI	6.0 ± 0.1	***	5.5 ± 0.2	**
LV ESD (mm)	Sham	2.6 ± 0.3		2.8 ± 0.3	
	MI	5.2 ± 0.1	*	4.7 ± 0.2	*
LVED Wall thickness (mm)	Sham	1.25 ± 0.07		1.19 ± 0.08	
	MI	1.39 ± 0.09		1.23 ± 0.06	
LVES Wall thickness (mm)	Sham	1.51 ± 0.08		1.32 ± 0.14	
	MI	1.50 ± 0.08		1.37 ± 0.06	
LVED Wall thickness/diameter	Sham	0.30 ± 0.02		0.28 ± 0.01	
	MI	0.23 ± 0.01	*	0.23 ± 0.01	*
LVES Wall thickness/diameter	Sham	0.62 ± 0.08		0.53 ± 0.07	
	MI	0.29 ± 0.02	*	0.30 ± 0.02	*
LV dP/dtP30 (mm Hg/s)	Sham	6310 ± 413		6102 ± 349	
	MI	4370 ± 260	**	4555 ± 305	**
LV dP/dtmax (mm Hg/s)	Sham	7321 ± 437		6995 ± 403	
	MI	4755 ± 298	***	5450 ± 251	**
LV dP/dtmin (mm Hg/s)	Sham	-6984 ± 605		-6322 ± 687	
	MI	-3820 ± 313	***	-4321 ± 514	*
Tau (ms)	Sham	9.5 ± 1.3		10.1 ± 0.9	
	MI	13.2 ± 1.5		10.8 ± 1.4	
Fractional shortening (%)	Sham	38 ± 3		36 ± 2	
	MI	13 ± 1	***	16 ± 2	***

Date are given as mean±SEM. *P<0.05, **P<0.01, ***P<0.001 vs. corresponding sham; †P<0.01 vs. wild-type (WT) myocardial infarction (MI). MAP = mean arterial pressure, LVSP = left ventricular systolic pressure, LVEDP = left ventricular end-diastolic pressure, HR = heart rate, LV EDD = left ventricular end-diastolic dimension, LV ESD = left ventricular end-systolic dimension. n=7 (WT sham), 8 (WT MI), 6 (TG sham), 7 (TG MI).

Analysis of cardiac remodelling and function after myocardial infarction

Echocardiographic and haemodynamic measurements were performed to evaluate cardiac remodelling and function 3 weeks after MI induction. The LV weight/tibia length ratio did not differ for sham-operated WT and TG mice (WT: 46± 2 versus TG: 47±2 mg/cm) (Table 1). All other measured parameters were comparable in sham-operated wild-type and transgenic mice (Table 1). However, 3 weeks after MI, infarct-induced cardiac hypertrophy was prevented by CNP over-expression in cardiomyocytes. While LV mass was significantly increased in wild-type mice, no significant increase in LV weight was observed in mice with elevated CNP expression (ΔLV/tibia: WT: 8.6±1.2 versus TG: 1.8± 1.3 mg/cm; $P<0.01$) (Figure 3A). The RV/tibia length ratio was also significantly increased in wild-type mice (Table 1), but CNP over-expression in cardiomyocytes prevented MI induced RV hypertrophy (ΔRV/tibia: WT: 2.4±0.8 versus TG: 1.4±0.5 mg/cm; $P<0.01$) (Figure 3A). It should be noted that lung wet weight was significantly higher in wild-type mice after MI than in sham-operated mice (sham: 158±7 versus MI: 198±11 mg/g; $P<0.05$), while the increase was not statistically different in the TG line (sham: 169±10 versus MI: 189±10 mg/g) (Table 1).

Cardiac function was significantly impaired in both MI groups except for HR and Tau. Whereas MAP was significantly decreased after MI in the wild-type group as described for this experimental model,[21,22] it did not differ in transgenic mice (Table 1). Moreover, indices of LV systolic and diastolic function, as dP/dt_{max} and dP/dt_{min}, fractional shortening, and LVEDD indicated better performance in transgenic mice,

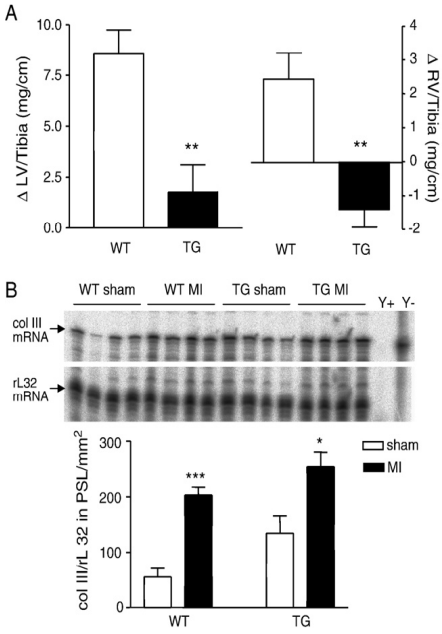

Figure 3. CNP over-expression in cardiomyocytes prevents cardiac hypertrophy.

(A) Left-ventricle (LV) and right-ventricle (RV) hypertrophy as indicated by ΔLV/tibia length and ΔRV/tibia length (mg/cm) were prevented in transgenic mice (TG). $^{**}P<0.01$ versus wild-type (WT) controls, n=7 (TG) or n=8 (WT). (B) Upper panel: RNase-protection assay showing collagen type III (col III) expression in non-infarcted left ventricles of TG and WT control mice 3weeks after myocardial infarction (MI) or sham operation. Lower panel: Quantification of col III expression in non-infarcted left ventricular myocardium normalized by rL32 mRNA levels.

although they did not reach statistical significance (Table 1).

Quantification of parameters of cardiac failure

To investigate whether CNP had other beneficial effects besides preventing cardiac hypertrophy, we examined mRNA expression levels of collagen type III and BNP, which are associated with fibrosis and cardiac failure. In the non-infarcted LV, mRNA levels of collagen type III were significantly elevated after MI in both groups without a significant inter-group difference. However, the increase in the transgenic group was less pronounced than in the wild-BNP mRNA, which is known to be upregulated in the ventricles under pathophysiological conditions like cardiac failure, was significantly elevated after MI in wild-type group, while the increase in the transgenic group did notreach statistical significance (data not shown).

Histological analysis

H.-E. staining was carried out to prove whether the prevented cardiac hypertrophy also had an impact on histological structure and inflammation after myocardial infarction. Normal cardiomyocytes were evident in both sham-operated lines (Figure 4A and B). However, there was prominent muscular degeneration and a moderate mononuclear inflammatory infiltration in the infracted area of wildtype mice 3 weeks after MI compared to only mild degeneration of myofibrils and no mononuclear inflammatory infiltrate in the transgenic group (Figure 4C and D).

Ladewig staining was used to assess the extent of cardiac fibrosis. No fibrosis was observed in sham-operated mice of either group (Figure 5A and B). Marked fibrosis (blue stain) was observed in the infarcted area of both groups that underwent myocardial infarction surgery. However, confirming the findings of H.-E. staining, there were more abundant muscle fibers (muscle = red stain) preserved in the TG group than in the wild-type controls (Figure 5C and D).

Discussion

In this study, we generated transgenic mice selectively over-expressing rat CNP in cardiomyocytes, we confirmed the over-expression on mRNA and peptide levels, and demonstrated that cardiomyocyte-restricted CNP over-expression does not reduce infarct size produced by 1-hour global myocardial ischaemia and 23-hour reperfusion, but prevents cardiac hypertrophy 3 weeks post-myocardial infarction.

CNP mediates most of its physiological actions (e.g. vasorelaxation) via interaction with its receptor NPRB. However, it was also recently identified as

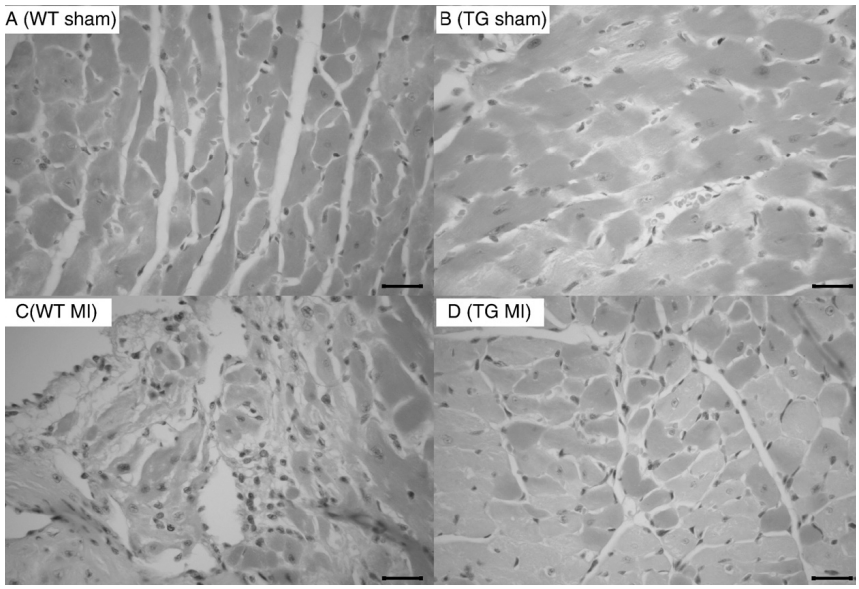

Figure 4. Cardiac morphological changes after myocardial infarction.

Normal-appearing cadiomyocytes were found in sham-operated wild-type (WT) and transgenic (TG) mice (A and B). There was prominent muscular degeneration and a moderate mononuclear inflammatory infiltrate in the infarcted area of wildtype animals (C), while only mild degeneration of myofibrils was observed in the infarcted transgenic group, and there is no mononuclear inflammatory infiltrate (D). Representative photographs stained with hematoxylin and eosin (H.-E.) are given. Bar=100 μm.

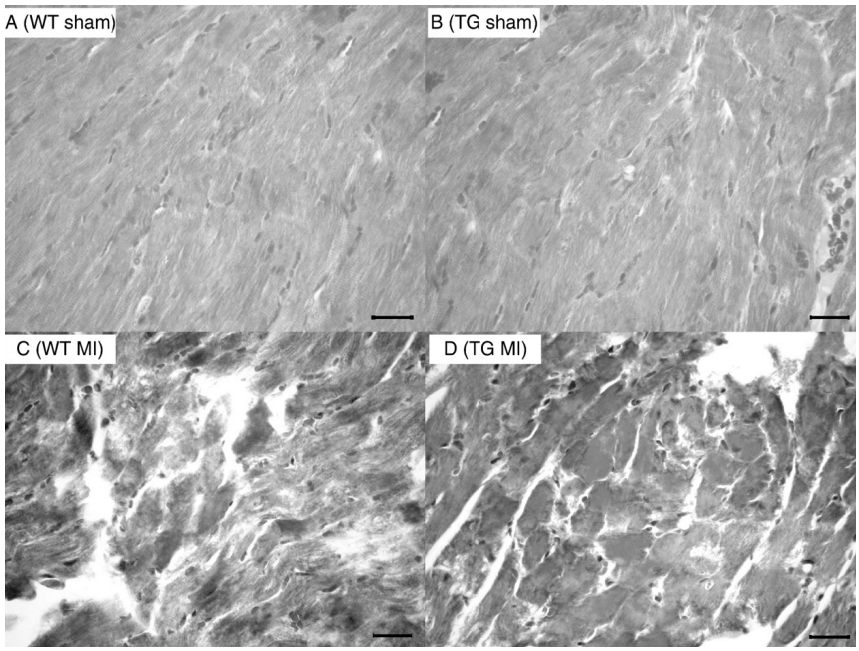

Figure 5. Histological analysis to determine the effect of CNP over-expression on cardiac fibrosis.

Normal myofibrils (red staining) were found in sham-operated wild-type (WT) and transgenic (TG) mice with no detectable fibrosis (blue staining) (A and B). Marked fibrosis and few muscle fibers were observed in the infarcted area of wild-type mice (C). Infarcted transgenic mice showed still marked fibrosis but more abundant myofibrils (D). Representative Ladewig staining photographs are given. Bar=100 μm.

an endothelium derived hyperpolarizing factor (EDHF) in rat mesenteric arteries involving activation of the clearance receptor NPRC.[23] This CNP/NPRC signalling pathway has been implicated in the regulation of coronary blood flow and the reduction of I/R-induced infarct size in isolated perfused Langendorff rat hearts.[12] In contrast to this described cardioprotective role of CNP against I/R, we did not observe a significant difference between transgenic mice over-expressing CNP in cardiomyocytes and their controls in our studies. Species-specific differences, independent experimental protocols, and unequal origin of CNP elevation may account for this discrepancy. Firstly, Langendorff preparations from rat hearts were used in previous studies, whereas we investigated transgenic mice *in vivo*. However, we do not consider this to be the main reason for the different findings, since the rat and mouse share similar signalling pathways in cardiac remodelling. Secondly, mice were subjected to 1 h of ischaemia and 23 h of reperfusion in our in vivo studies, but a protocol of 25 min of global ischaemia and 120 min of reperfusion was used in the previous *ex vivo* experiments. Thirdly, CNP originated from the cardiomyocytes in our transgenic animals and thus acted in an autocrine way; however, CNP was supplied intravascularly in retrogradely perfused hearts in the Hobbs study, thereby exposing the endothelium to the highest concentrations. Thus, the discrepancy between Hobbs *et al* and our findings could result from the need to stimulate NPRC on endothelial cells in order to mediate the beneficial effects of CNP in an I/R model, which did not occur in our transgenic mice.

Pre-ischaemic infusion of ANP or BNP has been shown to elicit protective effects against I/R in isolated rat hearts.[24,25] However, the possible mechanisms for the cardioprotective effects of the three natriuretic peptides on I/R seem to be controversial. NPRC activation is thought to be responsible for the cardioprotective role of CNP as mentioned above, and the selective agonist of NPRC (cANF4–23) also reduces infarct size and maintains coronary perfusion pressure.[12] Studies of ANP and BNP effects show the involvement of cGMP generated via the NPRA receptor in the reduction of infarct size.[24,25] Interestingly, increased cGMP supply in transgenic mice selectively over-expressing endothelial nitric oxide synthase (eNOS) in cardiomyocytes was also implicated in attenuating myocardial I/R injury in an *ex vivo* model.[26] Thus, although we can exclude the beneficial effect of CNP over-expression on I/R in our *in vivo* model based on infarct size measurement, the demonstrated cGMP increase in CNP-transgenic hearts may still indicate cardioprotective effects against I/R in an *ex vivo* model. Consequently, further studies need to evaluate the possible effects of CNP in different I/R models and their relevance under *in vivo* conditions.

Although we did not see beneficial effects of CNP on I/R injury in our *in vivo* model, cardiomyocyte-restricted overexpression of this peptide identified significantly improved cardiac remodelling 3 weeks after myocardial infarction. Both MI-induced LV and RV hypertrophy were prevented in our transgenic mice. The results provide clear *in vivo* evidence that CNP is a potent antihypertrophic agent after MI. This agrees in part with a previous report by Soeki and colleagues in rats.[13] *In vivo* administration of CNP via osmotic mini-pumps significantly improved cardiac function and led to less pronounced cardiac hypertrophy and fibrosis in rats 2 weeks post-MI.[13] In our present study, however, the improved cardiac remodelling in TG mice did not result in significantly improved cardiac function compared to their infarcted wild-type controls, although impairment of cardiac function compared to the sham group was less pronounced in transgenic mice than in the infarcted wild-type mice, probably due to inhibited cardiac remodelling. However, while Soeki *et al* applied CNP by mini-pump, we exclusively elevated cardiac CNP concentrations, showing for the first time that the local cardiac CNP mediates the antihypertrophic effects.

The prevention of hypertrophy by CNP over-expression in our transgenic mice that underwent myocardial infarction, agrees with data on the antihypertrophic effects of its receptor NPRB, as recently reported by Langenickel *et al.*[27] Transgenic rats expressing a dominant-negative mutant of NPRB (NPR-B ΔKC) developed progressive, blood pressure-independent cardiac hypertrophy.[27] Histological assessment and echocardiography revealed cardiac hypertrophy in these NPR-B ΔKC transgenic rats, which was aggravated with age accompanied by increasing cardiac markers of heart failure. Interestingly, there was no evidence for increased interstitial or perivascular fibrosis in these rats, supporting our finding of non-significant differences in the grade of stimulated fibrosis post-MI in CNP-transgenic mice compared to wild-type controls. Furthermore, chronic volume overload by an infrarenal aortocaval shunt in 8-week-old rats resulted in exaggerated cardiac hypertrophy in NPR-B ΔKC transgenic rats 6 weeks after surgery.[27] Although the affinity of CNP to NPRA is much less than to the NPRB receptor,[28] and CNP does not increase cGMP accumulation in cells expressing human NPRA,[11,29] we cannot finally exclude that transgenic CNP mediates part of its antihypertrophic effect via NPRA. Nevertheless, the combination of data from Langenickel *et al* and our findings provides clear evidence that the CNP/NPRB axis is implicated in the regulation of cardiomyocyte growth but not in cardiac fibrosis.

Furthermore, a limitation of Langenickel *et al*'s findings was the increased heart rate in the NPR-B ΔKC transgenic rats compared to controls, which was

possibly caused by reduced CNP actions on the central nervous system facilitating baroreflexes by downregulation of functional NPRB. Therefore, they could not discriminate whether central or peripheral alterations were responsible for the cardiac hypertrophy observed in this animal model. Since our transgenic mice did not show altered systemic blood pressure and heart rate and specifically over-express CNP in the heart, we can finally define the cardiac CNP/NPRB axis to be crucial in preventing cardiac hypertrophy.

Although we cannot conclusively prove exocytosis of transgene CNP to the extracellular space of cardiomyocytes, new research has shown that intracellular compartmentation of a peptide ligand allows interaction with its receptor and thus stimulation of receptor signalling.[30] Furthermore, that the CNP/NPRB axis is responsible for the cardiac CNP effects is also supported by cell-based studies. CNP modulates the growth, proliferation, and hypertrophy of smooth muscle cells, cardiomyocytes and fibroblasts,[31–33] and cGMP is implicated in all of these studies when CNP binds to its receptor NPRB. Additionally, our data on increased cGMP in the hearts of CNP-transgenic mice is congruent with findings in isolated rat and rabbit cardiomyocytes generating cGMP after CNP stimulation.[34,35]

The importance of the CNP/NPRB axis is furthermore illustrated by a series of studies about nitric oxide synthase, supporting the concept that cGMP, a second messenger not stimulated by interaction of CNP with NPRC, is a key player in the local regulation of cardiac remodelling and function after myocardial infarction.[36–39]

In conclusion, the present study indicates that the CNP/NPRB/cGMP signalling pathway plays an important role in the local regulation of cardiac hypertrophy under pathophysiological conditions. Thus, modulation of this pathway represents a potent new therapeutic target for the treatment of patients with myocardial infarction or hypertension-induced cardiac hypertrophy.

References

1. Sutton MG, Sharpe N. *Left ventricular remodeling after myocardial infarction: pathophysiology and therapy.* Circulation 2000;101:2981–8.
2. Pfeffer MA, Braunwald E. *Ventricular remodeling after myocardial infarction. Experimental observations and clinical implications.* Circulation 1990;81:1161–72.
3. Levin ER, Gardner DG, Samson WK. *Natriuretic peptides.* N Engl J Med 1998;339:321–8.
4. Ogawa Y, Itoh H, Nakao K. *Molecular biology and biochemistry of natriuretic peptide family.* Clin Exp Pharmacol Physiol 1995;22:49–53.
5. Walther T, Stepan H. *C-type natriuretic peptide in reproduction, pregnancy and fetal development.* J Endocrinol 2004;180:17–22.
6. Nakao K, Ogawa Y, Suga S, Imura H. *Molecular biology and biochemistry of the natriuretic peptide system. II: natriuretic peptide receptors.* J Hypertens 1992;10:1111–4.
7. Anand-Srivastava MB, Sehl PD, Lowe DG. *Cytoplasmic domain of natriuretic peptide receptor-C inhibits adenylyl cyclase. Involvement of a pertussis toxin-sensitive G protein.* J Biol Chem 1996;271:19324–9.
8. Maack T, Suzuki M, Almeida FA, *et al*. *Physiological role of silent receptors of atrial natriuretic factor.* Science 1987;238:675–8.
9. Nussenzveig DR, Lewicki JA, Maack T. *Cellular mechanisms of the clearance function of type C receptors of atrial natriuretic factor.* J Biol Chem 1990;265:20952–8.
10. Wei CM, Heublein DM, Perrella MA, *et al*. *Natriuretic peptide system in human heart failure.* Circulation 1993;88:1004–9.
11. D'Souza SP, Davis M, Baxter GF. *Autocrine and paracrine actions of natriuretic peptides in the heart.* Pharmacol Ther 2004;101:113–29.
12. Hobbs A, Foster P, Prescott C, Scotland R, Ahluwalia A. *Natriuretic peptide receptor-C regulates coronary blood flow and prevents myocardial ischemia/reperfusion injury: novel cardioprotective role for endothelium-derived C-type natriuretic peptide.* Circulation 2004;110:1231–5.
13. Soeki T, Kishimoto I, Okumura H, *et al*. *C-type natriuretic peptide, a novel antifibrotic and antihypertrophic agent, prevents cardiac remodeling after myocardial infarction.* J Am Coll Cardiol 2005;45:608–16.
14. Morkin E. *Control of cardiac myosin heavy chain gene expression.* Microsc Res Tech 2000;50:522–31.
15. Walther T, Balschun D, Voigt JP, *et al*. *Sustained long term potentiation and anxiety in mice lacking the Mas protooncogene.* J Biol Chem 1998;273:11867–73.
16. Stepan H, Leitner E, Bader M, Walther T. *Organ-specific mRNA distribution of C-type natriuretic peptide in neonatal and adult mice.* Regul Pept 2000;95:81–5.
17. Stepan H, Walther D, Faber R, Walther T. *Detection of C-type natriuretic peptide in fetal circulation.* J Perinat Med 2000;28:118–21.
18. Tarnavski O, McMullen JR, Schinke M, *et al*. *Mouse cardiac surgery: comprehensive techniques for the generation of mouse models of human diseases and their application for genomic studies.* Physiol Genomics 2004;16:349–60.
19. van den Bos EJ, Mees BM, de Waard MC, de Crom R, Duncker DJ. *A novel model of cryoinjury-induced myocardial infarction in the mouse: a comparison with coronary artery ligation.* Am J Physiol Heart Circ Physiol 2005;289: H1291–300.
20. Kurrelmeyer KM, Michael LH, Baumgarten G, *et al*. *Endogenous tumor necrosis factor protects the adult cardiac myocyte against ischemic-induced apoptosis in a murine model of acute myocardial infarction.* Proc Natl Acad Sci U S A 2000;97:5456–61.
21. Tschope C, Westermann D, Dhayat N, *et al*. *Angiotensin AT2 receptor deficiency after myocardial infarction: its effects on cardiac function and fibrosis depend on the stimulus.* Cell Biochem Biophys 2005;43:45–52.
22. Kinugawa S, Tsutsui H, Hayashidani S, *et al*. *Treatment with dimethylthiourea prevents left ventricular remodeling and failure after experimental myocardial infarction in mice: role of oxidative stress.* Circ Res 2000;87:392–8.
23. Chauhan SD, Nilsson H, Ahluwalia A, Hobbs AJ. *Release of C-type natriuretic peptide accounts for the biological activity of endotheliumderived hyperpolarizing factor.* Proc Natl Acad Sci U S A 2003;100:1426–31.
24. Okawa H, Horimoto H, Mieno S, *et al*. *Preischemic infusion of alphahuman atrial natriuretic peptide elicits myoprotective effects against ischemia reperfusion in isolated rat hearts.* Mol Cell Biochem 2003;248:171–7.
25. D'Souza SP, Yellon DM, Martin C, *et al*. *B-type natriuretic peptide limits infarct size in rat isolated hearts via KATP channel opening.* Am J Physiol Heart Circ Physiol 2003;284:H1592–600.
26. Brunner F,Maier R,AndrewP, *et al*. *Attenuation of myocardial ischemia/ reperfusion injury in mice with myocyte-specific overexpression of endothelial nitric oxide synthase.* Cardiovasc Res 2003;57:55–62.
27. Langenickel TH, Buttgereit J, Pagel-Langenickel I, *et al*. *Cardiac hypertrophy in transgenic rats expressing a dominant-negative mutant of the natriuretic peptide receptor B.* Proc Natl Acad Sci U S A 2006;103:4735–40.
28. Suga S, Nakao K, Hosoda K, *et al*. *Receptor selectivity of natriuretic peptide family, atrial natriuretic peptide, brain natriuretic peptide, and C-type natriuretic peptide.* Endocrinology 1992;130:229–39.
29. Koller KJ, Lowe DG, Bennett GL, *et al*. *Selective activation of the B natriuretic peptide receptor by C-type natriuretic peptide (CNP).* Science 1991;252:120–3.
30. Schuijt MP, Danser AH. *Cardiac angiotensin II: an intracrine hormone?* Am J Hypertens 2002;15:1109–16.
31. Furuya M, Yoshida M, Hayashi Y, *et al*. *C-type natriuretic peptide is a growth inhibitor of rat vascular smooth muscle cells.* Biochem Biophys Res Commun 1991;177:927–31.
32. Rosenkranz AC, Woods RL, Dusting GJ, Ritchie RH. *Antihypertrophic actions of the natriuretic peptides in adult rat cardiomyocytes: importance of cyclic GMP.* Cardiovasc Res 2003;57:515–22.

33. Horio T, Tokudome T, Maki T, *et al*. *Gene expression, secretion, and autocrine action of C-type natriuretic peptide in cultured adult rat cardiac fibroblasts.* Endocrinology 2003;144:2279–84.
34. Nir A, Zhang DF, Fixler R, *et al*. *C-type natriuretic peptide has a negative inotropic effect on cardiac myocytes.* Eur J Pharmacol 2001;412:195–201.
35. Zhang Q, Moalem J, Tse J, Scholz PM,Weiss HR. *Effects of natriuretic peptides on ventricular myocyte contraction and role of cyclic GMP signaling.* Eur J Pharmacol 2005;510:209–15.
36. Janssens S, Pokreisz P, Schoonjans L, *et al*. *Cardiomyocyte-specific overexpression of nitric oxide synthase 3 improves left ventricular performance and reduces compensatory hypertrophy after myocardial infarction.* Circ Res 2004;94:1256–62.
37. Liu YH, Carretero OA, Cingolani OH, *et al*. *Role of inducible nitric oxide synthase in cardiac function and remodeling in mice with heart failure due to myocardial infarction.* Am J Physiol Heart Circ Physiol 2005;289:H2616–23.
38. Jones SP, Greer JJ, van Haperen R, *et al*. *Endothelial nitric oxide synthase overexpression attenuates congestive heart failure in mice.* Proc Natl Acad Sci U S A 2003;100:4891–6.
39. Takimoto E, Champion HC, Li M, *et al*. *Chronic inhibition of cyclic GMP phosphodiesterase 5A prevents and reverses cardiac hypertrophy.* Nat Med 2005;11:214–22.

Chapter 7

Prior exercise training improves survival, infarct healing and left ventricular function after myocardial infarction

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Submitted

Abstract

Regular physical activity in patients with established coronary heart disease reduces the incidence of cardiac events. However, there is limited information on the effects of regular physical activity prior to an acute myocardial infarction (MI) on mortality, left ventricular (LV) remodeling and dysfunction after MI. Consequently, we investigated the effects of exercise training prior to an acute MI on survival and LV remodeling and dysfunction, and investigated whether exercise prior and following MI provides superior protection compared to either exercise intervention alone. C57Bl/6J mice were subjected to voluntary exercise or sedentary housing. Two weeks later MI was induced, after which exercise was either stopped (EX-MI-SED, SED-MI-SED) or continued (EX-MI-EX, SED-MI-EX). Exercise initiated after MI had no effect on survival, infarct size and LV remodeling, but reduced fibrosis and apoptosis in remote myocardium and attenuated global LV dysfunction, and decreased LV backward failure. When exercise was exclusively performed prior to MI, post-MI mortality decreased from 40% in SED-MI-SED to 17% in EX-MI-SED. Furthermore, infarct area was thicker accompanied by thickening of the subendocardial rim of surviving cardiomyocytes, while fibrosis and apoptosis of the remote region and global LV systolic dysfunction and pulmonary congestion were blunted. Surprisingly, when exercise training was initiated prior to MI and continued after MI, the ameliorating effects on LV dysfunction and pulmonary congestion of either pre-MI or post-MI exercise regimen alone were lost. The latter may in part be related to the markedly increased daily exercise load in the first week post-MI in EX-MI-EX versus SED-MI-EX mice.

Exercise either prior to MI or after MI both blunted LV dysfunction, while exercise prior to MI also improved survival likely due to improved scar healing. These results demonstrate that even when regular physical activity fails to prevent an acute MI it can still act to improve cardiac function and survival after MI.

Introduction

Physical inactivity has been proposed to be an independent risk factor for cardiovascular disease.[1,2] Indeed, prospective epidemiological data indicate that moderate (e.g. walking) and vigorous exercise in healthy subjects are associated with substantial reductions in the incidence of cardiovascular events.[3,4] The beneficial effects of exercise extends to patients with established coronary heart disease in which regular physical activity also reduces the incidence of cardiac events and all-cause mortality.[5] Furthermore, there is evidence that exercise training initiated after a cardiac event, such as myocardial infarction (MI), ameliorates left ventricular (LV) remodeling and dysfunction (chapter 2 of this thesis)[6-13] and clinical outcome.[14,15]

In contrast, there is substantially less information available as to whether prior exercise training affords any protection in situations where, despite regular exercise, a major cardiovascular event like MI does occur. Animal studies suggest that prior exercise can precondition the myocardium, thereby protecting the heart against irreversible damage produced by ischemia-reperfusion in rats[16-19] and dogs.[20] In addition, prior exercise training may modulate post-infarct remodeling independent of any myocardial preconditioning effect. Thus, two studies in rats using a permanent coronary artery ligation (in which preconditioning cannot limit acute myocardial necrosis[21,22] showed that infarct size and, likely as a consequence, LV remodeling were reduced by prior swim training.[23,24] The authors ascribed the reduction in infarct size to the observed increase in myocardial vascularization. An increased myocardial vascularity is indeed observed in swim trained rats.[25] In contrast, myocardial vascularization following treadmill running is not consistently associated with increased vascularization or enhanced collateral blood flow.[25] These different effects of swimming versus running may well be explained by the markedly different hemodynamic responses in rodents to swimming versus running.[26] Furthermore, forced swim training often results in diving bradycardia and intermittent hypoxia[27] when animals are submerged, both of which are known to stimulate angiogenesis.[28,29] In contrast, studies into the effects of prior exercise training by treadmill running on LV remodeling after MI have not been performed to date. Consequently, the present study was undertaken to test the hypothesis that exercise training prior to an acute MI is associated with improved survival and attenuated LV remodeling and dysfunction after MI. For this purpose we used a voluntary treadmill exercise training protocol that has negligible effects on LV geometry and function per se, but attenuates LV and myocardial dysfunction when initiated after MI produced by a permanent coronary artery ligation.[13]

Materials and Methods

Experiments complied with The Guide for Care and Use of Laboratory Animals of the National Institutes of Health (NIH Publication No. 86-23, Revised 1996) and were approved by the Erasmus MC Animal Care Committee.

Animals

A total of 123 wild type C57Bl/6J mice of either sex (~12-weeks old) entered the study and were randomly assigned to one of five experimental groups. Two weeks before induction of MI mice were either sedentary housed or exposed to voluntary exercise training by wheel running, where after the exercise training protocol was either stopped (SED-MI-SED, EX-MI-SED) or continued (SED-MI-EX, EX-MI-EX) for 8-weeks. Sham-operated mice (Sham) were used as controls.

Experimental procedures

All experimental procedures have previously been published in detail.[13,30] In brief, mice were weighed, sedated with 4% isoflurane, intubated and pressure-controlled ventilated with O_2/N_2 (1:2, vol/vol) containing ~2.5% isoflurane for anesthesia. MI was produced by permanent ligation of the left-anterior-descending-coronary-artery (LAD) with a 7-0 silk suture (BBraun).[13,30] Sham animals underwent the operation without infarct induction. Eight-weeks after entering the study, hemodynamic measurements were performed under anesthesia. M-mode LV echocardiography (Prosound SSD-4000, ALOKA) was performed and LV diameters at end-diastole (LVEDD) and end-systole (LVESD) were measured, and LV fractional shortening calculated.[13,30] A 1.4F microtipped pressure transducer catheter (SPR-671, Millar Instruments) was inserted into the right carotid artery and advanced into the LV for measurement of LV pressure and its first derivative dP/dt.[13,30]

After sacrifice, right ventricular (RV) and LV weight, tibial-length (TL) and lung fluid weight were determined. The LV (n=10/group) was fixed overnight in freshly prepared 4% paraformaldehyde in PBS and embedded in paraffin and used for histological analysis (see below). The operator was blinded to the experimental group during the analysis.

Infarct remodeling

The LV was cut along the long axis into 4μm sections and Masson's trichrome-staining was used for analysis of the infarct region. Endocardial infarct circumference was demarcated and its length determined. Minimal infarct thickness was measured at the shortest distance between endocardium and epicardium. Infarct area

was demarcated and the area measured. In the infarct area at four places the subendocardial rim of surviving cardiomyocytes and the total infarct thickness were measured and averaged. The percentage cardiomyocyte thickness of the total infarct thickness was calculated. The operator was blinded to the experimental group during the analysis.

Remodeling of the remote myocardium

Capillary density

Capillaries were detected using lectin staining. Briefly, short-axis LV paraffin sections were deparaffinized, cleared and hydrated to tris-buffered saline, pH 7.4, using a descending series of ethanol. Sections were treated with trypsin, pH 7.0, for 20 min at 37°C, washed and treated with hydrogen peroxide 3% for 10 min at room temperature to inhibit the endogenous peroxide. Sections were incubated overnight at 4°C with peroxidase-conjugated lectin from Bandeira simplicifolia (Sigma). The horseradish peroxidase was activated by incubation for 1-2 min with a diaminobenzidine commercial kit (Dako). Sections were washed, counterstained with haematoxillin, dehydrated with graded ethanol solutions, cleared in xylene and mounted. Capillaries were detected as brown endothelial cells and capillary density was determined as the number of vessels per mm^2.

Cardiomyocyte cross-sectional area and collagen content

Four micron thick paraffin LV sections were stained with Masson's trichrome for analysis of collagen content and cardiomyocyte cross-sectional area (CSA) measurements within the remote non-infarcted myocardium. Four fields were randomly selected in two sections of eight mice per group and photographed using an Olympus BH 20 microscope (Olympus Corporation) at a magnification of x400. Within each field, segments representing connective and muscle tissue were identified and manually traced with a digitizing pad and computer image analysis software (Clemex Vision PE 3.5) to calculate the traced area. Collagen content was calculated in each field as the sum of all connective tissue areas divided by the sum of all connective tissue and muscle areas and averaged for each animal. Cardiomyocyte CSA was measured by tracing the outline of cardiomyocytes showing the nucleus in each field and averaged for each animal.

Apoptosis

To localize DNA degradation in remote non-infarcted LV, we used the In Situ Cell Death Detection Kit (Roche Diagnostics, Germany), which is a modification of the

TUNEL assay (3' terminal deoxynucleotide transferase (TdT)-mediated dUTP nick end labeling). In brief, paraffin sections were deparaffinized, cleared and hydrated to phosphate-buffered saline (PBS), pH 7.4, using a descending series of ethanol. Sections were treated with Proteinase-K for 30min at 37°C, washed in PBS, treated with the TUNEL reaction mixture and incubated in the dark for 1h at 37°C, followed by washes in PBS. Both positive (DNaseI-treated sections) and negative controls (no TDT included in the reaction mixture) were included. Sections were covered with Vectashield including DAPI and directly photographed using fluorescence microscopy. TUNEL positive cells emit red fluorescence and all nuclei were blue. Apoptosis was determined as the number of TUNEL-positive nuclei per 10^5 nuclei.

Statistics

Data were analyzed using two-way ANOVA, followed by post-hoc testing with Student-Newman-Keuls. Survival was analyzed by Kaplan-Meier method and log-rank (Mantel-Cox) test. Significance was accepted when $P<0.05$. Data are mean±SEM.

Results

Survival and exercise

SED-MI-SED mice demonstrated 60% survival compared to Sham mice (Figure 1A), which significantly improved to 83% in EX-MI-SED mice where exercise was started 2 weeks before MI ($P<0.05$). In contrast, exercise after MI had no significant effect on survival. After induction of MI SED-MI-EX mice initially ran significantly shorter distances per day compared to EX-MI-EX mice, in which exercise had already been started 2 weeks prior to MI (Figure 1B), so that over the 8 weeks post-MI phase, EX-MI-EX mice ran a total of 456±35 km compared to 313±23 km in SED-MI-EX mice ($P<0.05$).

Global LV function

MI resulted in marked LV remodeling, reflected in LV hypertrophy and LV dilation, and LV dysfunction, reflected in lower LV systolic pressure, $LVdP/dt_{P30}$, fractional shortening (FS) and $LVdP/dt_{min}$ and higher Tau and LV end-diastolic pressure compared to Sham mice (Table 1, Figure 2). The LV dysfunction resulted in pulmonary congestion (increase in lung fluid weight and RV hypertrophy). Exercise after MI had no effect on LV remodeling, but improved $LVdP/dt_{P30}$ and fractional shortening, and alleviated pulmonary congestion. Similarly, exercise training prior to MI had no effect on LV remodeling but also improved $LVdP/dt_{P30}$ and fractional

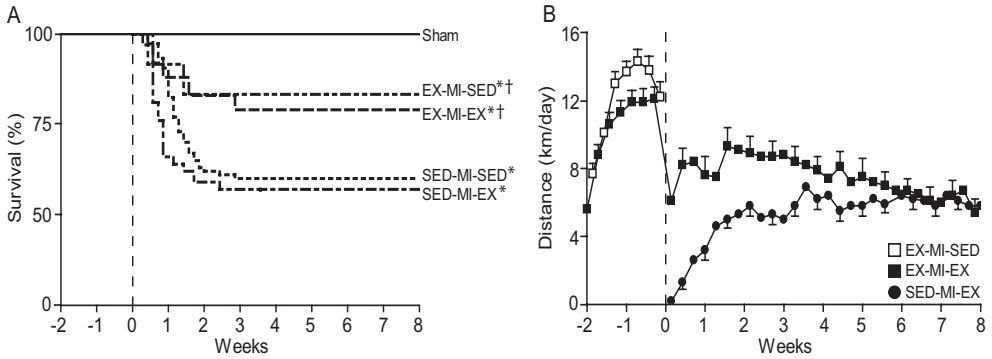


Figure 1.

Kaplan-Meier survival curves for all five groups. Numbers of mice entering the study: Sham (n=49), SED-MI-SED (n=44), SED-MI-EX (n=58), EX-MI-SED (n=12), EX-MI-EX (n=24) (A). Daily running distance in SED-MI-EX (n=26), EX-MI-SED (n=19) and EX-MI-EX (n=19) mice that survived the entire 8-week follow-up period (B). *P<0.05 versus Sham, †P<0.05 versus corresponding sedentary mice before MI.

Table 1: LV Anatomical and Functional Data

		Sham		MI	
		Sed	Ex	Sed	Ex
Animal numbers	Wt	41	27	40	23
	eNOSTg	43	21	30	18
Body weight (g)	Wt	27±1	25±1	25±1	24±1
	eNOSTg	26±1	24±1	25±1	25±1
LV weight (mg)	Wt	97±3	94±2	113±2*	112±3*
	eNOSTg	98±3	98±4	117±4*	123±4*†
RV weight (mg)	Wt	26±1	25±1	33±1*	29±1*‡
	eNOSTg	27±1	26±1	32±1*	35±2*†
Heart rate (bpm)	Wt	526±6	515±11	514±7	523±7
	eNOSTg	531±5	529±6	529±9	496±10*†
MAP (mmHg)	Wt	75±2	80±3	71±2	71±2*
	eNOSTg	69±2†	66±3†	61±2*†	63±2†
LVSP (mmHg)	Wt	97±2	99±3	84±2*	85±2*
	eNOSTg	88±2†	83±2†	75±2*†	74±2*†
LV dP/dt_{P30} (mmHg/s)	Wt	7070±180	6960±210	4870±200*	5620±220*‡
	eNOSTg	6920±220	6280±340	4380±210*†	4280±240*†
Fractional shortening (%)	Wt	39±1	44±1	8±1*	12±1*‡
	eNOSTg	38±1	36±2†	12±1*†	9±1*†‡
LV dP/dt_{min} (mmHg/s)	Wt	-7520±370	-7860±440	-4650±240*	-4840±310*
	eNOSTg	-6780±280	-5560±400†‡	-4040±240*†	-3920±250*†
Tau (ms)	Wt	8.7±0.6	8.6±0.7	12.4±1.0*	13.5±1.1*
	eNOSTg	9.0±0.7	10.6±1.2	11.8±1.0*	12.3±1.2
LVEDP (mmHg)	Wt	6.9±0.6	4.6±0.6‡	9.8±0.9*	8.0±0.9*
	eNOSTg	5.6±0.6	6.6±1.1	8.5±1.1*	8.2±1.4

Wt,wild type; eNOSTg,eNOS transgenic; LV,left ventricle; RV,right ventricle; MAP,mean arterial pressure; LVSP,left ventricular systolic pressure; LVEDP,left ventricular end diastolic pressure. *P<0.05 vs corresponding sham; †P<0.05 vs corresponding Wt; ‡P<0.05 vs corresponding Sedentary.

shortening and ameliorated pulmonary congestion. In contrast, in EX-MI-EX mice in which exercise was started 2 weeks before MI and continued after induction of MI, LV dilation and dysfunction were slightly aggravated compared to EX-MI-SED mice, so that LV FS, $LVdP/dt_{P30}$, and RV and lung fluid weights were no longer different from SED-MI-SED mice.

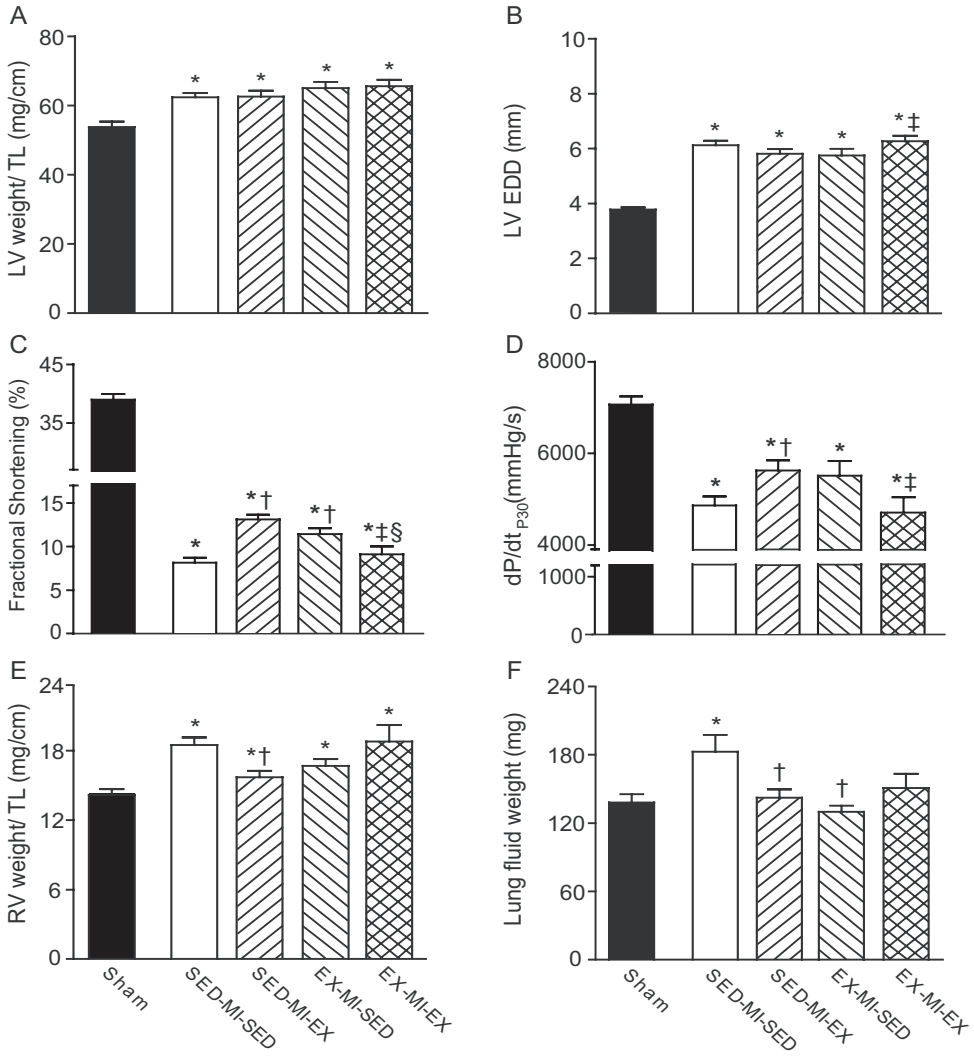


Figure 2.

Effects of exercise on relative LV mass (A), LV end-diastolic diameter (B), LV fractional shortening as measure for global LV pump function (C), LV dP/dt_{P30} as measure for global LV contractility (D), relative RV mass (E), lung fluid weight (F). *P<0.05 versus Sham, †P<0.05 versus corresponding SED-MI-SED, ‡P<0.05 versus SED-MI-EX, §P<0.05 versus EX-MI-SED.

Infarct remodeling

Exercise after MI had no effect on infarct remodeling, reflected in an unchanged infarct length, minimal and average thickness and total area (Figure 3A, 4A-C). In contrast, exercise training prior to infarction resulted in an increased infarct thickness, and hence total infarct area, irrespective of whether mice continued to exercise after MI. Interestingly, we also observed that the subendocardial rim of surviving cardiomyocytes within the infarct area was thicker in both EX-MI-SED and EX-MI-EX groups (Figure 4D).

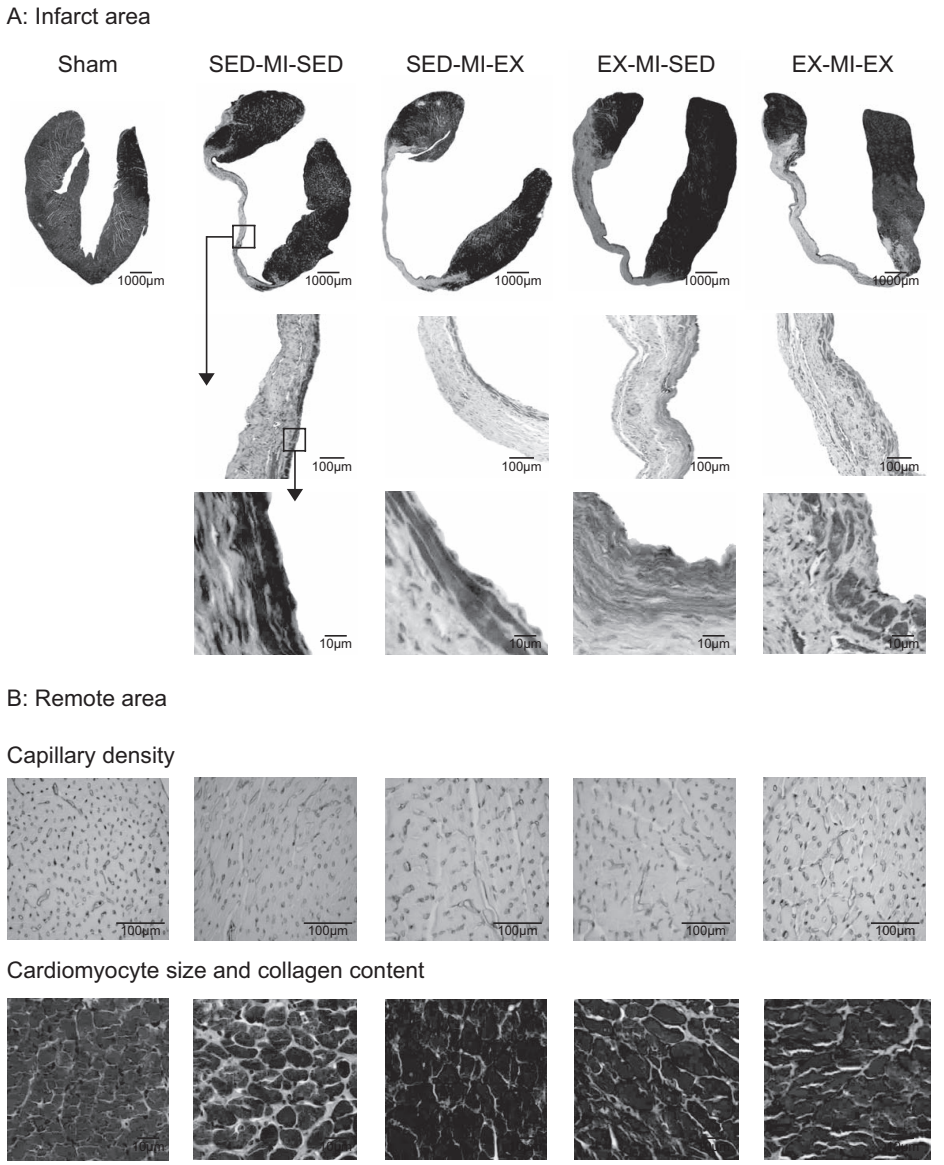


Figure 3.
Macroscopic and microscopic representative examples of LV infarct (A) and remote (B) myocardium.

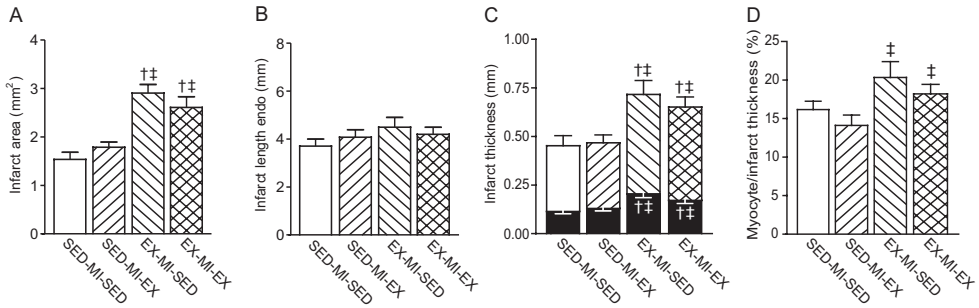


Figure 4.

Infarct remodeling reflected in infarct area (A), endocardial infarct length (B), minimal (black bars) and average (white bars) infarct thickness (C), and % cardiomyocyte layer within the infarct area (D). †P<0.05 versus corresponding SED-MI-SED, ‡P<0.05 versus SED-MI-EX.

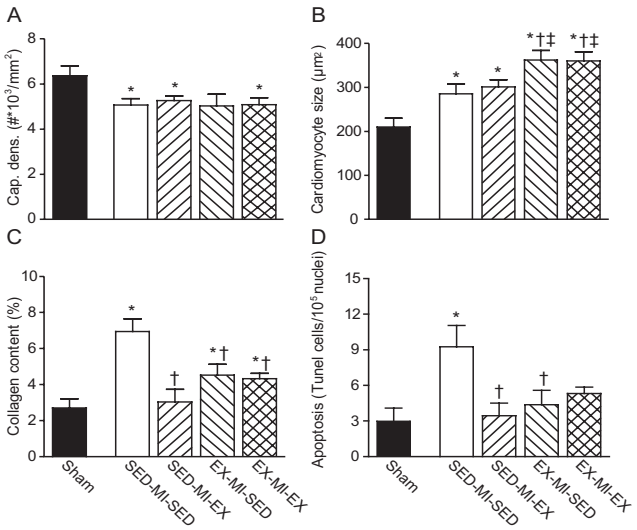


Figure 5.

The effects of exercise on capillary density (A), cardiomyocyte cross sectional area (B), collagen volume fraction (C) and apoptosis (D) in non-infarcted remote myocardium. *P<0.05 versus Sham, †P<0.05 versus corresponding SED-MI-SED.

Remodeling of the remote myocardium

Capillary density

After MI, capillary density (Figure 3B) in remote non-infarcted myocardium decreased by ~20% (Figure 5A). None of the exercise regimen had an effect on capillary density.

Cardiomyocyte cross-sectional area

CSA of myocytes in the remote surviving myocardium was increased after MI by 35% (Figure 3B, 5B). Exercise after MI did not affect cardiomyocyte size. In contrast, in mice in which exercise was started 2 weeks before MI, cardiomyocyte CSA had increased further compared to SED-MI-SED at 8 weeks after MI, irrespective of whether exercise was continued or stopped after MI.

Collagen content

MI increased interstitial collagen content in remote surviving myocardium (Figure 3B, 5C). Exercise after MI normalized collagen content. A similar decrease in collagen content was noted in EX-MI-SED. However, combining exercise prior to and after MI did not result in a further reduction in interstitial collagen content, compared to EX-MI-SED or SED-MI-EX.

Apoptosis

MI resulted in an increased number of TUNEL positive nuclei in remote surviving myocardium (Figure 5D). Exercise after MI reduced the number of TUNEL positive nuclei to sham levels. A similar decrease in TUNEL positive nuclei was noted in EX-MI-SED mice. However, the combination of exercise prior to and after MI failed to significantly (P=0.066) reduce the number of TUNEL positive nuclei, compared to SED-MI-SED mice.

Discussion

MI results in LV remodeling that serves to restore LV pump function. However, despite the apparent appropriateness of LV remodeling to maintain cardiac pump function early after MI, remodeling is an independent risk factor for the development of congestive heart failure.[31] In chapter 2 of this thesis we reported that exercise training after MI attenuates LV pump dysfunction in mice.[13] The present study investigated the impact of 2 weeks of exercise training prior to an acute MI, i.e. a higher level of physical fitness at the time of MI, on post-MI survival, LV remodeling and dysfunction in mice. The main findings were that: *(i)* exercise after MI had no adverse effect on survival, infarct size, LV dimensions and hypertrophy, while improving LV dysfunction and backward failure; *(ii)* exercise started before MI improved survival, infarct remodeling and global LV dysfunction; *(iii)* exercise started 2 weeks before MI in addition to exercise after MI only improved survival, but had no effects on LV dysfunction with a small improvement on LV remodeling. The implications of these findings will be discussed.

Effects of exercise training on LV remodeling and dysfunction after MI

In agreement with previous findings in our laboratory (chapter 2 of this thesis),[13,30] we observed in the present study that a large MI induced with a permanent coronary artery ligation (comprising ~45% of LV mass) caused 40% mortality in mice, that occurred principally during the first 2 weeks post-MI. Eight weeks after induction of MI, significant LV remodeling had occurred, characterized by LV chamber dilation

and myocardial hypertrophy, as well as increased interstitial collagen deposition and apoptosis, and decreased capillary density in remote surviving myocardium. MI also produced marked LV dysfunction, characterized by decrements in LV pump function (fractional shortening) and indices of systolic (dP/dt_{P30}) and diastolic (dP/dt_{min} and τ) function, resulting in LV backward failure reflected in pulmonary edema and RV hypertrophy.[13,30]

The effects of exercise initiated after MI on LV remodeling and dysfunction remain incompletely understood. Thus, several studies in humans reported contradictory effects of training on LV remodeling after a MI.[6,11,12,32-38] Careful inspection of these studies suggests that after a small MI, exercise has no detrimental effect[33,37] or even improves[6,11,12] LV geometry and function, independent of whether exercise was started late, i.e. ~1 year,[6,11] or early, i.e. <2 months,[12,33,37] after MI. In contrast, in patients with a large MI, exercise had a beneficial effect on ejection fraction (EF) and LV volumes but only when started late after MI.[6,11] However, when exercise after a large MI is started at a time when LV remodeling is still ongoing (<3-4 months after MI), the majority of studies reported that exercise has either no,[32,33,37,38] or even a detrimental[35,36] effect on LV dimensions and/or EF. Similar to these clinical studies, studies in rats indicate that exercise started late (>3 weeks) after a moderate to large MI, encompassing 35-50% of LV mass, at a time when infarct healing is complete, does not aggravate,[39,40] or even blunts[8-10] LV dilation[8] and hypertrophy.[8-10,39,40] In contrast, when started <1 week after a moderate to large MI,[7,41-44] exercise resulted in variable outcomes with beneficial,[7] no,[41,43] or detrimental[42,44] effects on LV remodeling. These rodent studies lend further support to the concern that early exercise may have detrimental effects on LV remodeling after a large MI, although interpretation is hampered by the fact that late exercise studies in rats principally employed treadmill running,[8,11,26,40] while early exercise studies predominantly used swimming.[10,41-43] This is important because the exercise responses to swimming are markedly different from those to treadmill running.[27,45] In contrast to these studies employing early swim training in rats, we observed (in a previous6 and in the present study) that exercise training through voluntary wheel running, starting gradually early after a large MI (comprising ~45% of LV mass), did not aggravate post-MI mortality, LV remodeling, capillary rarefaction and cardiomyocyte hypertrophy of remote myocardium, and even reduced myocardial interstitial fibrosis and apoptosis, and blunted LV dysfunction and LV backward failure. Taken together, rodent studies suggest that exercise training by daily treadmill running initiated either early or late after a large MI is beneficial.

Effects of prior exercise on LV remodeling and dysfunction after MI

There is a lack of clinical data regarding the effects of prior exercise training on outcome after a MI. Animal studies suggest that exercise training can precondition the myocardium, thereby protecting the heart against irreversible damage produced by ischemia-reperfusion in rats[16-19] and dogs (Table 2).[20] In addition, exercise training may modulate post-infarct remodeling independent of any preconditioning effect. Thus, two studies in rats using a permanent coronary artery ligation reported that 5-7 weeks of prior swim training had no effect on post-MI mortality, but reduced absolute and relative infarct size and attenuated LV remodeling, as determined at 2 days[24] or 4 weeks[23] after induction of MI. Training-induced increases in capillary[24] and arteriolar[23] densities were observed in the remote region, which lead the authors to speculate (collateral blood flow was not actually measured) that prior exercise training may have resulted in increased collateral blood flow to the area at risk thereby limiting infarct size. Although myocardial vasculature is known to be enhanced by swim training of young male rats,[25] there is no evidence to suggest that collateral vessel growth is stimulated by exercise training in healthy hearts. Thus, studies pertaining to exercise training of healthy dogs, swine or rats have consistently shown that exercise training does not increase innate collateral blood flow capacity in healthy hearts.[25] An alternative explanation may be that prior exercise training affected healing and remodeling of the infarct area (contracture) thereby leading to a reduction of infarct area.[23,24]

In contrast to the findings in rats, we observed that exercise training of mice via voluntary treadmill running reduced post-MI mortality from ~40% to ~20% and blunted LV dysfunction, but did not enhance vascularity of remote myocardium or limit infarct size as measured 8 weeks later. Interestingly, we observed that while the subendocardial infarct circumference was unaltered, infarct thickness was increased which was accompanied by a slightly thicker subendocardial rim of myocytes in the infarct area, consistent with exercise-induced modulation of infarct healing. In this respect it should be noted that mortality in C57Bl/6J mice after permanent coronary artery ligation is mainly caused by cardiac rupture of the infarct area within the first 2 weeks after MI.[46] It is therefore tempting to speculate that the increased infarct thickness acted to prevent cardiac rupture thereby enhancing post-MI survival in mice that had been subjected to prior exercise training. In addition, a reduction in fatal arrhythmias in the early phase following induction of MI may also have contributed to improved survival.[47]

The precise mechanism for the blunted LV dysfunction in EX-MI-SED mice cannot be determined from the present study. There is evidence that the hearts of exercise

Tabel 2: Literature overview

Authors	Year	Species	Exercise		MI induction	Follow-up	IS	LV remodeling	LV function
			Type	Duration					
Ischemia-Reperfusion									
Powers *et al* [16]	1998	rat, ♀ SD	forced treadmill	90min/day 4days/wk, 10wks	20 min CAO	10 min	-	HW/BW ↑	↑ LVSP
Yamashita *et al* [17]	1999	rat, ♂ Wistar	forced treadmill	27-30m/min, 25-30min one session	20 min CAO	48 h	50%→20% 0.5 en 48h after ex	-	-
Domenech *et al* [20]	2002	dog, ♀,♂	forced treadmill	5min, 5-9km/h 2x/day	60 min CAO 10min(ep)/24h(lp) after ex	4.5 h	24%→5% ep 24%→ 13% lp	-	↔ HR, SAP, DA
Brown *et al* [18]	2003	rat, ♀ SD	forced treadmill	35m/min, 20min >20wks	60 min CAO	2 h	32%→24%	LVW ↔	↑ coronary flow ↑ diastolic functi ↑ LV developed
Zhang *et al* [19]	2007	rat, ♂ SD	forced swim	3h/day 5days/wk, 8wks	30 min CAO	4 h	35%→27%	HW/BW ↑	↑ LVSP ↑ dP/dt
Permanent Ischemia									
McElroy *et al* [24]	1978	rat, ♂ SD	forced swim	60min/day 5days/wk, 5-6wks	permanent ligation	2 days	31%→22%	LVW ↔	-
Freimann *et al* [23]	2005	rat, ♂ SD	forced swim	90min/day 6days/wk, 7wks	permanent ligation	4 weeks	20%→11%	LVW ↔ LVEDD ↓	↑ FS
de Waard *et al*	present study	mouse, ♀,♂ C57Bl/6J	voluntary wheel running	12km/day 2wks	permanent ligation	8 weeks	↔	LVW ↔ LVEDD ↔	↑ FS ↑ dP/dt_{P30} ↓ pulmonary conges ↓ RVW

IR; ischemia-reperfusion, MI; myocardial infarction by permanent artery ligation, CAO; coronary artery occlusion, SD; Sprague-Dawley, LVW; left ventricular weight, LVEDD; left ventricular en dens.; arteriolar density, FS; fractional shortening, IS; infarct size, P; pressure, ep; early preconditioning, lp; late preconditioning

trained animals respond more effectively to stress. For example, trained rats have been shown to maintain or increase myocardial contractility more effectively in the face of a sustained pressure overload than sedentary animals.[48] Future studies are needed to establish the molecular mechanisms underlying the mitigation of LV dysfunction by pre-MI exercise, which should include interrogation of possible exercise-induced improvements of intact cardiomyocyte and myofilament function.[13]

Combining exercise before and after MI

Allowing mice in which exercise was started 2 weeks prior to MI to continue to exercise after induction of MI resulted in similar infarct remodeling, improvements in post-MI survival and reductions in collagen content of remote myocardium, as compared to trained mice that were sedentary housed after MI. In contrast, the mitigating effects of either pre-MI or post-MI exercise on LV dysfunction were lost when mice were subjected to voluntary exercise training both prior and after induction of MI. Although these observations are difficult to explain, it should be noted that mice that had been adapted to the running-wheel for already 2 weeks prior to induction of MI (EX-MI-EX), ran significantly longer daily distances immediately post-MI (~7 km/day during the first week) as compared to the initially shorter daily distances in SED-MI-EX mice during the first 4 weeks (~2 km/day during the first week). Consequently, it cannot be excluded that this markedly higher exercise load in the immediate post-MI phase offset some of the beneficial effects of post-MI exercise training. This may explain why studies in rats and humans showed a variable response to (forced) exercise training early after MI. Hence, future studies should address the influence of the early post-MI exercise load on LV dysfunction in more detail, e.g. by restricting voluntary exercise in EX-MI-EX mice in the very early post-MI phase, or by varying the forced exercise load during that time.

Clinical implications and conclusions

The beneficial effects of regular physical activity on preventing major cardiac events in healthy individuals and patients with coronary heart disease are now well established.[3-5] The results of the present study show that even when regular physical fails to prevent an acute myocardial infarction it can still act to improve cardiac function and survival after MI. These observations warrant an even greater emphasis on life style changes in patients with established coronary heart disease.

References

1. Wannamethee SG, Shaper AG. *Physical activity and cardiovascular disease.* Semin Vasc Med 2002;2:257-266.
2. Booth FW, Laye MJ, Lees SJ, Rector RS, Thyfault JP. *Reduced physical activity and risk of chronic disease: the biology behind the consequences.* Eur J Appl Physiol 2008;102:381-390.
3. Hakim AA, Petrovitch H, Burchfiel CM, Ross GW, Rodriguez BL, White LR, *et al. Effects of walking on mortality among nonsmoking retired men.* N Engl J Med 1998;338:94-99.
4. Manson JE, Greenland P, LaCroix AZ, Stefanick ML, Mouton CP, Oberman A, *et al. Walking compared with vigorous exercise for the prevention of cardiovascular events in women.* N Engl J Med 2002;347:716-725.
5. Thompson PD, Franklin BA, Balady GJ, Blair SN, Corrado D, Estes NA, 3rd, *et al. Exercise and acute cardiovascular events placing the risks into perspective: a scientific statement from the American Heart Association Council on Nutrition, Physical Activity, and Metabolism and the Council on Clinical Cardiology.* Circulation 2007;115:2358-2368.
6. Ehsani AA, Biello DR, Schultz J, Sobel BE, Holloszy JO. *Improvement of left ventricular contractile function by exercise training in patients with coronary artery disease.* Circulation 1986;74:350-358.
7. Oh BH, Ono S, Rockman HA, Ross J, Jr. *Myocardial hypertrophy in the ischemic zone induced by exercise in rats after coronary reperfusion.* Circulation 1993;87:598-607.
8. Orenstein TL, Parker TG, Butany JW, Goodman JM, Dawood F, Wen WH, *et al. Favorable left ventricular remodeling following large myocardial infarction by exercise training. Effect on ventricular morphology and gene expression.* J Clin Invest 1995;96:858-866.
9. Zhang LQ, Zhang XQ, Musch TI, Moore RL, Cheung JY. *Sprint training restores normal contractility in postinfarction rat myocytes.* J Appl Physiol 2000;89:1099-1105.
10. Wisloff U, Loennechen JP, Currie S, Smith GL, Ellingsen O. *Aerobic exercise reduces cardiomyocyte hypertrophy and increases contractility, Ca2+ sensitivity and SERCA-2 in rat after myocardial infarction.* Cardiovasc Res 2002;54:162-174.
11. Giannuzzi P, Temporelli PL, Corra U, Tavazzi L. *Antiremodeling effect of long-term exercise training in patients with stable chronic heart failure: results of the Exercise in Left Ventricular Dysfunction and Chronic Heart Failure (ELVD-CHF) Trial.* Circulation 2003;108:554-559.
12. Koizumi T, Miyazaki A, Komiyama N, Sun K, Nakasato T, Masuda Y, *et al. Improvement of left ventricular dysfunction during exercise by walking in patients with successful percutaneous coronary intervention for acute myocardial infarction.* Circ J 2003;67:233-237.
13. de Waard MC, van der Velden J, Bito V, Ozdemir S, Biesmans L, Boontje NM, *et al. Early exercise training normalizes myofilament function and attenuates left ventricular pump dysfunction in mice with a large myocardial infarction.* Circ Res 2007;100:1079-1088.
14. O'Connor GT, Buring JE, Yusuf S, Goldhaber SZ, Olmstead EM, Paffenbarger RS, Jr., *et al. An overview of randomized trials of rehabilitation with exercise after myocardial infarction.* Circulation 1989;80:234-244.
15. Taylor RS, Brown A, Ebrahim S, Jolliffe J, Noorani H, Rees K, *et al. Exercise-based rehabilitation for patients with coronary heart disease: systematic review and meta-analysis of randomized controlled trials.* Am J Med 2004;116:682-692.
16. Powers SK, Demirel HA, Vincent HK, Coombes JS, Naito H, Hamilton KL, *et al. Exercise training improves myocardial tolerance to in vivo ischemia-reperfusion in the rat.* Am J Physiol 1998;275:R1468-1477.
17. Yamashita N, Hoshida S, Otsu K, Asahi M, Kuzuya T, Hori M. *Exercise provides direct biphasic cardioprotection via manganese superoxide dismutase activation.* J Exp Med 1999;189:1699-1706.
18. Brown DA, Jew KN, Sparagna GC, Musch TI, Moore RL. *Exercise training preserves coronary flow and reduces infarct size after ischemia-reperfusion in rat heart.* J Appl Physiol 2003;95:2510-2518.
19. Zhang KR, Liu HT, Zhang HF, Zhang QJ, Li QX, Yu QJ, *et al. Long-term aerobic exercise protects the heart against ischemia/reperfusion injury via PI3 kinase-dependent and Akt-mediated mechanism.* Apoptosis 2007;12:1579-1588.
20. Domenech R, Macho P, Schwarze H, Sanchez G. *Exercise induces early and late myocardial preconditioning in dogs.* Cardiovasc Res 2002;55:561-566.
21. Murry CE, Jennings RB, Reimer KA. *Preconditioning with ischemia: a delay of lethal cell injury in ischemic myocardium.* Circulation 1986;74:1124-1136.
22. van den Doel MA, Gho BC, Duval SY, Schoemaker RG, Duncker DJ, Verdouw PD. *Hypothermia extends the cardioprotection by ischaemic preconditioning to coronary artery occlusions of longer duration.* Cardiovasc Res 1998;37:76-81.
23. Freimann S, Scheinowitz M, Yekutieli D, Feinberg MS, Eldar M, Kessler-Icekson G. *Prior exercise training improves the outcome of acute myocardial infarction in the rat. Heart structure, function, and gene expression.* J Am Coll Cardiol 2005;45:931-938.
24. McElroy CL, Gissen SA, Fishbein MC. *Exercise-induced reduction in myocardial infarct size after coronary artery occlusion in the rat.* Circulation 1978;57:958-962.
25. Duncker DJ, Bache RJ. *Regulation of coronary blood flow during exercise.* Physiological Reviews 2008;88 (in press).
26. Flaim SF, Minteer WJ, Clark DP, Zelis R. *Cardiovascular response to acute aquatic and treadmill exercise in the untrained rat.* J Appl Physiol 1979;46:302-308.
27. Baldwin KM. *Effects of chronic exercise on biochemical and functional properties of the heart.* Med Sci Sports Exerc 1985;17:522-528.
28. Hudlicka O, Brown MD, Walter H, Weiss JB, Bate A. *Factors involved in capillary growth in the heart.* Mol Cell Biochem 1995;147:57-68.
29. Hudlicka O, Price S. *The role of blood flow and/or muscle hypoxia in capillary growth in chronically stimulated fast muscles.* Pflugers Arch 1990;417:67-72.

30. van den Bos EJ, Mees BM, de Waard MC, de Crom R, Duncker DJ. *A novel model of cryoinjury-induced myocardial infarction in the mouse: a comparison with coronary artery ligation.* Am J Physiol Heart Circ Physiol 2005;289:H1291-1300.
31. Konstam MA, Udelson JE, Sharpe N. *Prevention and reversal of left ventricular remodeling: summation.* J Card Fail 2002;8:S506-511.
32. Dubach P, Myers J, Dziekan G, Goebbels U, Reinhart W, Vogt P, *et al. Effect of exercise training on myocardial remodeling in patients with reduced left ventricular function after myocardial infarction: application of magnetic resonance imaging.* Circulation 1997;95:2060-2067.
33. Giannuzzi P, Tavazzi L, Temporelli PL, Corra U, Imparato A, Gattone M, *et al. Long-term physical training and left ventricular remodeling after anterior myocardial infarction: results of the Exercise in Anterior Myocardial Infarction (EAMI) trial. EAMI Study Group.* J Am Coll Cardiol 1993;22:1821-1829.
34. Giannuzzi P, Temporelli PL, Corra U, Gattone M, Giordano A, Tavazzi L. *Attenuation of unfavorable remodeling by exercise training in postinfarction patients with left ventricular dysfunction: results of the Exercise in Left Ventricular Dysfunction (ELVD) trial.* Circulation 1997;96:1790-1797.
35. Jugdutt BI, Michorowski BL, Kappagoda CT. *Exercise training after anterior Q wave myocardial infarction: importance of regional left ventricular function and topography.* J Am Coll Cardiol 1988;12:362-372.
36. Kubo N, Ohmura N, Nakada I, Yasu T, Katsuki T, Fujii M, *et al. Exercise at ventilatory threshold aggravates left ventricular remodeling in patients with extensive anterior acute myocardial infarction.* Am Heart J 2004;147:113-120.
37. Otsuka Y, Takaki H, Okano Y, Satoh T, Aihara N, Matsumoto T, *et al. Exercise training without ventricular remodeling in patients with moderate to severe left ventricular dysfunction early after acute myocardial infarction.* Int J Cardiol 2003;87:237-244.
38. Sullivan MJ, Higginbotham MB, Cobb FR. *Exercise training in patients with severe left ventricular dysfunction. Hemodynamic and metabolic effects.* Circulation 1988;78:506-515.
39. Libonati JR. *Exercise and diastolic function after myocardial infarction.* Med Sci Sports Exerc 2003;35:1471-1476.
40. Musch TI, Moore RL, Leathers DJ, Bruno A, Zelis R. *Endurance training in rats with chronic heart failure induced by myocardial infarction.* Circulation 1986;74:431-441.
41. Alhaddad IA, Hakim I, Siddiqi F, Lagenback E, Mallavarapu C, Nethala V, *et al. Early exercise after experimental myocardial infarction: effect on left ventricular remodeling.* Coron Artery Dis 1998;9:319-327.
42. Gaudron P, Hu K, Schamberger R, Budin M, Walter B, Ertl G. *Effect of endurance training early or late after coronary artery occlusion on left ventricular remodeling, hemodynamics, and survival in rats with chronic transmural myocardial infarction.* Circulation 1994;89:402-412.
43. Hochman JS, Healy B. *Effect of exercise on acute myocardial infarction in rats.* J Am Coll Cardiol 1986;7:126-132.
44. Kloner RA, Kloner JA. *The effect of early exercise on myocardial infarct scar formation.* Am Heart J 1983;106:1009-1013.
45. Bernstein D. *Exercise assessment of transgenic models of human cardiovascular disease.* Physiol Genomics 2003;13:217-226.
46. Cavasin MA, Yang XP, Liu YH, Mehta D, Karumanchi R, Bulagannawar M, *et al. Effects of ACE inhibitor, AT1 antagonist, and combined treatment in mice with heart failure.* J Cardiovasc Pharmacol 2000;36:472-480.
47. Scheffer MG, Verdouw PD. *Decreased incidence of ventricular fibrillation after an acute coronary artery ligation in exercised pigs.* Basic Res Cardiol 1983;78:298-309.
48. Dowell RT, Cutilletta AF, Rudnik MA, Sodt PC. *Heart functional responses to pressure overload in exercised and sedentary rats.* Am J Physiol 1976;230:199-204.

Chapter 8

General discussion and summary

Pathological left ventricular (LV) remodeling after myocardial infarction (MI) is a compensatory mechanism, which serves to restore LV pump function to normal levels. Despite the apparent appropriateness of the process, LV remodeling after MI is an independent risk factor for the development of heart failure. The mechanisms underlying the progression from LV remodeling to overt heart failure remain incompletely understood, but recent evidence indicates that abnormalities in myofilament function and Ca^{2+}-handling contribute to LV dysfunction in the porcine heart, early after MI.[1] In contrast to pathological LV remodeling after MI, physiological LV remodeling produced by regular exercise training is associated with a decreased risk for heart failure. The effects of exercise on LV remodeling and dysfunction after MI are still incompletely understood, as several studies in humans and rats reported contradictory effects of training after a MI. The studies presented in this thesis were carried out to obtain a better understanding of the influences of regular exercise training on myocardial abnormalities associated with pathological LV remodeling and dysfunction in a mouse model of MI, in particular, the role of nitric oxide in the beneficial effects of exercise training.

In **chapter 2** of this thesis the impact of 8 weeks of exercise training by voluntary treadmill running, started within 24 hours after a large MI (comprising ~43% of LV mass), on LV remodeling and dysfunction in mice at the *in vivo*, cellular and molecular level were investigated. Eight weeks of exercise after a large MI had no adverse effect on LV dimensions and hypertrophy, while ameliorating LV dysfunction and backward failure. Furthermore, exercise training normalized the MI-induced myofilament dysfunction, which likely contributed to the exercise-induced improvement in unloaded shortening of isolated intact cardiomyocytes, as the $[Ca^{2+}]_i$ transient amplitude was not altered by exercise which was consistent with the lack of effect of exercise on SERCA2a and PLB expression. However, basal diastolic calcium concentrations were normalized by exercise training. Exercise likely mediated these effects via increased protein levels of the β_1-adrenergic receptor, cAMP and Na^+/Ca^{2+}-exchanger. However, in the future Na^+/Ca^{2+}-exchanger functional properties need to be determined to show whether the small increase in Na^+/Ca^{2+}-exchanger protein levels is indeed responsible for the exercise-induced reduction in diastolic calcium levels, or whether other mechanisms, including increased sarcolemmal Ca^{2+}-ATPase activity, also contribute.

Several studies suggest an important role of endogenous production of NO in the beneficial effects of exercise.[2] For example, in vivo studies have shown that exercise results in an increased vascular expression of eNOS.[3,4] Furthermore, exercise training in healthy individuals elevated NO bioavailability through a

variety of mechanisms including increased NOS enzyme expression and activity.[5] Such adaptations likely contribute to cardiovascular protection by regular physical exercise in individuals with elevated cardiovascular risk or established coronary artery disease. Consequently, in **chapter 3** we investigated the hypothesis that eNOS plays an obligatory role in the beneficial effects of exercise training on cardiac remodeling and dysfunction after MI. For this purpose we used wild type and homozygous ($eNOS^{-/-}$) as well as heterozygous ($eNOS^{+/-}$) eNOS-deficient mice. The extent of the MI-induced abnormalities in normal mice, such as LV remodeling, including cardiomyocyte hypertrophy and a reduction in capillary density, increased interstitial collagen content and apoptosis, as well as LV systolic and diastolic dysfunction, were not exaggerated in $eNOS^{+/-}$ or $eNOS^{-/-}$ mice. The beneficial effects of exercise after MI on LV systolic dysfunction, pulmonary congestion, interstitial fibrosis and apoptosis in normal mice were lost in $eNOS^{+/-}$ and $eNOS^{-/-}$ mice, while LV systolic dysfunction and pulmonary congestion in $eNOS^{+/-}$ were even aggravated by exercise. Therefore we conclude that the beneficial effects of exercise training after MI require full eNOS activity. It could be speculated that in the absence of eNOS, other factors, including prostacyclin[6] or nNOS,[7] may be upregulated or increased in $eNOS^{-/-}$ mice and act to compensate for the absence of eNOS, thereby preventing further worsening of cardiac remodeling and function after MI. If one eNOS allele is still functional ($eNOS^{+/-}$) and results in normal basal eNOS expression,[8] such compensatory mechanisms may not occur, leaving the heart more vulnerable to the effects of exercise. Future studies are needed to study the influence of potential compensating factors. The beneficial effects of exercise on cardiac function can be attributed, at least in part, to an exercise-induced upregulation of eNOS expression and activity. Therefore we investigated whether the beneficial effects of exercise after MI could be mimicked by increased levels of eNOS. We first studied in **chapter 4** the in vivo effects of increased eNOS expression by 10-12 folds on the cardiovascular system in a transgenic mouse model. Elevated eNOS expression resulted in a reduction of blood pressure, plasma cholesterol and atherosclerosis. Subsequently, this mouse model with elevated eNOS expression was used in **chapter 5** to investigate whether elevated eNOS expression mimicked the beneficial effects of exercise training on LV remodeling and dysfunction after MI. The results indicated that in mice with MI, single therapy of either exercise or elevated eNOS expression improved global LV fractional shortening and cardiomyocyte force development, reduced collagen content and attenuated pulmonary congestion, whereas only elevated eNOS expression improved survival. In view of the complementary mechanisms by which exercise

and elevated eNOS expression appear to exert beneficial effects we then tested in the second part of **chapter 5** the hypothesis that combined treatment with exercise and elevated eNOS expression had an added benefit, and investigated the underlying mechanisms. Contrary to our hypothesis, the results unexpectedly indicated that exercise in addition to elevated eNOS expression abolished the beneficial effects of either treatment alone on global cardiac function after MI. We demonstrated that this detrimental interaction is the result of increased oxidative stress due to eNOS 'uncoupling', since a shortage of substrate (L-arginine) and/or cofactors (BH4) of eNOS can result in 'uncoupling' of NOS thereby generating reactive oxygen species such as super oxide rather than NO. From this study it can be concluded that exercise training is not in every situation harmless, but can lead to increased oxidative stress. Several investigators have proposed a role for eNOS in patients with cardiovascular disease.[9,10] There is currently intense interest in the development of gene therapy for cardiovascular disease.[11] Our results indicate that in the future eNOS gene therapy in combination with physical exercise (as part of lifestyle changes) requires careful food supplementation of L-arginine and BH4 to avoid potential eNOS 'uncoupling' and increased oxidative stress. Therefore, pre-clinical animal studies are necessary to investigate in more detail the optimal conditions for physical exercise and food supplementations in order to use eNOS gene therapy for the treatment of pathological cardiac remodeling.

To obtain insight into the effect of enhanced cGMP on cardiac function, we used mice with overexpression of C-type natriuretic peptide (CNP). cGMP is synthesized from GTP by both soluble guanylyl cyclases (sGC) and particulate guanylyl cyclases (pGC), which are distinguished in function of their main subcellular location and activation. sGC are located mainly in the cytosol and activated by NO, while pGC are integral proteins of the cell membrane which are activated by for instance CNP. Consequently, we investigated in **chapter 6** whether enhanced cGMP through CNP activation of pGC can result in improved cardiac function after MI, similar to the improved LV function after MI in eNOS overexpressing mice. The results showed that cardiomyocyte-restricted elevated CNP expression prevents cardiac hypertrophy induced by MI with limited effect on LV dysfunction. In conclusion, the present study indicates that the CNP/cGMP signaling pathway plays an important role in the local regulation of cardiac hypertrophy under pathophysiological conditions. Thus, modulation of this pathway represents a potent new therapeutic target for the treatment of patients with MI or hypertension-induced cardiac hypertrophy.

Finally, physical inactivity has been proposed to be an independent risk factor for cardiovascular disease.[12,13] Indeed, prospective epidemiological data indicate that

moderate (e.g. walking) and vigorous exercise in healthy subjects are associated with substantial reductions in the incidence of cardiovascular events.[14,15] The beneficial effects of exercise extend to patients with established coronary heart disease in which regular physical activity also reduces the incidence of cardiac events and all-cause mortality.[16] Furthermore, there is evidence that exercise training initiated after a cardiac event, such as MI, ameliorates LV remodeling and dysfunction[17-24] (chapter 2 of this thesis) and clinical outcome.[25,26] In contrast, there is less information available as to whether prior exercise training affords any protection in situations where a major cardiovascular event like MI does occur. **Chapter 7** of this thesis showed that exercise either prior to MI or after MI both blunted LV dysfunction, while exercise prior to MI also improved survival likely due to improved scar healing. These results demonstrate that even when regular physical activity fails to prevent an acute MI it can still act to improve cardiac function and survival after MI. In contrast, the beneficial effects of either pre-MI or post-MI exercise on LV dysfunction were lost when mice were subjected to voluntary exercise training both prior and after induction of MI. Although this observation is difficult to explain, it should be noted that mice that had been adapted to the running-wheel for already 2 weeks prior to induction of MI (EX-MI-EX), ran significantly longer daily distances immediately after MI (~7 km/day during the first week) as compared to the initially shorter daily distances in mice, which didn't run before MI (SED-MI-EX), during the first 4 weeks (~2 km/day during the first week). Consequently, it cannot be excluded that this difference in immediate post-MI exercise load offset some of the beneficial effects of post-MI exercise training. Future studies should address the influence of early post-MI exercise load on LV dysfunction in more detail, e.g. by restricting voluntary exercise in EX-MI-EX mice within the first week after MI. Or alternatively, by a short adaptation period (1-2 days) to voluntary treadmill running, in the SED-MI-EX mice, to increase daily running distance immediately after MI. The precise mechanism for the blunted LV dysfunction in mice where exercise was started prior to MI cannot be determined from this thesis. There is evidence that the hearts of exercise-trained animals respond more effectively to stress. For example, trained rats have been shown to maintain or increase myocardial contractility more effectively in the face of a sustained pressure overload than sedentary animals.[27] Future studies are needed to establish the molecular mechanisms underlying the alleviation of LV dysfunction by pre-MI exercise, which should include examination of possible exercise-induced improvements of intact cardiomyocyte function.

Future perspectives

In response to a MI, cardiomyocytes undergo hypertrophic growth or apoptosis, responses associated with the development of cardiac pathologies. Research has been done into understanding of the stimuli, which promote these responses, and in identifying the underlying intracellular signaling pathways. These signaling pathways most likely modulate gene and protein expression to obtain the end-stage response. Pathological cardiac hypertrophy is accompanied by the induction of genes normally expressed during fetal development, such as atrial natriuretic factor, brain natriuretic peptide, skeletal α–actin and β-myosin heavy chain.[28,29] Recent investigation has centered on identifying the molecular signaling pathways and its transcriptional factors that regulate pathological cardiac myocyte hypertrophy, for instance calcineurin-nuclear factor of activated T-cells (NFAT) pathway[30] and myocyte enhancer factor-2 (MEF-2).[31] Insulin-like growth factor 1 (IGF1), and its downstream PI3-kinase pathway, has been implicated in the regulation of body and organ size during postnatal development and is released in response to exercise training.[32]

In our research, future studies are required to investigate in the early post-MI phase the signaling transduction and gene expression pathways, which are responsible for pathological remodeling and the differences and/or similarities in these pathways compared to physiological remodeling as a result of voluntary exercise training. By investigating the signaling pathways implicated in the regulation of gene expression, the transcription factors they regulate and the changes in gene expression involved in our large MI-model and voluntary exercise model, we can obtain a better understanding of the role of exercise training in these pathways. Furthermore, it is of relevance to know whether exercise training after MI can change the development of pathological remodeling into the beneficial physiological remodeling. Moreover, it is relevant to identify the pathways involved in the beneficial effects of exercise training prior to MI for future treatment or reversal of pathological remodeling in humans.

Finally, it will be of interest to perform meta-analysis of cohort studies to investigate the influence of physical fitness in the years prior to an acute MI on post-MI survival, infarct size and/or cardiac function among people without previous cardiovascular events.

References

1. van der Velden J, Merkus D, Klarenbeek BR, James AT, Boontje NM, Dekkers DH, Stienen GJ, Lamers JM, Duncker DJ. *Alterations in myofilament function contribute to left ventricular dysfunction in pigs early after myocardial infarction.* Circ Res 2004;95:e85-95.
2. Shen W, Zhang X, Zhao G, Wolin MS, Sessa W, Hintze TH. *Nitric oxide production and NO synthase gene expression contribute to vascular regulation during exercise.* Med Sci Sports Exerc 1995;27:1125-1134.
3. Delp MD, Laughlin MH. *Time course of enhanced endothelium-mediated dilation in aorta of trained rats.* Med Sci Sports Exerc 1997;29:1454-1461.
4. Woodman CR, Muller JM, Laughlin MH, Price EM. *Induction of nitric oxide synthase mRNA in coronary resistance arteries isolated from exercise-trained pigs.* Am J Physiol 1997;273:H2575-2579.
5. Maroun MJ, Mehta S, Turcotte R, Cosio MG, Hussain SN. *Effects of physical conditioning on endogenous nitric oxide output during exercise.* J Appl Physiol 1995;79:1219-1225.
6. Godecke A, Decking UK, Ding Z, Hirchenhain J, Bidmon HJ, Godecke S, Schrader J. *Coronary hemodynamics in endothelial NO synthase knockout mice.* Circ Res 1998;82:186-194.
7. Meng W, Ma J, Ayata C, Hara H, Huang PL, Fishman MC, Moskowitz MA. *ACh dilates pial arterioles in endothelial and neuronal NOS knockout mice by NO-dependent mechanisms.* Am J Physiol 1996;271:H1145-1150.
8. Kojda G, Cheng YC, Burchfield J, Harrison DG. *Dysfunctional regulation of endothelial nitric oxide synthase (eNOS) expression in response to exercise in mice lacking one eNOS gene.* Circulation 2001;103:2839-2844.
9. Cooney R, Hynes SO, Sharif F, Howard L, O'Brien T. *Effect of gene delivery of NOS isoforms on intimal hyperplasia and endothelial regeneration after balloon injury.* Gene Ther 2007;14:396-404.
10. Kupatt C, Hinkel R, von Bruhl ML, Pohl T, Horstkotte J, Raake P, El Aouni C, Thein E, Dimmeler S, Feron O, Boekstegers P. *Endothelial nitric oxide synthase overexpression provides a functionally relevant angiogenic switch in hibernating pig myocardium.* J Am Coll Cardiol 2007;49:1575-1584.
11. Khurana R, Martin JF, Zachary I. *Gene therapy for cardiovascular disease: a case for cautious optimism.* Hypertension 2001;38:1210-1216.
12. Booth FW, Laye MJ, Lees SJ, Rector RS, Thyfault JP. *Reduced physical activity and risk of chronic disease: the biology behind the consequences.* Eur J Appl Physiol 2008;102:381-390.
13. Wannamethee SG, Shaper AG. *Physical activity and cardiovascular disease.* Semin Vasc Med 2002;2:257-266.
14. Hakim AA, Petrovitch H, Burchfiel CM, Ross GW, Rodriguez BL, White LR, Yano K, Curb JD, Abbott RD. *Effects of walking on mortality among nonsmoking retired men.* N Engl J Med 1998;338:94-99.
15. Manson JE, Greenland P, LaCroix AZ, Stefanick ML, Mouton CP, Oberman A, Perri MG, Sheps DS, Pettinger MB, Siscovick DS. *Walking compared with vigorous exercise for the prevention of cardiovascular events in women.* N Engl J Med 2002;347:716-725.
16. Thompson PD, Franklin BA, Balady GJ, Blair SN, Corrado D, Estes NA, 3rd, Fulton JE, Gordon NF, Haskell WL, Link MS, Maron BJ, Mittleman MA, Pelliccia A, Wenger NK, Willich SN, Costa F. *Exercise and acute cardiovascular events placing the risks into perspective: a scientific statement from the American Heart Association Council on Nutrition, Physical Activity, and Metabolism and the Council on Clinical Cardiology.* Circulation 2007;115:2358-2368.
17. de Waard MC, van der Velden J, Bito V, Ozdemir S, Biesmans L, Boontje NM, Dekkers DH, Schoonderwoerd K, Schuurbiers HC, de Crom R, Stienen GJ, Sipido KR, Lamers JM, Duncker DJ. *Early exercise training normalizes myofilament function and attenuates left ventricular pump dysfunction in mice with a large myocardial infarction.* Circ Res 2007;100:1079-1088.
18. Ehsani AA, Biello DR, Schultz J, Sobel BE, Holloszy JO. *Improvement of left ventricular contractile function by exercise training in patients with coronary artery disease.* Circulation 1986;74:350-358.
19. Giannuzzi P, Temporelli PL, Corra U, Tavazzi L. *Antiremodeling effect of long-term exercise training in patients with stable chronic heart failure: results of the Exercise in Left Ventricular Dysfunction and Chronic Heart Failure (ELVD-CHF) Trial.* Circulation 2003;108:554-559.
20. Koizumi T, Miyazaki A, Komiyama N, Sun K, Nakasato T, Masuda Y, Komuro I. *Improvement of left ventricular dysfunction during exercise by walking in patients with successful percutaneous coronary intervention for acute myocardial infarction.* Circ J 2003;67:233-237.
21. Oh BH, Ono S, Rockman HA, Ross J, Jr. *Myocardial hypertrophy in the ischemic zone induced by exercise in rats after coronary reperfusion.* Circulation 1993;87:598-607.
22. Orenstein TL, Parker TG, Butany JW, Goodman JM, Dawood F, Wen WH, Wee L, Martino T, McLaughlin PR, Liu PP. *Favorable left ventricular remodeling following large myocardial infarction by exercise training. Effect on ventricular morphology and gene expression.* J Clin Invest 1995;96:858-866.
23. Wisloff U, Loennechen JP, Currie S, Smith GL, Ellingsen O. *Aerobic exercise reduces cardiomyocyte hypertrophy and increases contractility, Ca^{2+} sensitivity and SERCA-2 in rat after myocardial infarction.* Cardiovasc Res 2002;54:162-174.
24. Zhang LQ, Zhang XQ, Musch TI, Moore RL, Cheung JY. *Sprint training restores normal contractility in postinfarction rat myocytes.* J Appl Physiol 2000;89:1099-1105.
25. O'Connor GT, Buring JE, Yusuf S, Goldhaber SZ, Olmstead EM, Paffenbarger RS, Jr., Hennekens CH. *An overview of randomized trials of rehabilitation with exercise after myocardial infarction.* Circulation 1989;80:234-244.
26. Taylor RS, Brown A, Ebrahim S, Jolliffe J, Noorani H, Rees K, Skidmore B, Stone JA, Thompson DR, Oldridge N. *Exercise-based rehabilitation for patients with coronary heart disease: systematic review and meta-analysis of randomized controlled trials.* Am J Med 2004;116:682-692.
27. Dowell RT, Cutilletta AF, Rudnik MA, Sodt PC. *Heart functional responses to pressure overload in exercised and*

sedentary rats. Am J Physiol 1976;230:199-204.
28. Mercadier JJ, Lompre AM, Wisnewsky C, Samuel JL, Bercovici J, Swynghedauw B, Schwartz K. *Myosin isoenzyme changes in several models of rat cardiac hypertrophy.* Circ Res 1981;49:525-532.
29. Nadal-Ginard B, Mahdavi V. *Molecular basis of cardiac performance. Plasticity of the myocardium generated through protein isoform switches.* J Clin Invest 1989;84:1693-1700.
30. Wilkins BJ, Molkentin JD. *Calcium-calcineurin signaling in the regulation of cardiac hypertrophy.* Biochem Biophys Res Commun 2004;322:1178-1191.
31. Konhilas JP, Widegren U, Allen DL, Paul AC, Cleary A, Leinwand LA. *Loaded wheel running and muscle adaptation in the mouse.* Am J Physiol Heart Circ Physiol 2005;289:H455-465.
32. Neri Serneri GG, Boddi M, Modesti PA, Cecioni I, Coppo M, Padeletti L, Michelucci A, Colella A, Galanti G. *Increased cardiac sympathetic activity and insulin-like growth factor-I formation are associated with physiological hypertrophy in athletes.* Circ Res 2001;89:977-982.

Chapter 9

Nederlandse samenvatting

Pathologische linker ventrikel (LV) remodellering is een aanpassingsmechanisme dat optreedt om te compenseren voor de verstoorde pompfunctie van het hart die ontstaat na een hartinfarct. Hoewel dit proces een goedbedoelde poging is om de pompfunctie van het LV te herstellen, blijkt dat deze aanpassing een onafhankelijke risicofactor is voor het ontwikkelen van hartfalen. De onderliggende mechanismen die verantwoordelijk zijn voor de progressie van LV remodellering naar ernstig hartfalen worden nog niet volkomen begrepen. Recent onderzoek dat werd verricht in samenwerking met de afdeling Fysiologie van de VU universiteit in Amsterdam heeft aangetoond dat verstoringen in krachtontwikkeling van het contractiele apparaat en de afname in de hoeveelheid van de calciumpomp bijdragen aan LV disfunctie als gevolg van een hartinfarct. Niet alleen na een hartinfarct treedt remodellering van het hart op maar ook na langdurige en intensieve inspanningstraining. Echter, in tegenstelling tot de pathologische LV remodellering zoals die optreedt na een hartinfarct leidt fysiologische LV remodellering als gevolg van regelmatige inspanningstraining niet tot een verhoogde kans op hartfalen, maar resulteert zelfs in verbeterde LV functie en wordt geassocieerd met een afname van het risico tot het ontwikkelen van hartfalen. Er is nog veel onbekend over de effecten van inspanningstraining op remodellering en disfunctie van het LV na een hartinfarct. Studies in mensen en knaagdieren beschrijven tegengestelde effecten van inspanningstraining na een hartinfarct. In dit proefschrift is getracht om beter inzicht en begrip te krijgen in de invloed van regelmatige inspanningstraining op afwijkingen van het hart die geassocieerd worden met pathologische LV remodellering en disfunctie als gevolg van een acuut hartinfarct. In het bijzonder hebben we de rol van stikstofmonoxide (NO) in de positieve effecten van inspanningstraining bestudeerd in een muismodel.

In **hoofdstuk 2** van dit proefschrift werden de effecten van 8 weken vrijwillige inspanningstraining welke werd gestart binnen 24 uur na een acuut hartinfarct (overeenkomend met ~43% van de LV massa), op LV remodellering en disfunctie in muizen onderzocht op in vivo, cellulair en moleculair niveau. Acht weken inspanningstraining na een hartinfarct had geen nadelig effect op LV dimensies en hypertrofie, terwijl LV disfunctie en 'backward failure' verbeterde. Bovendien, verbeterde inspanningstraining de door het hartinfarct geïnduceerde disfunctie van het contractiele apparaat. Dit laatste droeg waarschijnlijk bij aan de verbetering in verkorting van geïsoleerde intacte hartspiercellen, aangezien de amplitude van de $[Ca^{2+}]_i$ transient niet veranderde als gevolg van inspanningstraining. Dit laatste kwam overeen met het gebrek aan effect van inspanningstraining op SERCA2a and phospholamban expressie. Inspanningstraining verbeterde tevens basale

diastolische calcium concentraties. Deze effecten van inspanningstraining werden waarschijnlijk gemedieerd via toegenomen eiwitexpressie van de β_1-adrenerge receptor, cAMP en Na^+/Ca^{2+}-'exchanger'. Vervolgproeven zullen gedaan moeten worden om de functionele eigenschappen van de Na^+/Ca^{2+}-'exchanger' te bepalen en om aan te tonen dat de kleine toename in eiwitexpressie van de Na^+/Ca^{2+}-'exchanger' verantwoordelijk was voor de door inspanningstraining geïnduceerde afname in diastolisch calcium, of dat andere mechanismen zoals een toename in sarcolemmale Ca^{2+}-ATPase activiteit ook hieraan bijdroegen.

Verschillende studies suggereren een belangrijke rol voor de productie van endogeen NO in de positieve effecten van inspanningstraining. In vivo studies hebben bijvoorbeeld aangetoond dat inspanningstraining resulteert in een toename in de vasculaire expressie van eNOS, het enzym dat verantwoordelijk is voor de produktie van NO. Tevens blijkt inspanningstraining in gezonde individuen de beschikbaarheid van NO te verhogen via verschillende mechanismen, waaronder een toename in eNOS enzym expressie en activiteit. Deze aanpassingen dragen waarschijnlijk bij aan de bescherming door regelmatige inspanningstraining in individuen met verhoogde kans op cardiovasculaire aandoeningen of coronair vaatlijden. Daarom hebben we in **hoofdstuk 3** de hypothese onderzocht dat eNOS een vereiste rol speelt in de positieve effecten van inspanningstraining op cardiale remodellering en disfunctie na een hartinfarct. Om dit te onderzoeken hebben we wild type, homozygote ($eNOS^{-/-}$) als ook heterozygote ($eNOS^{+/-}$) eNOS-deficiente muizen gebruikt. Het grootste deel van de cardiale afwijkingen die ontstonden na een hartinfarct in normale muizen, zoals remodellering van het LV, waaronder cardiomyocyte hypertrofie en de afname in capillaire dichtheid, toename in interstitieel collageen en apoptose, als ook LV systolische en diastolische functiestoornissen, verergerden niet in $eNOS^{+/-}$ of $eNOS^{-/-}$ muizen. De positieve effecten van inspanningstraining na een hartinfarct op LV systolische disfunctie, pulmonaire congestie, interstitieel fibrose en apoptose in normale muizen gingen verloren in $eNOS^{+/-}$ en $eNOS^{-/-}$ muizen, terwijl LV systolische disfunctie en pulmonaire congestie in $eNOS^{+/-}$ zelfs verergerden als gevolg van inspanningstraining. Daarom kunnen we concluderen dat volledige eNOS expressie vereist is voor de voordelen van inspanningstraining na een hartinfarct. Het zou mogelijk kunnen zijn dat in de afwezigheid van eNOS in $eNOS^{-/-}$ muizen, andere factoren, zoals prostacycline of nNOS, opgereguleerd worden of toegenomen zijn. Op deze manier wordt er gecompenseerd voor de afwezigheid van eNOS en verdere verslechtering van cardiale remodellering en functie na een hartinfarct voorkomen. Wanneer slechts één eNOS allel functioneel is, in $eNOS^{+/-}$ muizen, en zorgt voor normale basale eNOS expressie, zullen zulke

compensatoire mechanismen waarschijnlijk niet optreden en zo het hart meer kwetsbaar maken voor de effecten van inspanningstraining. Om hiervan zeker te zijn zullen vervolgproeven gedaan moeten worden om de invloed van potentiële compensatiefactoren te bestuderen. De positieve effecten van inspanningstraining op de cardiale functie kunnen op zijn minst gedeeltelijk worden toegeschreven aan een door inspanningstraining geïnduceerde toename in eNOS expressie en activiteit. Daarom hebben we onderzocht of de positieve effecten van inspanningstraining na een hartinfarct kunnen worden nagebootst door toegenomen spiegels van eNOS. Als eerste bestudeerden we in **hoofdstuk 4** de in vivo effecten van een 10-12 keer verhoogde eNOS expressie op het cardiovasculaire systeem in een transgeen muismodel. Verhoogde eNOS expressie zorgde voor een afname in bloeddruk, plasma cholesterol en atherosclerose. Vervolgens werd dit muismodel met verhoogde eNOS expressie gebruikt in **hoofdstuk 5** om te onderzoeken of verhoogde eNOS expressie de positieve effecten van inspanningstraining op LV remodellering en disfunctie na een hartinfarct kon nabootsen. De resultaten duidden erop dat in een muis met een hartinfarct, enkelvoudige therapie van inspanningstraining of verhoogde eNOS expressie globale LV fractionele verkorting en de kracht ontwikkeling van cardiomyocyten verbeterde, collageen afnam en pulmonaire congestie verminderde, hoewel alleen verhoogde eNOS expressie de overlevingskans van de muizen na een hartinfarct verbeterde. Gezien de complementaire mechanismen waarlangs inspanningstraining en verhoogde eNOS expressie positieve effecten bleken te hebben, testten we in het tweede deel van **hoofdstuk 5** de hypothese dat inspanningstraining gecombineerd met verhoogde eNOS expressie, als behandelingstherapie, een additioneel voordeel zou hebben en onderzochten de onderliggende mechanismen. In contrast met onze hypothese lieten de resultaten onverwacht zien dat inspanningstraining samen met verhoogde eNOS expressie de positieve effecten van beide behandelingen apart op globale cardiale functie na een hartinfarct deden verdwijnen. We toonden aan dat deze nadelige interactie het resultaat was van verhoogde oxidatieve stress, als gevolg van eNOS 'ontkoppeling', aangezien een tekort van substraat (L-arginine) en/of co-factoren (BH4) van eNOS kunnen resulteren in eNOS 'ontkoppeling' en daardoor worden reactieve zuurstof radicalen, zoals super oxide, in plaats van NO gevormd. Uit deze studie kon worden geconcludeerd dat inspanningstraining niet in elke situatie ongevaarlijk is, maar kan leiden tot een toename in oxidatieve stress. Verscheidene onderzoekers hebben gesuggereerd dat eNOS een positieve rol zou kunnen spelen in patienten met cardiovasculaire aandoeningen. Momenteel is er veel interesse naar de ontwikkeling van gentherapie in de behandeling

voor cardiovasculaire ziekten. De resultaten uit onze studie laten zien dat, in de toekomst, eNOS gentherapie in combinatie met fysieke inspanning (als onderdeel van veranderingen in leefstijl) zorgvuldig uitgebalanseerde voedingsmiddelen supplementen, als L-arginine en BH4, vereist om potentiële eNOS 'ontkoppeling' en verhoogde oxidatieve stress te voorkomen. Pre-klinische proefdierstudies zullen nodig zijn om in meer detail de optimale condities van fysieke inspanningstraining in combinatie met voedingsmiddelen supplementen te bepalen en zo eNOS gentherapie voor de behandeling van pathologische cardiale remodellering te kunnen gaan gebruiken.

Om inzicht te verkrijgen in de effecten van verhoogd cGMP op de functie van het hart, hebben we muizen gebruikt die 'C-type natriuretic peptide' (CNP) tot overexpressie brengen. cGMP wordt gesynthetiseerd uit GTP door 'soluble guanylyl cyclases' (sGC) en door 'particulate guanylyl cyclases' (pGC), deze 'cyclases' worden onderscheiden door de functie van hun belangrijkste subcellulaire lokatie en activatie. sGC zijn hoofdzakelijk gelokaliseerd in het cytosol en worden geactiveerd door NO, terwijl pGC integrale eiwitten van het celmembraan zijn die geactiveerd worden door bijvoorbeeld CNP. In **hoofdstuk 6** is onderzocht of verhoogd cGMP via CNP activatie van pGC, in muizen met CNP overexpressie, resulteerde in verbeterde functie van het hart na een hartinfarct. De resultaten lieten zien dat verhoogde CNP expressie, die beperkt was tot de cardiomyocyten, door een hartinfarct geïndiceerde cardiale hypertrofie beperkt en weinig effect heeft op LV disfunctie. Deze studie geeft dus aan dat het CNP/cGMP signaaltransductiepad een belangrijke rol speelt in de locale regulatie van cardiale hypertrofie onder pathofysiologische omstandigheden. Modulatie van dit signaaltransductiepad zou een potent nieuw therapeutisch doel kunnen zijn voor de behandeling van patienten met een hartinfarct of door hypertensie geïnduceerde hypertrofie van het hart.

Ten slotte wordt er gesuggereerd dat fysieke inactiviteit een onafhankelijke risicofactor is voor het ontwikkelen van cardiovasculaire aandoeningen. Epidemiologische studies laten inderdaad zien dat matige (b.v. wandelen) en heftige inspanningstraining in gezonde mensen wordt geassocieerd met een substantiele afname in de incidentie van cardiovasculaire aandoeningen. De positieve effecten van inspanningstraining strekken zich uit tot patienten met ernstige hartziekten, waarbij regelmatige fysieke activiteit de incidentie van cardiale aandoeningen en sterfte door andere oorzaken dan cardiale aandoeningen reduceerd. Verder is het bewezen dat wanneer inspanningstraining gestart wordt na een cardiaal event, bijvoorbeeld een hartinfarct, LV remodellering en disfunctie (hoofdstuk 2 van dit proefschrift) zullen verbeteren. Er is echter weinig bekend over de

beschermende effecten van inspanningstraining wanneer deze al wordt gestart voordat een cardiaal event, zoals een hartinfarct, plaats vindt. **Hoofdstuk 7** van dit proefschrift beschrijft dat wanneer inspanningstraining gestart werd voorafgaand of na een hartinfarct, LV disfunctie verminderde, terwijl inspanningstraining voorafgaand aan een hartinfarct ook de overlevingskans verbeterde, dit laatste was waarschijnlijk het gevolg van verbeterde genezing van het infarct(litteken)weefsel. Deze resultaten laten zien dat zelfs wanneer regelmatige fysieke activiteit niet een acuut hartinfarct kan voorkomen, het wel kan zorgen voor verbeterde functie van het hart en overlevingskans na een hartinfarct. In contrast hiermee gaan de positieve effecten van inspanningstraining, wanneer deze start voor of na een hartinfarct, op LV disfunctie verloren in muizen die werden blootgesteld aan inspanningstraining zowel voor als na het optreden van een hartinfarct (EX-MI-EX). Hoewel deze waarneming moeilijk te verklaren is, moeten we er rekening mee houden dat de muizen die al 2 weken hebben kunnen wennen aan de tredmolen voor het optreden van het hartinfarct (EX-MI-EX), direct na het hartinfarct langere afstanden liepen per dag (~7 km/dag gedurende de eerste week) in vergelijking met de in eerste instantie kortere afstanden (~2 km/day during the first week) die per dag werden afgelegd door muizen die niet voor het hartinfarct gewend waren aan de tredmolen (SED-MI-EX). Daarom kunnen we niet uitsluiten dat deze verschillen in acute inspanningstrainingbelasting direct na het hartinfarct sommige van de positieve effecten van inspanningstraining na het hartinfarct te niet doen. Toekomstige studies zullen zich in meer detail moeten richten op de invloed van inspanningstrainingbelasting direct na het hartinfarct op LV disfunctie. Eeen mogelijkheid is om vrijwillige inspanningstraining te beperken in EX-MI-EX muizen in de eerste week na een hartinfarct. Een alternatief zou kunnen zijn om de SED-MI-EX muizen voor een korte periode (1-2 dagen) te laten wennen aan de tredmolen, om zo de dagelijkse loopafstand direct na een hartinfarct te laten toenemen. Het preciese mechanisme voor verbeterde LV disfunctie in muizen waarbij inspanningstraining werd gestart voor een hartinfarct kan niet worden bepaald uit de resultaten die gepresenteerd worden in dit proefschrift. Het is bewezen dat harten van dieren die getraind zijn meer effectief reageren op stress. In getrainde ratten is bijvoorbeeld aangetoond dat de contractiliteit van het hart behouden blijft of zelfs toeneemt na een langdurige periode van drukbelasting, in vergelijking met sedentair gehuisveste ratten. Voor onze studie zullen vervolgproeven nodig zijn om de moleculaire mechanismen aan te tonen die bijdragen aan de verbeterde LV disfunctie door inspanningstraining wanneer die gestart wordt voor een hartinfarct. Dit houdt ook in dat mogelijke inspanningstraining geïnduceerde verbeteringen in

functie van intacte hartspiercellen moeten worden bestudeerd.

Toekomstig onderzoek

Hartspiercellen ondergaan hypertrofe groei of apoptose na het optreden van een hartinfarct. Deze reactie wordt gekoppeld aan de ontwikkeling van ziekten van het hart. Er is veel onderzoek gedaan naar de prikkels die verantwoordelijk zijn voor het aanzetten van deze processen en om de onderliggende signaaltransductiepaden te identificeren. Deze signaaltransductiepaden zijn zeer waarschijnlijk verantwoordelijk voor het modificeren van gen- en eiwitexpressie. Pathologische hypertrofie van het hart gaat gepaard met het induceren van genen die normaal gesproken tot uiting komen gedurende de embryonale ontwikkeling, bijvoorbeeld 'atrial natriuretic factor', 'brain natriuretic peptide', 'skeletal α–actin' en 'β-myosin heavy chain'. Recent onderzoek is gericht op het identificeren van de moleculaire signaaltransductiepaden en de transcriptie factoren die pathologische hypertrofie van de hartspiercellen reguleren, bijvoorbeeld 'calcineurin-nuclear factor', 'activated T-cells (NFAT) pathway' en 'myocyte enhancer factor-2 (MEF-2)'. 'Insulin-like growth factor 1 (IGF1)', en zijn 'downstream' PI3-kinase pad, blijkt betrokken te zijn bij de regulatie van lichaams- en orgaangrootte gedurende postnatale ontwikkeling en komt vrij in respons op inspanningstraining.

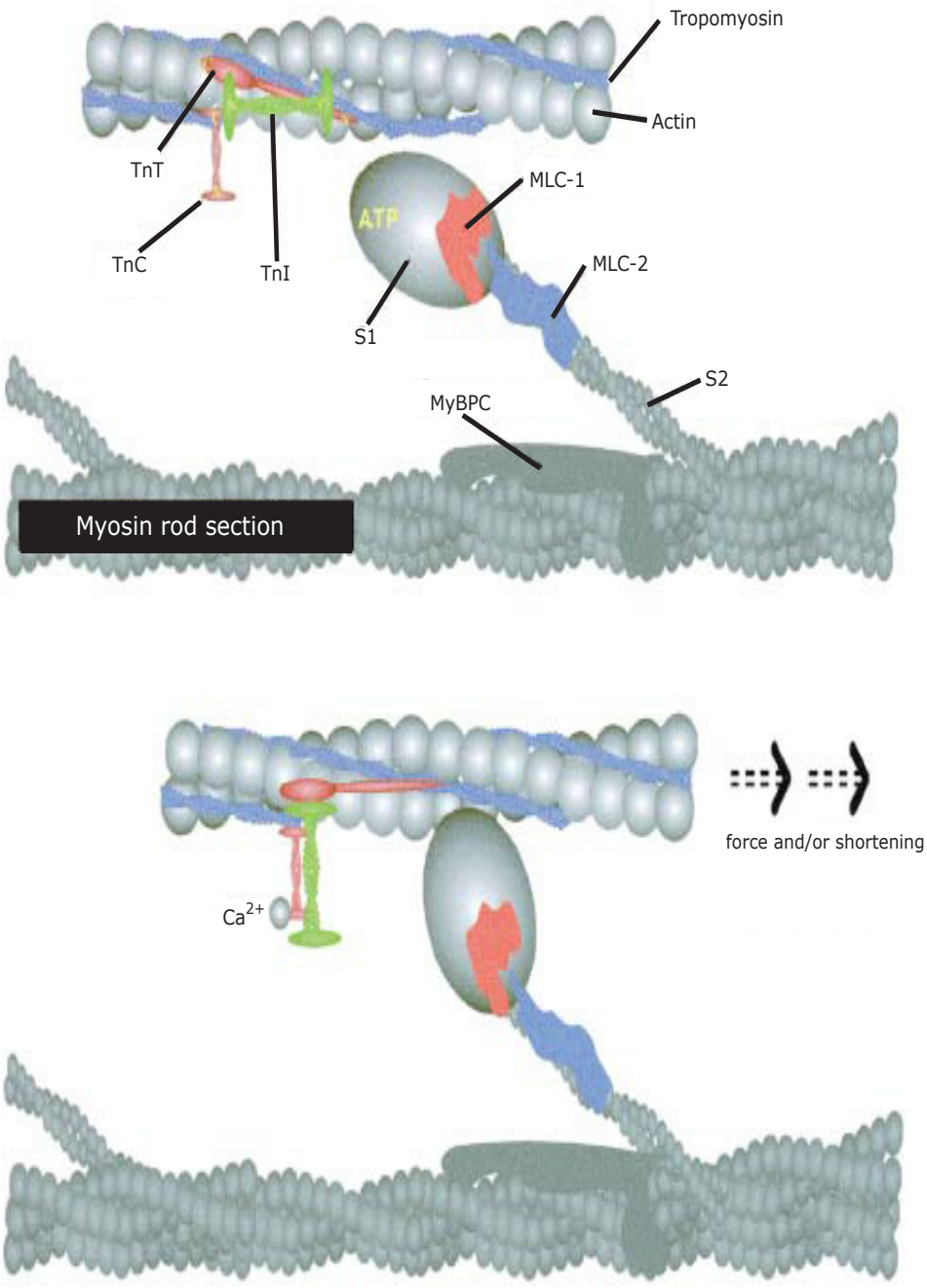
Binnen ons onderzoek zijn toekomstige studies nodig om in de vroege fase na een hartinfarct de signaaltransductiepaden en genexpressie die verantwoordelijk zijn voor pathologische remodellering van het hart te bestuderen. Ook zullen de verschillen en/of overeenkomsten tussen deze paden moeten worden vergeleken met fysiologische remodellering van het hart als gevolg van inspanningstraining. Door te onderzoeken welke signaaltransductiepaden betrokken zijn in de regulatie van de genexpressie, de transcriptiefactoren die ze reguleren en de veranderingen die optreden in de genexpressie die betrokken zijn in ons model van een groot hartinfarct en ons model voor inspanningstraining, kunnen we de rol van inspanningstraining op deze paden beter begrijpen. Verder is het relevant om te weten of inspanningstraining na een hartinfarct de ontwikkeling van pathologische remodellering positief kan beïnvloeden in de richting van de goede fysiologische remodellering. Bovendien is het interessant om de paden te identificeren die betrokken zijn bij de positieve effecten van inspanningstraining wanneer deze gestart wordt voordat een hartinfarct plaats vindt, om zo in de toekomst patienten te kunnen behandelen tegen pathologische remodellering.

Tot slot is het interessant om een meta-analyse van cohort studies uit te voeren, om zo de invloed van fysieke fitheid in de jaren voordat een acuut hartinfarct

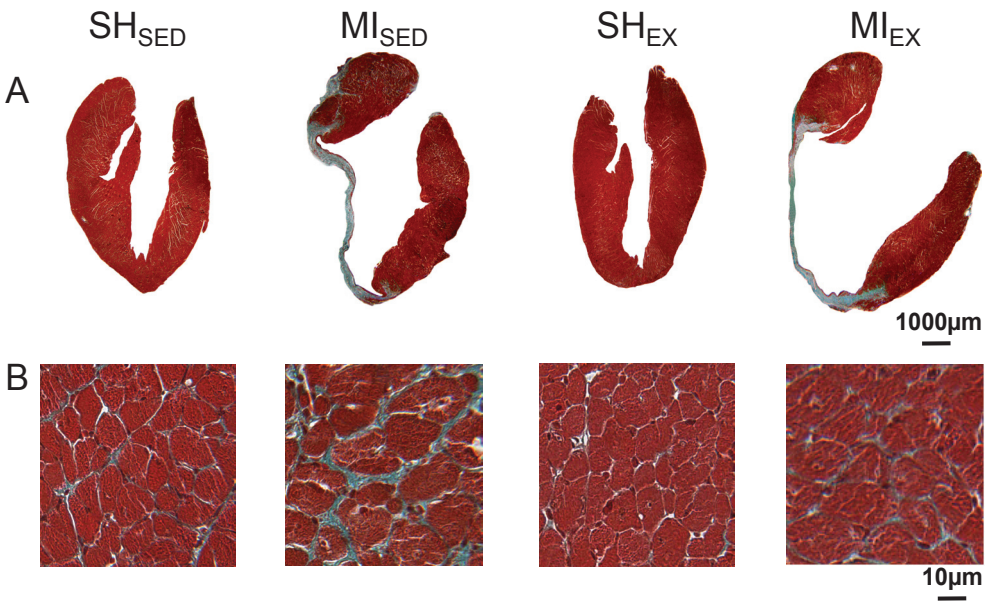
optreedt op de overlevingskans, infarct grootte en/of functie van het hart te onderzoeken onder mensen die niet eerder een cardiovasculair event hebben meegemaakt.

Chapter 10

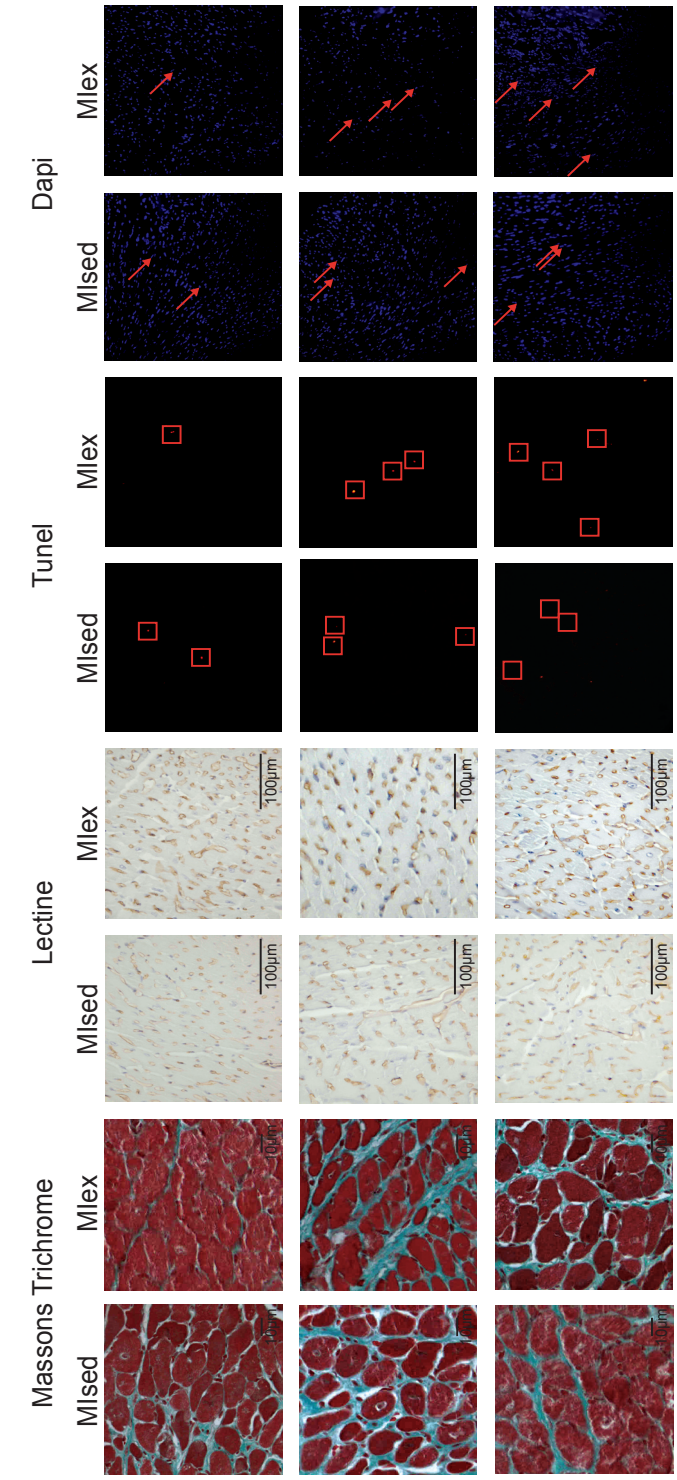
Colour section



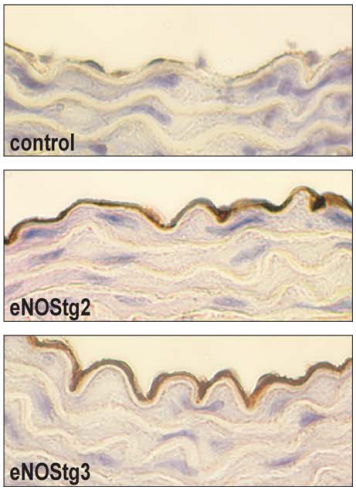
Chapter 1
Figure 2: Sarcomere structure.
The major proteins that are involved in contractile activation and regulation are shown in diastole (top panel) and systole (bottom panel).[20] Abbreviations: TnI: troponin I, TnT: troponin T, TnC: troponin C, MLC: myosin light chain, globular head portion (S1) and hinged stalk region (S2) of myosin, MyBPC: myosin binding protein C, Ca^{2+}: calcium.



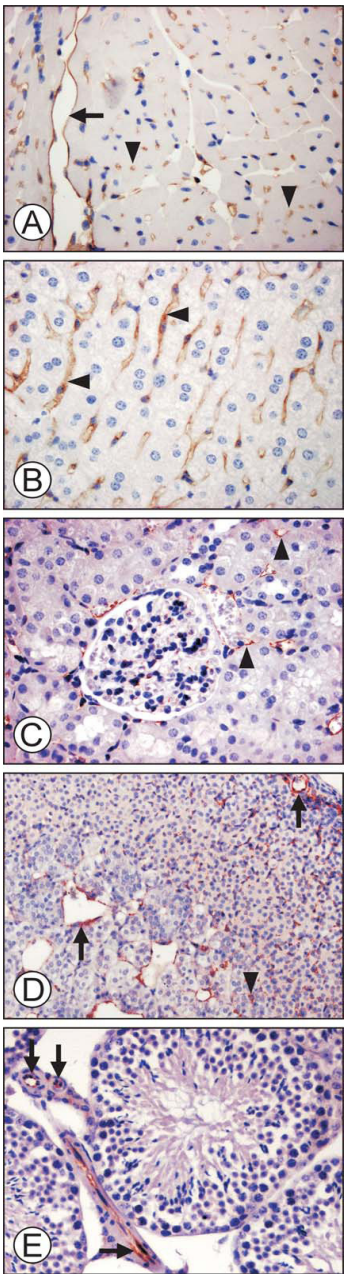
Chapter 2
Figure 2:
A, Masson's trichrome staining showing the green/blue infarct area in longitudinal cross-section of the LV, 8 weeks after permanent LAD ligation. MI includes the anterior wall and apical part of the LV. B, Magnifications of the Masson's trichrome image showing collagen and myocyte CSA in viable myocardium (remote zone in MI mice).



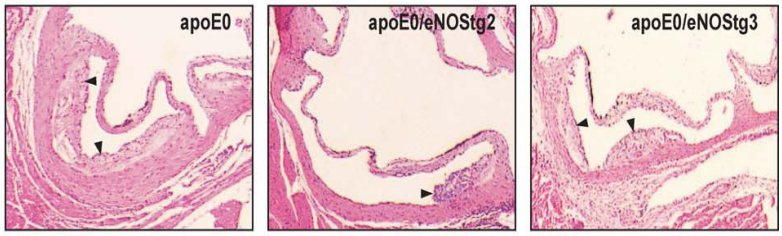
Chapter 3
Figure 5:
Representative histological examples from Masson's trichrome, lectin, TUNEL and Dapi staining in MI_{SED} and MI_{EX} mice.



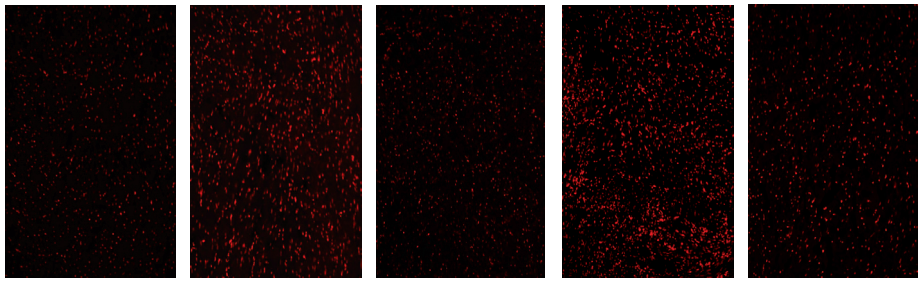
Chapter 4
Figure 1C:
Immunohistochemistry on aortas from control, eNOStg2, or -3 mice. Aortas were collected after in situ fixation. Paraffin sections were incubated with antihuman eNOS antibody and a peroxidase-conjugated secondary antibody. Original magnification, x630.



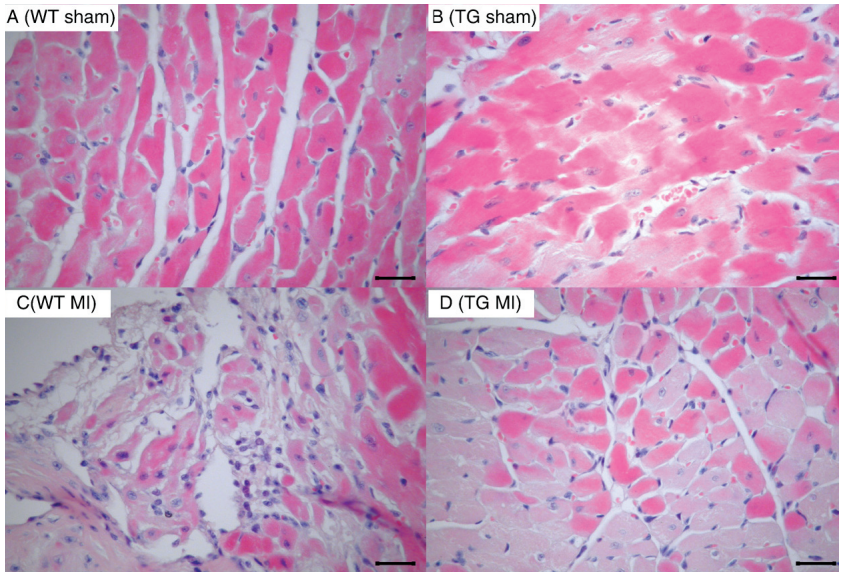
Chapter 4
Figure 2: Expression pattern of human eNOS in transgenic mice
Organs from eNOStg2 mice were collected after in situ fixation. Paraffin sections were incubated with antihuman eNOS antibody and a peroxidase-conjugated secondary antibody. A, heart. B, liver. C, kidney. D, adrenal. E, testis tissue. Arrowheads indicate representative immunoreactive capillaries; arrows indicate larger blood vessels. Original magnification, ×400.



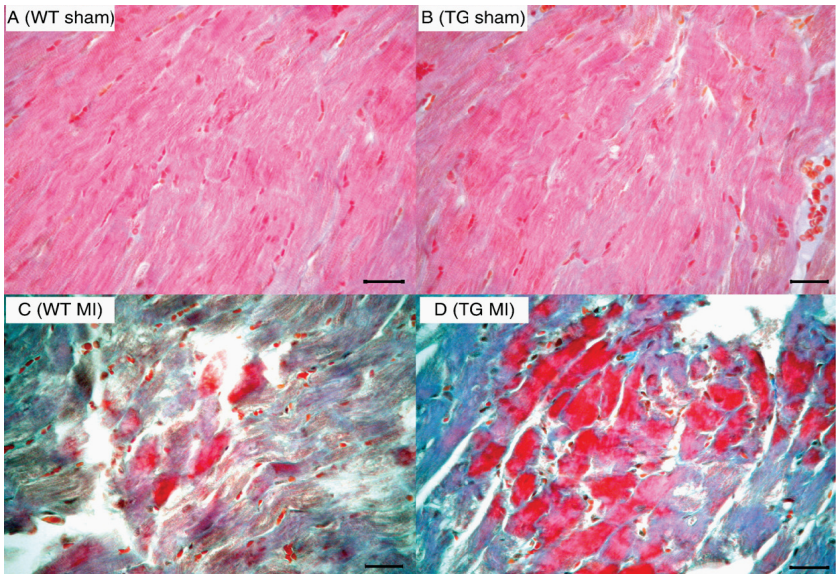
Chapter 4
Figure 4B:
Representative photomicrographs of hematoxylin and eosin-stained paraffin sections with lesion areas in the aortic valves (arrowheads). Original magnification, ×25.



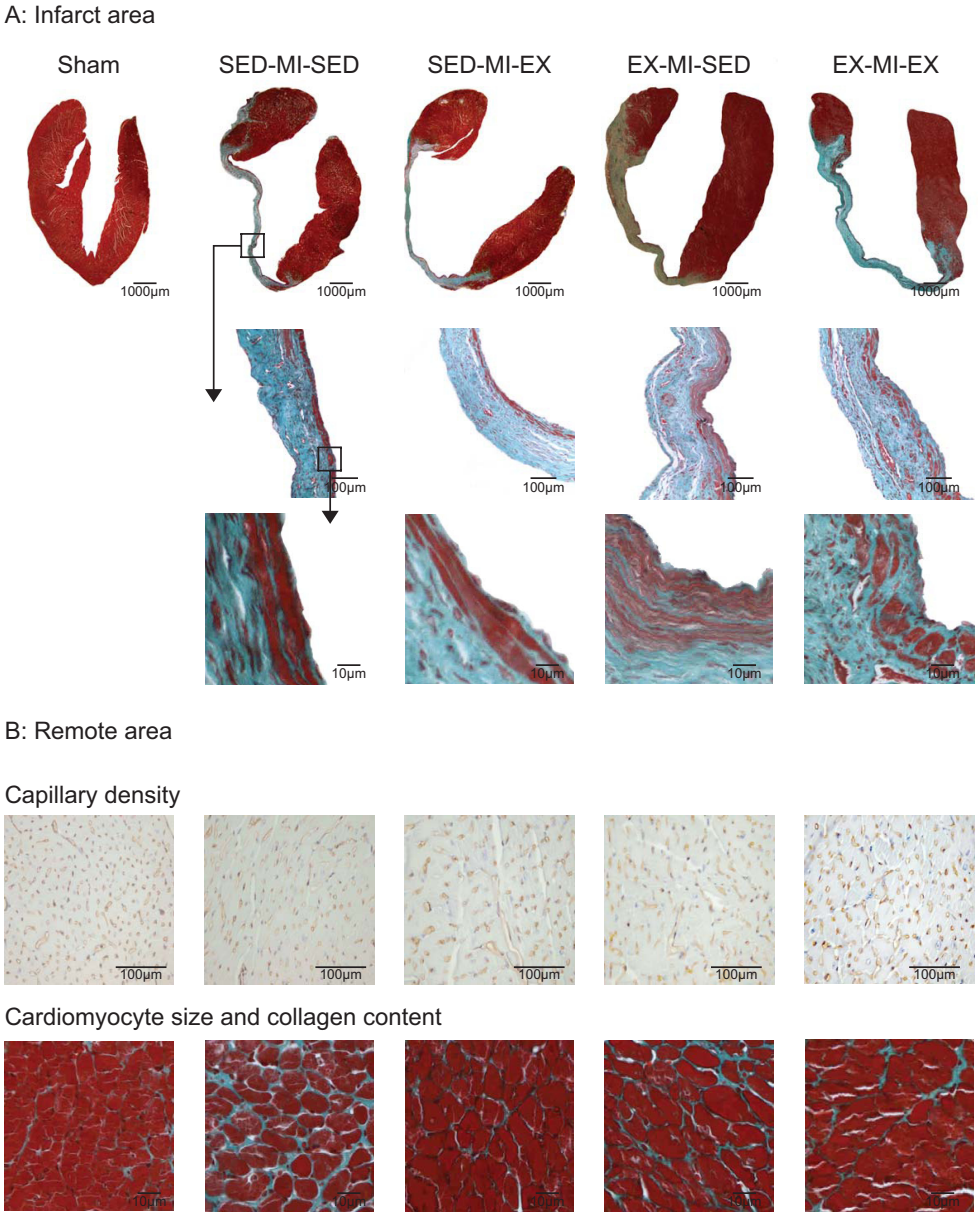
Chapter 5
Figure 5B:
Representative examples of dihydroethidium fluorescence (x200).



Chapter 6
Figure 4. Cardiac morphological changes after myocardial infarction.
Normal-appearing cadiomyocytes were found in sham-operated wild-type (WT) and transgenic (TG) mice (A and B). There was prominent muscular degeneration and a moderate mononuclear inflammatory infiltrate in the infarcted area of wildtype animals (C), while only mild degeneration of myofibrils was observed in the infarcted transgenic group, and there is no mononuclear inflammatory infiltrate (D). Representative photographs stained with hematoxylin and eosin (H.-E.) are given. Bar=100 μm.



Chapter 6
Figure 5. Histological analysis to determine the effect of CNP over-expression on cardiac fibrosis.
Normal myofibrils (red staining) were found in sham-operated wild-type (WT) and transgenic (TG) mice with no detectable fibrosis (blue staining) (A and B). Marked fibrosis and few muscle fibers were observed in the infarcted area of wild-type mice (C). Infarcted transgenic mice showed still marked fibrosis but more abundant myofibrils (D). Representative Ladewig staining photographs are given. Bar=100 μm.



Chapter 7
Figure 3:
Macroscopic and microscopic representative examples of LV infarct (A) and remote (B) myocardium.

Dankwoord

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Monique

List of publications

Full papers

1. van Haperen R, **de Waard MC**, van Deel ED, Mees BME, Kutryk M, van Aken T, Hamming J, Grosveld F, Duncker DJ, de Crom R. *Reduction of blood pressure, plasma cholesterol, and atherosclerosis by elevated endothelial nitric oxide.* J Biol Chem. 2002 Dec 13;277(50):48803-48807
2. van Haperen R, Cheng C, Mees BME, van Deel E, **de Waard MC**, van Damme LC, van Gent T, van Aken T, Krams R, Duncker DJ, de Crom R. *Functional expression of endothelial nitric oxide synthase fused to green fluorescent protein in transgenic mice.* Am J Pathol. 2003 Oct;163(4):1677-86
3. Duncker DJ, van Haperen R, van Deel E, **de Waard MC**, Mees BME, de Crom R. *Endothelial nitric oxide synthase in cardiovascular homeostasis and disease.* Physiological genomics of the critically Ill mouse. 2004: 291-309
4. van den Bos EJ, Mees BME, **de Waard MC**, de Crom R, Duncker DJ. *A novel model of cryoinjury-induced myocardial infarction in the mouse: A comparison with coronary artery ligation.* Am J Physiol Heart Circ Physiol. 2005 Sep; 289(3):H1291-300
5. Cheng C, van Haperen R, **de Waard MC**, van Damme LCA, Tempel D, Hanemaaijer L, van Cappelen GWA, Bos J, Slager CJ, de Crom R, Krams R. *Shear stress affects the intracellular distribution of eNOS: direct demonstration by a novel in vivo technique.* Blood. 2005 Dec 1; 106(12):3691-8
6. Hanada K, Vermeij M, Garinis GA, **de Waard MC**, Kunen MGS, Myers l, Maas A, Duncker DJ, Meijers C, Dietz HC, Kanaar R, Essers J. *Perturbations of vascular homeostasis and aortic valve abnormalities in Fibulin-4 deficient mice.* Circ. Res. 2007 Mar 16; 100(5):738-46
7. **de Waard MC**, van der Velden J, Boontje NM, Dekkers DHW, Schoonderwoerd K, Schuurbiers JCH, de Crom R, Stienen GJM, Lamers JMJ, Duncker DJ. *Early exercise training normalizes myofilament function and attenuates left ventricular pump dysfunction in mice with a large myocardial infarction.* Circ. Res. 2007 Apr 13;100(7):1079-1088
8. Wang Y*****, **de Waard MC***, Sterner-Kock A, Stepan H, Schultheiss HP, Duncker DJ, Walther T. *****:both authors contributed equally to this work. *Cardiomyocyte-restricted over-expression of C-type natriuretic peptide prevents cardiac hypertrophy induced by myocardial infarction in mice.*

Eur. J. Heart Failure. 2007 Apr 2;9: 548-557

9. van Deel ED, Merkus D, van Haperen R, **de Waard MC**, de Crom R, Duncker DJ. *Vasomotor control in mice overexpressing human endothelial nitric oxide synthase.*
   Am J Physiol Heart Circ Physiol. 2007 Aug;293(2):H1144-53
10. Boer C, **de Waard MC**, Beurmans H, Gan C, Vonk Noordergraaf A, Duncker DJ. *Septal and biventricular remodeling in pulmonary hypertension:general concepts in mouse and human hearts.*
    Submitted
11. van Til N, Stok M, Aerts F, Visser T, Kroos M, **de Waard MC**, Jacobs E, Willart M, van der Wegen P, Scholte B, Lambrecht B, Verstegen M, Duncker DJ, van der Ploeg A, Reuser A, Wagemaker G. *Lentiviral gene therapy of hematopoietic stem cells results in phenotype correction in a mouse model of Pompe disease.*
    Submitted
12. **de Waard MC**, van der Velden J, Boontje NM, Dekkers DHW, van Haperen R, Lamers JMJ, de Crom R, Duncker DJ. *Detrimental effect of combined exercise and eNOS-overexpression on cardiac remodeling and dysfunction after myocardial infarction.*
    Previsionally accepted in AJP (invited first revision)
13. van den Bos EJ, **de Waard MC**, Davis BH, Bergen J, Taylor DA, Duncker DJ. *Mechanisms and dynamics of myoblast death after transplantation into a myocardial infarction.*
    Submitted
14. **de Waard MC**, van Haperen R, de Crom R, Duncker DJ. *Beneficial effects of exercise training after myocardial infarction requires full eNOS activity.*
    Submitted
15. **de Waard MC**, Nieuwenhuizen IM, Severijnen L, Papoutsi T, Chaudhry B, Duncker DJ, Oostra BA, Willemsen R, Mientjes EJ. *Cardiac failure is unlikely the cause of neonatal lethality in Fxr1 knockout mice.*
    In preparation to be submitted in 2008
16. **de Waard MC** and Duncker DJ. *Prior exercise training improves survival, infarct healing and left ventricular function after myocardial infarction.*
    Submitted
17. Bito V, **de Waard MC**, Ozdemir S, Lenaerts I, Biesmans L, Duncker DJ, Sipido KR. *Exercise training partially reverses cellular remodeling and contractile function after myocardial infarction*
    In preparation to be submitted in 2008

Abstracts

1. van Deel ED, **de Waard MC**, Stubinitsky R, Verdouw PD, Duncker DJ. *Influence of anesthesia and fluid-supplementation on heart rate and aortic blood pressure and their stability in FVB mice.* Basic Research in Cardiology 2000; 95:522
2. Cheng C, van Haperen R, **de Waard MC**, de Crom R, Krams R. *eNOS expression responds to changes in shear stress: direct demonstration in a novel in vivo mouse model.* Circulation 2003; 108;IV-151
3. **de Waard MC**, van Deel ED, Merkus D, de Vos A, van Haperen R, de Crom R, Duncker DJ. *Exercise training and eNOS overexpression improve left ventricular pump function after myocardial infarction via different mechanisms.* Circulation 2003; 108:IV-94
4. Cheng C, **de Waard MC**, van haperen R, Tempel D, van der Baan A, Krams R, de Crom R. *In vivo induction of low shear stress and oscillatory shear stress causes atherosclerosis.* Circulation 2004; 110: III-39
5. **de Waard MC**, van Deel ED, Merkus D, van HaperenR, de Crom R, Duncker DJ. *Beneficial effect of exercise training on cardiac function is lost in mice that overexpress endothelial NO synthase.* Circulation 2004; 110:III-299
6. van Deel ED, **de Waard MC**, Merkus D, van Haperen R, de Crom R, Duncker DJ. Altered systemic vasomotor control in mice overexpressing endothelial nitric oxide synthase. The Faseb J 2005; 19:A1244
7. Boer C, **de Waard MC**, Beurmans H, Gan C, Vonk Noordergraaf A, Duncker DJ. *Septal and biventricular remodeling in pulmonary hypertension: general concepts in mouse and human hearts.* The Faseb J 2005; 19:A1332-A1333
8. **de Waard MC**, van der Velden J, Boontje NM, de Crom R, Duncker DJ. *Exercise training ameliorates left ventricular dysfunction after myocardial infarction by preventing myofilament dysfunction.* Circulation 2005; 112:II-279
9. **de Waard MC**, van Haperen R, de Crom R, Duncker DJ. *Beneficial effects of exercise training after myocardial infarction are lost in mice lacking one eNOS allele.* Circulation 2005; 112:II-257
10. Hanada K, Vermeij M, Garinis G, **de Waard MC**, Kunen M, Duncker DJ, Meijers C, Kanaar R, Essers J. *Reduction of Fibulin-4 expression affects elastogenesis and results in aortic aneurysm and dissection.* Circulation 2005; 97:1201
11. Wang Y, **de Waard MC**, Sterner-Kock A, Duncker DJ, Walther T. *Cardiomyocyte-restricted over-expression of C-type natriuretic peptide prevents cardiac hypertrophy induced by myocardial infarction in mice.* Circulation 2006; 114: II_124-II_125
12. Bito V, Ozdemir S, Biesmans L, **de Waard MC**, van der Velden J, Duncker

DJ, Sipido KR. *Early exercise training after myocardial infarction improves cardiomyocyte function.* Eur Heart J 2007; 28:226-227

13. van Deel ED, **de Waard MC**, Merkus D, Duncker DJ. *Exercise training ameliorates left ventricular dysfunction after myocardial infarction but not left ventricular dysfunction in pressure overload induced cardiac hypertrophy.* The Faseb J 2007; 21:lb430
14. **de Waard MC** and Duncker DJ. *Exercise training started before myocardial infarction improves survival but aggravates left ventricular dysfunction.* Circulation 2007; 116:II_311-II_312

Curriculum Vitae

Monique Chantal de Waard werd op 2 juni 1978 geboren in Hardinxveld-Giessendam. In 1996 behaalde zij haar HAVO diploma aan het Willen de Zwijger College in Papendrecht. In september 1996 startte ze, in Delft, aan de Hogeschool Rotterdam met de opleiding Hoger Laboratorium Onderwijs en studeerde in juni 2000 af in de richting Medische Biologie. In het laatste jaar van deze studie liep ze stage bij Experimentele Cardiologie, een onderzoeksafdeling van het Thoraxcentrum onderdeel van het Erasmus MC, toen onder leiding van Prof.Dr. P.D. Verdouw, waar ze werkte aan het opzetten en ontwikkelen van technieken voor de cardiovasculaire karakterisatie van transgene muizen met verschillende genconstructen voor de overexpressie van humaan endothelial NO-synthase. In september 2000 begon ze als research analist op diezelfde afdeling en vanaf begin 2004 doet Monique promotie onderzoek bij Experimentele Cardiologie, onder leiding van Prof.Dr. D.J. Duncker, naar de beschermende effecten van inspanningstraining bij muizen met een hartinfarct en de rol van stikstof oxide hierin. Tijdens deze periode in 2007 behaalde zij de ‘Master of Science in Clinical Research’ met de specialisatie ‘Cardiovascular Sciences’ aan de cardiovasculaire research school ‘COEUR’.