DOCTORAL (Ph.D.) THESIS

ALTERATIONS IN MYOCARDIAL FUNCTION AND CONTRACTILE PROTEINS DURING HUMAN HEART FAILURE

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CONTENTS

INTRODUCTION

Heart failure is the final common pathway to death in cardiovascular disease, including pressure overload (i.e. hypertension), volume overload (i.e. mitral regurgitation), ischaemia-reperfusion injury, myocardial infarction, and inherited or acquired cardiomyopathies (Drexler & Hasenfuss, 2004). Heart failure is a major health care problem and one of the most frequent reasons for patients to be admitted to hospital. The incidence of heart failure is increasing rapidly (1% of the population in the Western world), particularly with the aging of the population. Despite significant advances in its treatment, the prognosis of heart failure remains poor.

The performance of the heart as a pump depends on the coronary circulation, the function of the myocytes and the composition of the extracellular matrix. At the level of the myocyte the contraction-relaxation cycle is controlled by excitation-contraction (EC) coupling.

Physiology of cardiac contraction and relaxation

Excitation–contraction (EC) coupling

The term EC coupling describes the process that converts electrical depolarization of the plasma membrane to contraction of the cardiomyocyte. Depolarization of the plasma membrane during the cardiac action potential causes the activation of voltage-gated L-type calcium (Ca^{2+}) channels (LTCC, or dihydropyridine receptors) in the sarcolemmal membrane encompassing the transverse (T) tubules. Additional Ca^{2+} can enter via the T-type Ca^{2+} channels (TTCC) or the Na^{+}/Ca^{2+} exchanger (NCX) in its reverse mode. The ensuing Ca^{2+} influx then triggers a much greater Ca^{2+} release from the sarcoplasmic reticulum (SR) via ryanodine receptors (RyR2) through a process called Ca^{2+}-induced Ca^{2+} release (CICR). The release of Ca^{2+} from the SR in the cardiomyocyte initiates contraction of the heart during *systole* (*Figure 1*) (Bers, 2002).

The approximately tenfold increase in cytoplasmic Ca^{2+} concentration during systole (from ~0,1μM to ~1μM) results in actin–myosin crossbridge formation and contraction of the myocyte.

Myocardial relaxation during *diastole* is initiated by the removal of Ca^{2+} from the cytoplasm, which results in deactivation of the contractile machinery. Cytosolic Ca^{2+} is pumped back into the SR by sarcoplasmic reticulum ATP-ase (SERCA2a). The activity of

this enzyme is regulated by the binding of phospholamban (PLB). In its non-phosphorylated form, PLB inhibits SERCA2a activity, whereas phosphorylation of PLB reverses the inhibition. Cytosolic Ca^{2+} can also be expelled from the cardiomyocyte via the sarcolemmal NCX (forward mode).

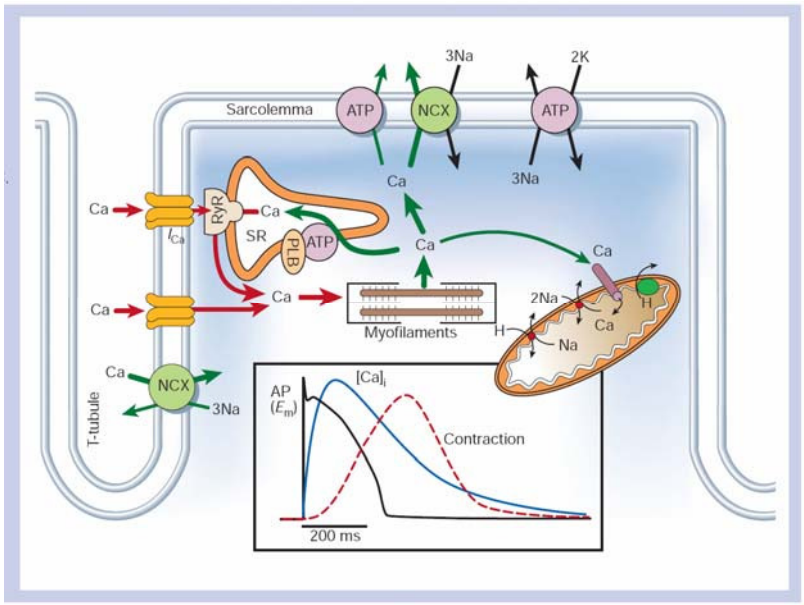

Figure 1. Ca^{2+} transport in ventricular myocytes. Inset shows the time course of an action potential, Ca^{2+} transient and contraction measured in a rabbit ventricular myocyte at 37°C. NCX, Na^{+}/Ca^{2+} exchange; ATP, ATPase; PLB, phospholamban; SR, sarcoplasmic reticulum (Bers, DM., (2002) *Nature*, Vol. 415, 198-205.).

Composition, function and regulation of the myocardial contractile system

The contractile machinery is composed of the thick (myosin) and thin (actin) filaments, the thin filament regulatory system and cytoskeleton components. The fundamental contractile unit in the myocardium is the sarcomere, which spans from Z-line to Z-line (*Figure 2A*). The Z-lines appear to be the anchor proteins at which intermediate filaments of the cytoskeleton are connected to actin filaments (Drexler & Hasenfuss, 2004).

The *thick filament* is composed of myosin, of which each molecule has a long α-helical tail and a globular head (*Figure 2B*). The heads form crossbridges, which interact with the thin filament, containing the site of ATP hydrolysis, and have two light chains associated with each head. The *thin filament* is composed of two chains of the globular protein G-actin, which form the helical, double-stranded actin polymer. In the groove between the actin strands a long flexible protein, tropomyosin is located. Furthermore, at every seventh actin there is a troponin complex attached to tropomyosin. The *tropomyosin-troponin system* represents the regulatory unit of the contractile machinery. The troponin complex is made up of three

subunits: troponin T (TnT, or the tropomyosin-binding subunit), troponin C (TnC, or the Ca^{2+} binding subunit) and troponin I (TnI or the inhibitory subunit) (Zot & Potter, 1987).

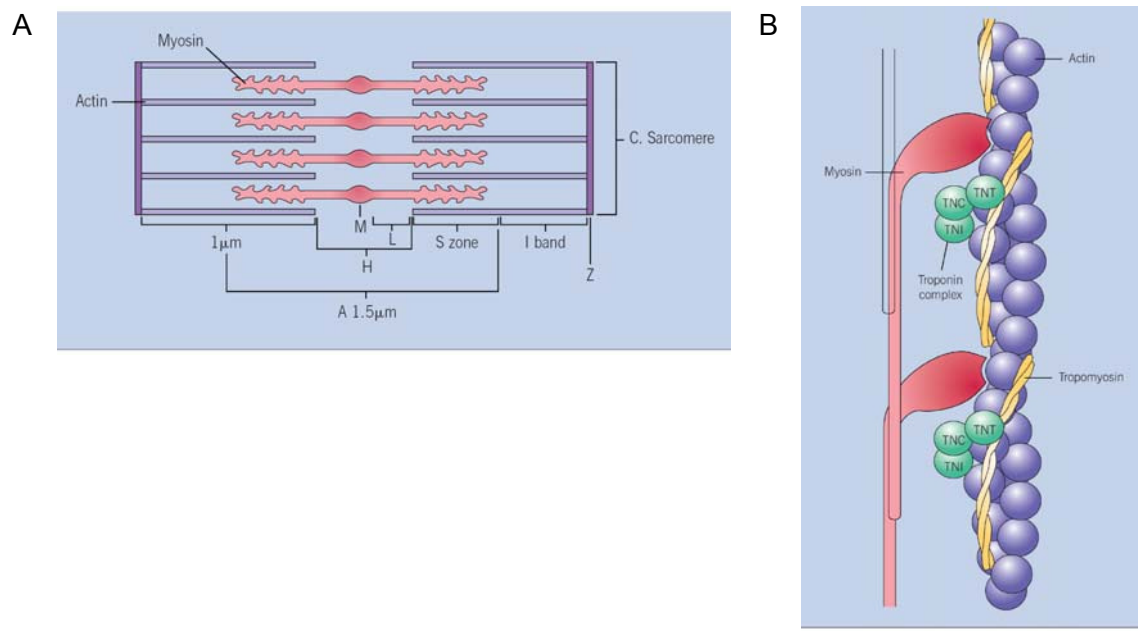

Figure 2. A. Structure of the sarcomere. Thick filaments, composed of myosin, are localized to the A-band. In the I-band, only thin C filaments are present. B. Interaction between thick (myosin) and thin (actin) filaments. Tn, troponin (© 2004 Elsevier Ltd - Cardiology 2E, edited by Crawford, DiMarco and Paulus).

Under relaxed conditions the actin-crossbridge relation is blocked because of the steric blocking position of tropomyosin. TnT is tightly bound to tropomyosin and TnI is tightly bound to actin. The interaction of TnC and TnI is weak. Activation occurs when Ca^{2+} binds to the regulatory site of TnC. This promotes a cascade of changes in protein-protein interactions that result in strong TnC-TnI, weakened TnI-actin, weakened TnT-tropomyosin interaction and a movement of tropomyosin that finally promotes strong interaction between actin and myosin, resulting in crossbridge formation (Palmiter & Solaro, 1997).

The contractile force of the myocardium results from the number of actin-myosin crossbridge interactions per unit of time. This number depends on the amount of Ca^{2+} bound to TnC, which is a function of Ca^{2+} availability and the affinity of TnC to Ca^{2+}, as well as on the behaviour of the individual crossbridge cycle. Augmentation of Ca^{2+} affinity to TnC, increased recruitment of crossbridges per number of Ca^{2+} bound and prolongation of the attachment period of the individual crossbridge cycle are termed *Ca^{2+}-sensitization* or *increased Ca^{2+}-sensitivity*.

At the level of the myocyte, contractile performance is regulated mainly by four different mechanisms.

I. *Alteration in sarcomere length (preload)*: the underlying principle is that the maximum force depends on the degree of overlap between thick and thin filaments. However, it was shown that an increase in sarcomere length also increased sarcoplasmic reticulum Ca^{2+} release as well as Ca^{2+} sensitivity of the myofilaments. This relationship between sarcomere length and force underlies the *Frank-Starling law*, whereby increased end-diastolic volume leads to increased systolic contraction.

II. *β-adrenoceptor stimulation*: under physiologic conditions, the sympathetic nervous system plays a central role in the response of the heart to acute stress. Circulating or locally released catecholamines bind to myocardial β_1- and β_2-adrenoceptors. β-adrenoceptors couple to adenylyl cyclase through stimulatory G proteins (G_S). Stimulation of adenylyl cyclase by G_S results in production of cyclic AMP. Cyclic AMP binding to protein kinase A (PKA) activates this enzyme, which subsequently results in phosphorylation of target proteins. In the myocardium, PKA phosphorylates ion channels and proteins that are involved in Ca^{2+} homeostasis; contractile proteins and metabolic enzymes, and it may translocate to the nucleus to regulate gene transcription. β-adrenoceptor stimulation increases myocardial performance by the following effects. Increased Ca^{2+} influx through L-type Ca^{2+} channels after beta-adrenoceptor stimulation results in increased Ca^{2+}-induced Ca^{2+} release from the SR through RyRs, of which the activity may also be regulated by PKA-dependent phosphorylation (Witcher *et al.*, 1991). Increased rate and degree of Ca^{2+} activation of contractile proteins result in increased contractile force and rate of force development of the myocardium (Hasenfuss *et al.*, 1994). In addition, the rate of relaxation is considerably increased because of phosphorylation of the thin filament regulatory protein, TnI and PLB. The former decreases Ca^{2+} affinity of the myofilaments and increases dissociation of Ca^{2+}. Cyclic-AMP-dependent phosphorylation of PLB results in stimulation of the SERCA.

III. *Frequency-dependent regulation of contractile force:* An increase in frequency of contraction increases contractile force by an increase in trans-sarcolemmal Ca^{2+} influx and Ca^{2+} release from the SR. This important regulatory mechanism is termed the force-frequency relation, strength-interval relation or *Treppe* (staircase) phenomenon.

IV. *Peptide hormones* such as angiotensin and endothelin, as well as cytokines, influence contraction and relaxation of the heart via other signalling pathways.

Definition, symptoms and pathophysiology of heart failure

Several definitions of heart failure have been outlined over the years, although none has been generally satisfying. This reflects the complexity of this syndrome, which is characterized by reduced cardiac output and increased venous return, and is accompanied by molecular abnormalities that cause progressive deterioration of the failing heart.

Symptoms consistent with heart failure may be categorized into those related to pulmonary congestion (dyspnea on exertion, orthopnea, paroxysmal nocturnal dyspnea), systemic venous congestion (peripheral oedema, ascites, abdominal pain/nausea) and low cardiac output (decreased exercise tolerance/fatigue, change in mental status/confusion). The New York Heart Association (NYHA) classification system is commonly used to categorize the severity of symptoms (*Table 1*).

Class I	No symptoms with ordinary activity
Class II	Mild limitation of physical activity; symptoms with ordinary physical activity
Class III	Marked limitation of physical activity; symptoms with less than ordinary physical activity
Class IV	Symptoms with any physical activity, or at rest

Table 1. New York Heart Association classification system.

Human heart failure has many underlying causes, the frequencies of which have changed considerably over the years. At present, the leading cause is coronary heart disease, which accounted for 67% of failure cases during the 1980s according to the Framingham heart study (Levy *et al.*, 2002). Most of these patients also had a history of arterial hypertension (57%). Valvular heart disease underlies failure in about 10% of the patients, and 20% of heart failure cases are attributable to primary myocardial diseases, of which dilated cardiomyopathy predominates. Regardless of the original cardiac abnormality, the advanced heart failure syndrome presents a complex picture, including disturbed myocardial function, ventricular remodeling (Gerdes *et al.*, 1992), altered hemodynamics, neurohumoral activation (Francis *et al.*, 1990), cytokine overexpression (Baumgarten *et al.*, 2002) and endothelial dysfunction (Zelis & Flaim, 1982) .

Traditionally, heart failure has been defined as a pathophysiologic state in which an abnormality of cardiac function is responsible for the inability of the heart to pump an adequate volume of blood to meet the requirements of the metabolizing tissues. This definition reflects the concept that *impaired contractility* and the reduced ability of the heart

to eject blood are responsible for the clinical syndrome. However, many patients have structural cardiac alterations that impair systolic and diastolic function but do not have clinical signs of heart failure, because compensatory mechanisms maintain cardiac output and peripheral perfusion. Therefore, heart failure is now considered more as a cardiocirculatory disorder than simply as a disease of the heart.

On the basis of a better understanding of systolic and diastolic function and the observations of systolic dysfunction in experimental and clinical heart failure, the concept of mechanical pump failure dominated the 1960s and 1970s and prompted pharmacological approaches to increase cardiac contractility. The presence of *systemic vasoconstriction* suggested that circulatory failure was an important component of the disease, and consequently vasodilator treatment was introduced.

During the past 10 years experimental and clinical studies have demonstrated that heart failure is also characterized by *increased neurohumoral activation*, particularly of the sympathetic nervous system and the renin-angiotensin-aldosterone system. Increased neurohumoral activity is now regarded as a major pathophysiologic component contributing to the symptoms and progression of heart failure. Although activation of the adrenergic and renin-angiotensin system is effective for short-term compensation, the sustained neurohormonal activation is associated with long-term adverse consequences, including progressive left ventricular dysfunction, remodelling, pump failure and reduced survival. Although the precise way in which hemodynamic and neurohumoral factors interact to cause progression of heart failure remains undefined, there is some evidence that several mechanisms are involved, including energy depletion, increased ventricular wall stress, altered cardiac gene expression, elevated oxidative stress, myocyte necrosis and apoptosis (*Figure 3*).

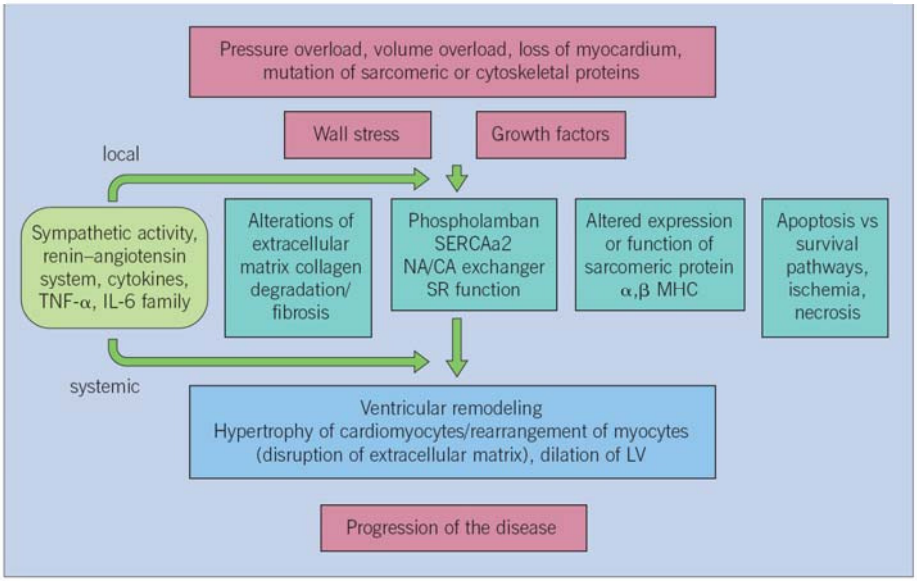

Figure 3. Pathophysiology of heart failure. IL, interleukin; LV, left ventricle; SERCA, SR Ca^{2+}-ATPase; SR, sarcoplasmic reticulum; TNF, tumor necrosis factor (© 2004 Elsevier Ltd - Cardiology 2E, edited by Crawford, DiMarco and Paulus).

More recently, there is increasing evidence to implicate cytokine (tumor necrosis factor (TNF)-α, interleukin (IL)-6) activation in heart failure, introducing the concept that heart failure involves an *inflammatory component* that may have both functional and structural consequences.

Structural and functional myocardial protein alterations in heart failure

At the level of the myocardium characteristic functional, biochemical and molecular alterations that occur in end-stage heart failure have been described. During heart failure, cardiac contractility is impaired by abnormalities in the structure and function of molecules responsible for the rhythmic release and reuptake of Ca^{2+} within the myocytes (Beuckelmann *et al.*, 1992; Hasenfuss *et al.*, 1992). Disturbed function of the failing myocardium could also result from altered response of the contractile machinery to Ca^{2+} or from altered function of the actin-myosin crossbridge cycle itself (Hajjar & Gwathmey, 1992). Controversy exists regarding myofilament Ca^{2+} sensitivity in end-stage heart failure, which was suggested to be unchanged (Hajjar *et al.*, 1988), decreased (Schwinger *et al.*, 1994) or increased (Wolff *et al.*, 1996). Moreover, alterations primarily in the myofilamentary proteins have been implicated in myocardial injuries that develop during reperfusion following ischaemia (Wang & Zweier, 1996), or as a consequence of exposure to the above described inflammatory cytokines (Ferdinandy *et al.*, 2000; Finkel *et al.*, 1992). During end-stage human heart failure, a number of alterations have been reported that affect the expression (Morano *et al.*, 1997; van der Velden *et al.*, 1999) and phosphorylation (Bodor *et al.*, 1997; van der Velden *et al.*, 2003b; Wolff *et al.*, 1996) of the contractile proteins. These changes may contribute to both the systolic and the diastolic dysfunction observed in end-stage failing hearts (Perez *et al.*, 1999).

Peroxynitrite and heart failure

Increased levels of nitric oxide and reactive nitrogen species, e.g. peroxynitrite, was suggested to contribute to the development of congestive heart failure (Haywood *et al.*, 1996; Mihm *et al.*, 2001a; Ziolo *et al.*, 2001). It was previously demonstrated in different animal preparations, that peroxynitrite modulated myocardial proteins via the formation of nitrotyrosine (Reiter *et al.*, 2000), and the amount of nitrated proteins correlated with the reduction in cardiac pump function (Ferdinandy *et al.*, 2000; Weinstein *et al.*, 2000). In

addition, a decreased efficiency of the heart to utilize ATP for work has been described following treatment of working rat hearts with peroxynitrite or cytokine (Ferdinandy *et al.*, 1999; Schulz *et al.*, 1997), pointing to the contractile process as a potential mediator of the peroxynitrite-induced mechanical dysfunction. The peroxynitrite-induced reduction of myofibrillar Ca^{2+}-responsiveness was found to be linked to the activation of the cGMP-dependent protein kinase pathway (Brunner & Wolkart, 2003). Alternatively, nitration of the 40 kDa myofibrillar isoform of creatine kinase was suggested as a mechanism responsible for the disturbed conversion of ATP to mechanical work in the hearts of doxorubicin-treated mice and the peroxynitrite-treated cardiac trabeculae of rats (Mihm *et al.*, 2003; Weinstein *et al.*, 2000). It is important to consider that peroxynitrite-induced nitrotyrosine formation is not restricted to a single myofibrillar protein, either in animal or in human myocardial preparations (Kanski *et al.*, 2005; Mihm *et al.*, 2001b; Mihm *et al.*, 2002). Hence, the mechanical dysfunction will depend on the extent of tyrosine nitration in a set of affected myocardial proteins and their functional and/or structural consequences.

Nevertheless, the mechanism by which nitrated myocardial proteins decrease the myocardial contractile function in the human heart in particular remains obscure.

Diastolic dysfunction, diastolic heart failure

Cardiovascular diseases such as hypertension, coronary artery disease, and cardiomyopathies often lead to systolic and diastolic ventricular dysfunction. Nearly all patients with systolic dysfunction have some degree of concomitant diastolic dysfunction, specifically, impaired relaxation and variable decreases in ventricular compliance. Moreover, patients with normal ejection fraction (EF) can display marked impairment in diastolic function (*isolated diastolic dysfunction*). Diastolic left ventricular (LV) dysfunction refers to a condition in which abnormalities in mechanical function are present during diastole. This condition is increasingly recognized (Redfield *et al.*, 2003), as evident from a population based survey, in which diastolic LV dysfunction was observed five times more often than systolic LV dysfunction (Fischer *et al.*, 2003).

In contrasts to LV diastolic dysfunction, diastolic heart failure describes a clinical syndrome. Heart failure with preserved LVEF is frequently referred to as *diastolic heart failure* (DHF) in opposition to systolic heart failure, which stands for heart failure with reduced LVEF (Grossman, 1991; Kitzman *et al.*, 2002; Redfield, 2004; Zile & Brutsaert, 2002). From a conceptual perspective, diastolic heart failure occurs when the ventricular chamber is unable to accept an adequate volume of blood during diastole, at normal diastolic

pressures and at volumes sufficient to maintain an appropriate stroke volume. These abnormalities are caused by a decrease in ventricular relaxation and/or an increase in ventricular stiffness (Zile & Brutsaert, 2002).

DHF is currently diagnosed in as much as 49% of heart failure patients (Cleland *et al.*, 2002). The diagnosis of primary diastolic heart failure requires three obligatory conditions to be simultaneously satisfied: 1. presence of signs or symptoms of congestive heart failure; 2. presence of normal or only mildly abnormal LV systolic function; 3. evidence of abnormal LV relaxation, filling, diastolic distensibility or diastolic stiffness (European study group, 1998).

Despite the increased recognition of both DHF and diastolic LV dysfunction, their pathophysiology remains incompletely understood. Whether heart failure with preserved LVEF results from diastolic LV dysfunction (Zile *et al.*, 2004) or from subtle systolic LV dysfunction, unappreciated by a routine LVEF measurement (Yu *et al.*, 2002) and possibly exacerbated by high arterial impedance (Kawaguchi *et al.*, 2003), is still a matter of debate. Furthermore, explanations proposed for diastolic LV dysfunction are divergent ranging from high LV myocardial stiffness (Grossman, 1991; Zile *et al.*, 2004) to pericardial or right ventricular constraint (Morris-Thurgood & Frenneaux, 2000; Pak *et al.*, 1996). Moreover, the relative importance of myocardial fibrosis and of high cardiomyocyte resting tension for LV myocardial stiffness remains undefined (Kass *et al.*, 2004).

Failure to resolve these controversies concerning DHF and diastolic LV dysfunction could arise from a lack of myocardial biopsy or necropsy material (Kass *et al.*, 2004; Redfield, 2004), which would allow clinical and hemodynamic features to be confronted with cellular and molecular myocardial properties.

Ca^{2+}-sensitizers in heart failure

Ca^{2+}-sensitizers represent a new class of inotropic drugs. They improve myocardial performance by directly acting on contractile proteins without increasing intracellular Ca^{2+} load. Thus, they avoid the undesired effects of an increased intracellular Ca^{2+} load. Ca^{2+}-sensitizers may enhance myocardial performance without increasing myocardial oxygen consumption and without provoking fatal arrhythmias (Lehmann *et al.*, 2003). The therapeutic consequences, however, are not understood in detail.

Isometric force production and its Ca^{2+}-sensitivity are determined by the cooperative interplay between the Ca^{2+} regulation on the thin filaments and strongly bound force-generating cross-bridges (Brenner, 1988). Ca^{2+} regulation and force generation, on the other

hand, are impaired by accumulating intracellular metabolites (i.e. H^+ and inorganic phosphate (P_i)) during ischaemia (Kentish, 1991; Regnier *et al.*, 1995). Hence, ischaemic metabolites may also influence pharmacological Ca^{2+}-sensitization.

EMD 57033, the (+) enantiomer of 5-[1-(3,4-dimethoxybenzoyl)-1,2,3,4-tetrahydro-6-quinolyl]-6-methyl-3,6-dihyro-2H-1,3,4-thiadiazin-2-one), EMD 53998, interferes with the force-generating actin-myosin interactions (Solaro *et al.*, 1993) and antagonises the effects of P_i (Strauss *et al.*, 1992). In multicellular preparations of failing human hearts, the positive inotropy of EMD 57033 was accompanied by a pronounced negative lusitropic effect (Hajjar *et al.*, 1997; Holubarsch *et al.*, 1998). This negative lusitropy was associated with an EMD 57033-evoked Ca^{2+}-independent force component (Palmer *et al.*, 1995) in porcine skinned cardiac trabeculae. However, the development of this Ca^{2+}-independent force was not consistently observed in human myocardial preparations (Hajjar *et al.*, 1997; Herzig *et al.*, 1996).

OR-1896, the (-) enantiomer of N-[4-(1,4,5,6-tetrahydro-4-methyl-6-oxo-3-pyridazinyl)phenyl] acetamide, an active metabolite of the recently clinically introduced cardiotonic agent levosimendan, the (-) enantiomer of {[4-(1,4,5,6-tetrahydro-4-methyl-6-oxo-3-pyridazinyl)phenyl]hydrazono} propanedinitrile, has been reported to possess a moderate Ca^{2+}-sensitizing property in intact rabbit papillary muscles (Takahashi *et al.*, 2000a), permeabilized myocyte-sized preparations of the guinea pig (Szilagyi *et al.*, 2004), and canine ventricular intact trabeculae both at the normal and at acidic pH (Takahashi *et al.*, 2000b; Takahashi & Endoh, 2002). Furthermore, it has been suggested that, due to its structural homology to levosimendan, OR-1896 facilitates force production by stabilising the Ca^{2+}-bound conformation of TnC (Takahashi *et al.*, 2000b). However, no experimental data have been presented on the Ca^{2+}-sensitizing effect of OR-1896 in human preparations yet.

AIMS

Within the present research we focussed on the function and structure of the contractile apparatus in human myocardium in health and under pathological conditions. Based on the previous research described in the *General introduction* the following aims were defined:

I. *Peroxynitrite and heart failure*

- To elucidate the relationship between peroxynitrite-induced protein nitration and Ca^{2+}-activated force production in isolated human cardiomyocytes.
- To characterize the effects of *in vitro* peroxynitrite treatment on Ca^{2+}-activated force production, its Ca^{2+}-sensitivity and on actin-myosin cross-bridge transitions.
- To identify myofilamentary proteins responsible for the peroxynitrite-induced contractile dysfunction.

II. *Diastolic dysfunction, diastolic heart failure*

- To identify the contribution of cardiomyocyte function, collagen content and myocardial protein composition to the *in vivo* diastolic LV dysfunction using endomyocardial biopsy material from patients with diastolic heart failure.
- To correlate *in-vitro* measured cardiomyocyte data with *in-vivo* hemodynamic indices of diastolic LV function.

III. *Ca^{2+}-sensitizers in heart failure*

- To compare the effect of two Ca^{2+}-sensitizers (OR-1896 and EMD 53998) under control conditions and under mimicked ischaemic conditions in cardiomyocytes of failing (NYHA Class IV) and non-failing human hearts.

MATERIALS and METHODS

2.1. Force measurements in single myocyte-sized preparations

2.1.1. Myocyte isolation and mounting

Frozen human myocardial tissue samples were first defrosted and mechanically disrupted in cell isolation solution (in mM: Mg^{2+} 1, KCl 145, EGTA 2, ATP 4, imidazole 10; pH 7.0). The suspension was incubated for 5 minutes in this solution supplemented with 0.3% or 0.2% Triton X-100 (Sigma, St. Louis, MO, USA) for explanted donor and failing heart samples and endomyocardial catheter biopsy samples, respectively. Thereafter, cells were washed twice in cell isolation solution and kept on ice for a maximum of 12 hours. Subsequently, a demembranated single cardiomyocyte (*Figure 4*) was mounted between two thin insect needles with silicone adhesive (Dow Corning, Midland, USA) while viewed under an inverted microscope (Axiovert 135, Zeiss, Germany). One needle was attached to a force transducer element (SensoNor, Horten, Norway) and the other to an electromagnetic motor (Aurora Scientific Inc., Aurora, Canada).

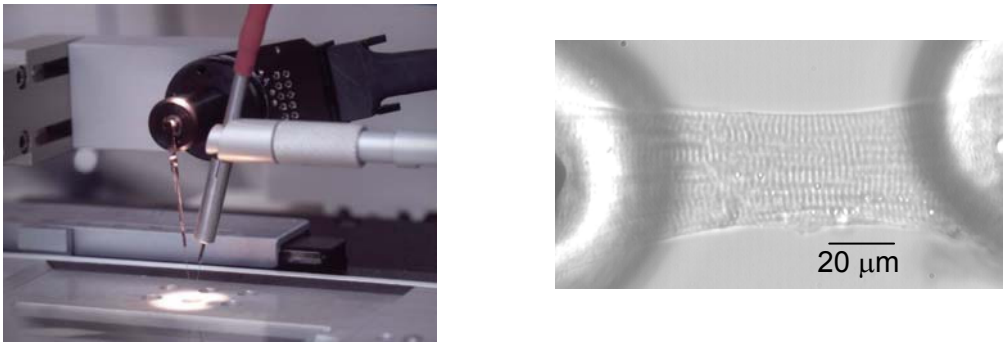

Figure 4. Left panel. The central part of the isolated myocyte set-up. Right panel. A single cardiomyocyte mounted between a sensitive force transducer and an electromagnetic motor.

2.1.2. Solutions

The compositions of the relaxing and activating solutions used during force measurements were calculated as described by Fabiato (Fabiato & Fabiato, 1979; Papp *et al.*, 2002). The pCa, i.e. -log[Ca^{2+}], values of the relaxing and activating solutions (pH 7.2) were 9 and 4.75 (or 4.5), respectively. Solutions with intermediate free [Ca^{2+}] levels were obtained by mixing activating and relaxing solutions. All the solutions for force measurements contained (in mM): Mg^{2+} 1, MgATP 5, phosphocreatine 15, and N,N-bis(2-hydroxyethyl)-2-

aminoethanesulfonic acid (BES) 100. The ionic equivalent was adjusted to 150 with KCl at an ionic strength of 186.

2.1.3. Experimental protocol

The measurements were performed at 15 ^{o}C, and the average sarcomere length was adjusted to 2.1-2.2 μm. Isometric force was measured after the myocyte had been transferred from the relaxing solution to a Ca^{2+}-containing solution (*Figure 5, left panel*). When a steady force level was reached, the length of the myocyte was reduced by 20% within 2 ms and then quickly restreched (slack test). As a result, the force first dropped from the peak isometric level to zero (difference = total peak isometric force (F_{total})) and then started to redevelop. The force redevelopment after the restretch was fitted to a single exponential in order to estimate the rate constant of force redevelopment (k_{tr}) at various [Ca^{2+}] levels (*Figure 5, right panel*). The passive force component ($F_{passive}$) was determined in relaxing solution following the Ca^{2+} contractures. The Ca^{2+}-activated isometric force (F_{active}) was calculated by subtracting the $F_{passive}$ from the F_{total}. F_{active} at submaximal levels of activation was normalized to that at maximal activation (F_{max}) in order to characterize the Ca^{2+}-sensitivity of isometric force production (pCa_{50}).

After the first maximal activation at pCa 4.75 (or 4.5), resting sarcomere length was readjusted to 2.1-2.2 μm, if necessary. The second maximal activation at pCa 4.75 (or 4.5) was used to calculate F_{max}. Cells were subsequently exposed to a series of solutions with intermediate pCa to construct the force-pCa relationship. When at the end of this series, reexposure to pCa 4.75 (or 4.5) yielded a value of F_{active} below 80% of its initial value, measurements were discarded.

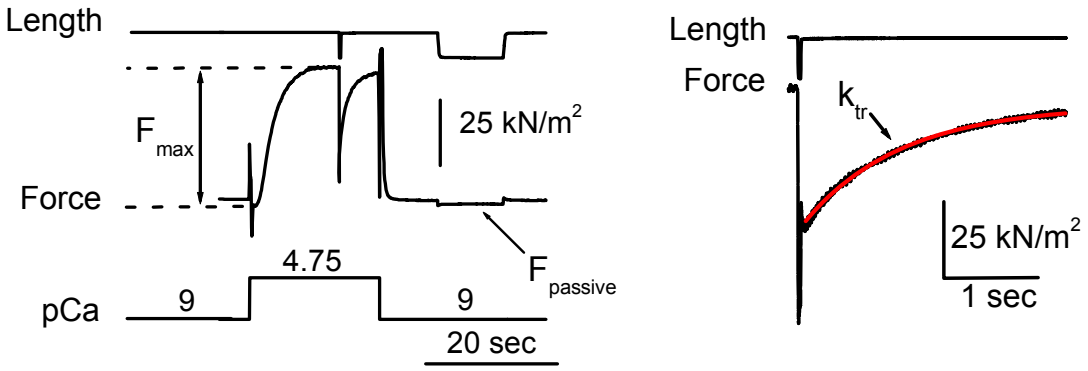

Figure 5. Left panel. Experimental protocol. Following transfer of the myocyte from the relaxing (pCa 9) to the activating solution (pCa 4.75 in this case) isometric force developed. When maximal force level (F_{max}) was attained cells were shortened by 20% of their original length for 20 ms and then restreched (slack-test). As a result force first dropped to zero then quickly redeveloped. Right panel. The k_{tr} parameter (~turnover rate of the actin-myosin cycle) was determined by fitting an exponential to the force redevelopment. A similar length change with longer slack duration was performed in relaxing solution to determine the passive force component ($F_{passive}$).

2.1.4. Ca^{2+}-sensitizers, peroxynitrite or enzyme administrations

EMD 53998 and OR-1896 were kindly provided by Orion Pharma (Espoo, Finland). The thiadiazinone derivative EMD 53998 consists of a racemic mixture of the (+) enantiomer (EMD 57033) and the (-) enantiomer (EMD 57439). The (+) enantiomer is the active Ca^{2+}-sensitizing compound, whereas the (-) enantiomer is mainly responsible for the phosphodiesterase inhibitor activity of the mixture (Solaro *et al.*, 1993). This inhibitory effect is not important in permeabilized preparations, as used in this study. Dimethylsulfoxide (DMSO, final concentration 0.1%), used as a solvent for the Ca^{2+}-sensitizers, did not modify the contractile function.

Peroxynitrite (Cat. No. 516620) with low (~0.1%) hydrogen peroxide content (Uppu & Pryor, 1996) was obtained from Calbiochem (San Diego, CA, USA). Before each experiment concentrated stock solutions of peroxynitrite (ranging from 10 μM to 10 mM) were prepared based on peroxynitrite concentration determination by absorbance measurements at 302 nm (Uppu & Pryor, 1996). The pH in stock solutions was adjusted to 11 (by KOH) to oppose peroxynitrite decomposition and the ionic equivalent was set by 150 mM KCl. A single volume of 20 μl from these stock solutions was rapidly introduced into a droplet (180 μl) of relaxing solution (pH 7.2, T=20^{o}C), which surrounded each myocyte preparation in the mechanical set-up. This approach resulted in nominal peroxynitrite concentrations ranging from 1 μM to 1000 μM, which decreased quickly because of spontaneous degradation (half-life: less than 3 s in this system). Peroxynitrite exposure was terminated following 60 s of incubation.

The catalytic subunit of protein kinase A (PKA, 100 units/ml, Sigma, batch 12K7495) was dissolved in relaxing solution containing 6 mM dithiothreitol (DTT, MP Biochemicals). After establishing the baseline force-pCa relationship, myocytes were incubated in this solution for 40 min at 22°C. Subsequently, a second force-pCa relationship was determined at 15°C.

2.2. Biochemical assays

2.2.1. Western immunoblot

Nitrotyrosine formation and α-actinin levels in peroxynitrite-treated (1-1000 μM) permeabilized myocyte preparations were assayed by Western immunoblotting following SDS-polyacrylamide gradient gel electrophoresis (6-18% gradient gels with 20 μg of protein homogenates in each lane). In parallel, the loading was visualized by silver staining (Giulian

et al., 1983), as described previously (Barta *et al.*, 2003). Following the electrophoresis step, myofibrillar proteins were transferred to nitrocellulose membranes, which were subsequently incubated with 5% non-fat dry milk and thereafter with primary antibodies (monoclonal anti-nitrotyrosine (Cayman Chemicals, Ann Arbor, MI, USA), dilution 1:5000; polyclonal anti-nitrotyrosine (Upstate, Charlottesville, VA, USA), dilution 1:7500; and anti-α-actinin (clone EA-53, Sigma, St. Louis, MO, USA), dilution 1:5000). For visualization, biotinylated secondary antibodies (Vector Laboratories, Burlingame, CA, USA) and enhanced chemiluminescence (Amersham Biosciences, Uppsala, Sweden) were used. For the evaluation of the immunoprecipitated samples, the whole resin-bound complexes were separated on 10% SDS-polyacrylamide gels and subsequently subjected to Western immunoblotting. Some of the assays were combined with the removal of the bound antibody complexes (stripping). To this end, membranes were washed with stripping buffer (2 mM DTT, 2% SDS, 400 mM NaCl, 20 mM Tris-HCl, pH 7.4) for 90 min at 60 °C. The membranes were then blocked and treated with primary and secondary antibodies as above.

2.2.2. Dot blot

1 µl volumes of all tissue samples prepared as indicated above and treated with 0-500 µM peroxynitrite were dotted onto nitrocellulose membranes. The total protein amounts and nitrotyrosine levels in the homogenates were next tested with anti-α-actinin and anti-nitrotyrosine antibodies, as described for the Western immunoblots. Dot intensities were quantified from unsaturated recordings by densitometry, using custom-prepared software. Nitrotyrosine levels were expressed relative to the dot intensity resulting from 500 µM peroxynitrite exposure.

2.2.3. Immunoprecipitation

Human permeabilized ventricular myocytes were treated with different concentrations of active or 500 µM decomposed peroxynitrite, and then homogenized in relaxing solution for the immunoprecipitation assays. The homogenates were centrifuged at 10 000 rpm for 10 min and the supernatants were used for further experiments (immunoprecipitation assays or dot blot). For immunoprecipitation, the assay mixture (1 ml each) contained: 250 µg protein of the tissue homogenate (treated with active or decomposed peroxynitrite), 2 µg antibody (anti-α-actinin (Sigma, St. Louis, MO, USA)) or mouse IgG (Zymed Laboratories, San Francisco, CA, USA), 2 µl protease inhibitor cocktail (Sigma, St. Louis, MO, USA), and 20 µl protein A/G agarose resin (Santa Cruz, Santa Cruz, CA, USA) in relaxing solution. The mixtures

were incubated at 4 °C overnight under continuous agitation. The resin-bound complexes were separated by centrifugation (1 000 rpm, 1 min) and washed three times. The washed pellets were boiled in SDS sample buffer (Quality Biologicals, Gaithersburg, MD, USA) for 10 min and then subjected to Western immunoblotting.

2.2.4. Examination of myocardial tissue properties in endomyocardial biopsies

The extent of interstitial fibrosis in the endomyocardial biopsy samples was determined on elastica von Gieson-stained sections of tissue placed in 5% formalin using an automated image analyzer (Prodit) and expressed as collagen volume fraction (CVF) (Bronzwaer *et al.*, 2003). Patients were classified as low, intermediate and high extent of interstitial fibrosis in accordance to a CVF of 0-5% (Class I), 5-10% (Class II) and 10-15% (Class III).

Expression of myosin heavy chain, desmin, actin, troponin T (TnT), tropomyosin, troponin I (TnI), myosin light chain 1 and 2 (MLC-1 and MLC-2) were analysed using one-dimensional SDS polyacrylamide gel electrophoresis as described previously (van der Velden *et al.*, 2003b). In order to detect degradation products of myofilament proteins, Western immunoblot analysis was performed using specific monoclonal antibodies against desmin (clone DE-U-10, Sigma), TnT (clone 2G3, Spectral Diagnostics), TnI (clone 8I-7, Spectral Diagnostics), MLC-1 and MLC-2 (clone F109.16A12 and F109.3E1, Alexis). Signals were visualized using a secondary horseradish peroxidase-labeled goat-anti-mouse antibody and enhanced chemiluminescence (Amersham Biosciences).

2.2.5. Enzyme-linked immunosorbent assay (ELISA)

ELISA was used to determine phosphorylation status of TnI using specific monoclonal antibodies against whole TnI and against dephosphorylated TnI. Biopsy samples were treated with 10% trichloro-acetic acid to preserve the phosphorylation status of myocardial proteins and homogenized in Tris-SDS buffer. Homogenates of 0.5 μg/μl total protein concentration were diluted in phosphate-buffered saline (PBS-HCl; pH 7.35), applied in triplicate to ELISA plates and incubated overnight at 4ºC. Aspecific binding sites were blocked with PBS-HCl containing 5% milk (Biorad) and 0.3% (v/v) Tween 20 (Sigma) for one hour at room temperature. Thereafter, both dephosphorylated and total TnI were identified using 1:500 dilutions of mouse antibody against human TnI dephosphorylated at the PKA phosphorylation sites (clone 22B11, Research Diagnostics) and mouse antibody against total TnI (clone 16A11, Research Diagnostics). Thereafter, TnI antibody binding was detected using polyclonal goat anti-mouse immunoglobulins/HRP (Dako Cytomation) as secondary

antibody. Following each antibody application, plates were washed with PBS-Tween. To visualize the residual immunocomplexes, plates were incubated for 5 minutes in darkness with 100µl of 0.1% (w/v) tetra-methyl-benzidine (TMB, Sigma) and 0.02% hydrogen peroxide (H_2O_2, Sigma) in 0.11 M sodium acetate (pH 5.5). The color reaction was stopped by adding 50µl of 1N H_2SO_4. The plates were read at 450 nm (Dynatech, MR 7000) relative to a blanc obtained by adding PBS to the wells instead of antigen in the first step. Variability of the triplicate measurements obtained for each biopsy sample was less than 5%. The signal intensity of dephosphorylated TnI was normalized to that of total TnI.

2.3. Data analysis

Ca^{2+}-force relations were fitted to a modified Hill equation:

$$F_{total} = F_{max}*[Ca^{2+}]^{nHill}/(Ca_{50}{}^{nHill}+[Ca^{2+}]^{nHill}) + F_{passive}$$

where F_{total} is the steady-state force at a given $[Ca^{2+}]$; F_{max} is the steady Ca^{2+}-activated force component at saturating $[Ca^{2+}]$; and $F_{passive}$ is the Ca^{2+}-independent force production at pCa 9. Ca_{50} (or pCa_{50}) corresponds to the $[Ca^{2+}]$ at which F_{total} -$F_{passive}$ = F_{max} /2 and reflects the midpoint of the relationship (a measure of Ca^{2+}-sensitivity of the contractile apparatus). The Hill constant (nHill), a measure of the steepness of the sigmoidal force-pCa relation.

Circumferential LV end-diastolic wall stress (σ) was computed using a thick wall ellipsoid model of the LV:

$$\sigma = PD/2h \times [1-(h/D)-(D^2/2L^2)]$$

where P is LV end-diastolic pressure, h is LV echocardiographically determined LV wall thickness, and D and L are LV short axis diameter and long axis length at the midwall (Paulus *et al.*, 1990).

The radial myocardial stiffness modulus (E) was calculated to assess myocardial material properties using:

$$E = \Delta\sigma_R/\Delta\varepsilon_R = \Delta P/(\Delta h/h) = -\Delta P/\Delta \ln h$$

and assuming the increment in radial stress ($\Delta\sigma_R$) to be equal but opposite in sign to the increment in ΔP at the endocardium, and the increment in radial strain ($\Delta\varepsilon_R$) to be equal to the increment in wall thickness (Δh) relative to the instantaneous wall thickness. Because $\Delta h/h = \Delta \ln h$, E equals the slope of a P vs. ln*h* plot (Bronzwaer *et al.*, 1991; Bronzwaer *et al.*, 2003). Agreement between E and diastolic LV stiffness indices derived from multiple beat analyses during caval occlusion has previously been reported in patients with dilated cardiomyopathy (Bronzwaer *et al.*, 2002).

Values are given as mean±SEM (or SD). Statistical significance was set at $P<0.05$ and was obtained for multiple comparisons between groups by analysis of variance (ANOVA) followed by a Bonferroni-test and for single comparisons by an unpaired Student's t-test. Monovariate and bivariate linear regression analyses were performed using SPSS (Version 9.0)

Chapter 3

PEROXYNITRITE-INDUCED α-ACTININ NITRATION AND CONTRACTILE ALTERATIONS IN ISOLATED HUMAN MYOCARDIAL CELLS

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Cardiovascular Research. (2005) in press

RESULTS

Patient characteristics, myocardial tissue samples

Healthy human hearts were obtained from general organ donor patients whose hearts were also explanted to obtain pulmonary and aortic valves for transplant surgery. These experiments complied with the Helsinki Declaration of the World Medical Association and were approved by the Albert Szent-Györgyi Medical University Ethical Review Board (No: 51-57/1997.OEj). The choice of tissue samples from individuals without known cardiovascular diseases minimized uncertainties related to tissue inhomogeneity. Left ventricular wall samples were obtained from the base. All biopsies were stored in cardioplegic solution composed of (in mM) NaCl 110, KCl 16, $MgCl_2$ 1.6, $CaCl_2$ 1.2, and $NaHCO_3$ 5, and kept at 4 ^oC for ~6-8 h before being frozen in liquid nitrogen. 6 donor hearts were used (3 men, 3 women, age = 39±5.1 years. The donors did not show any sign of cardiac abnormalities and did not receive any medication except of plasma volume expanders, dobutamine and furosemide. The cause of death included cerebral contusion due to accidents and cerebral haemorrhages, or subarachnoidal haemorrhages due to stroke.

Force measurements in single myocyte-sized preparations

Panel A of Figure 6 shows that peroxynitrite decreased the maximal isometric force production (F_{max} at pCa 4.75) in permeabilized human ventricular myocytes, and that the reduction in force development was larger in myocytes exposed to higher peroxynitrite concentrations. Incubation with decomposed peroxynitrite, on the other hand, had no effect on isometric force production (*Figure 6A, control*). To confirm specificity, dot blot analyses with nitrotyrosine-specific monoclonal and polyclonal antibodies were employed in parallel. These assays revealed increasing levels of protein nitration in response to higher peroxynitrite concentrations (*Figure 6B*). The results of statistical analyses of isometric force values (in 75 different myocytes) and nitrotyrosine levels (3 assays in myocyte suspensions) are illustrated in panel C of *Figure 6*. The maximal isometric Ca^{2+}-activated force (F_{max}: 28±2 kN/m^2) decreased to zero in a range of peroxynitrite concentrations (IC_{50}: 55±4 μM) in which the protein nitration level exhibited a dramatic increase.

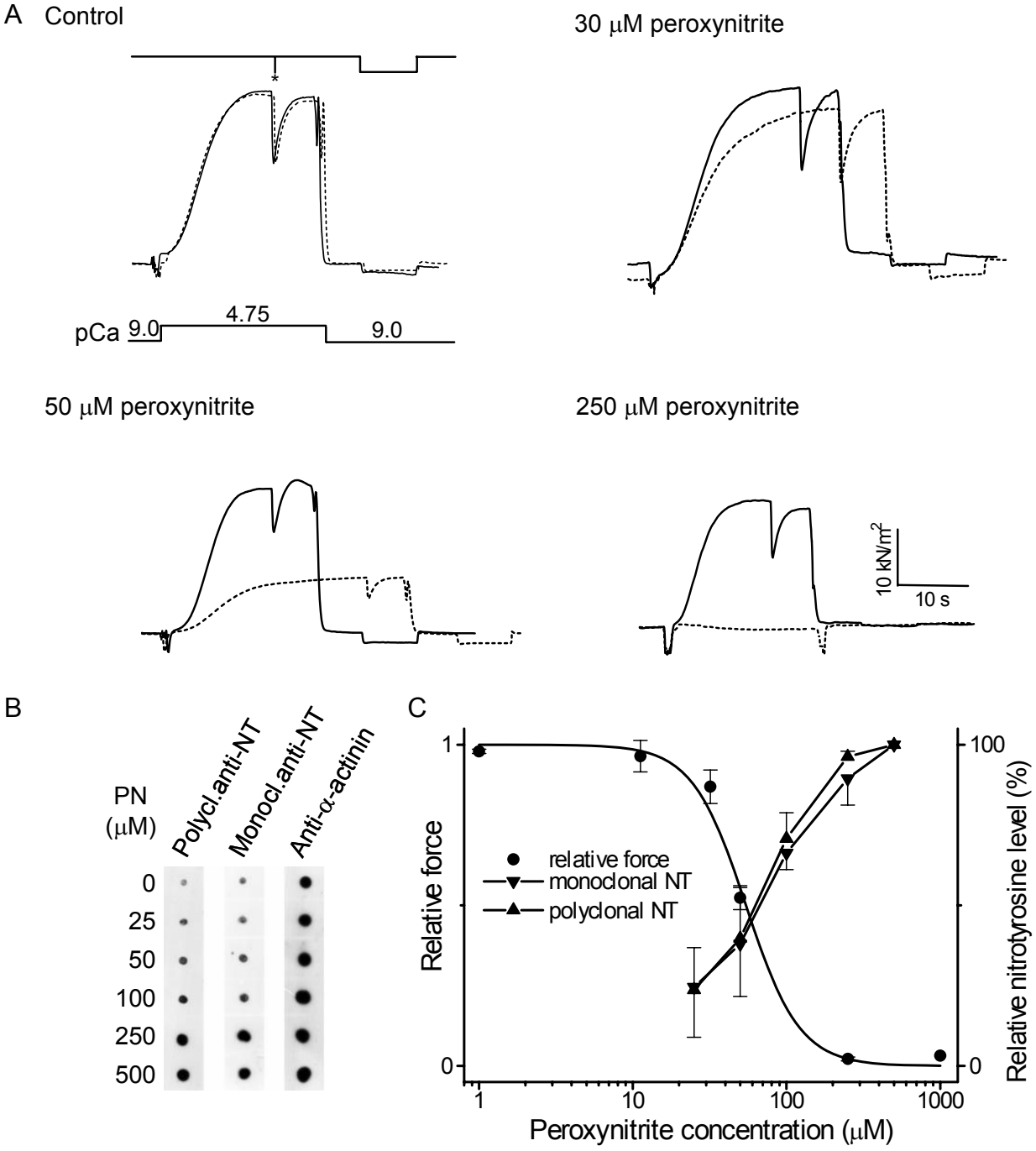
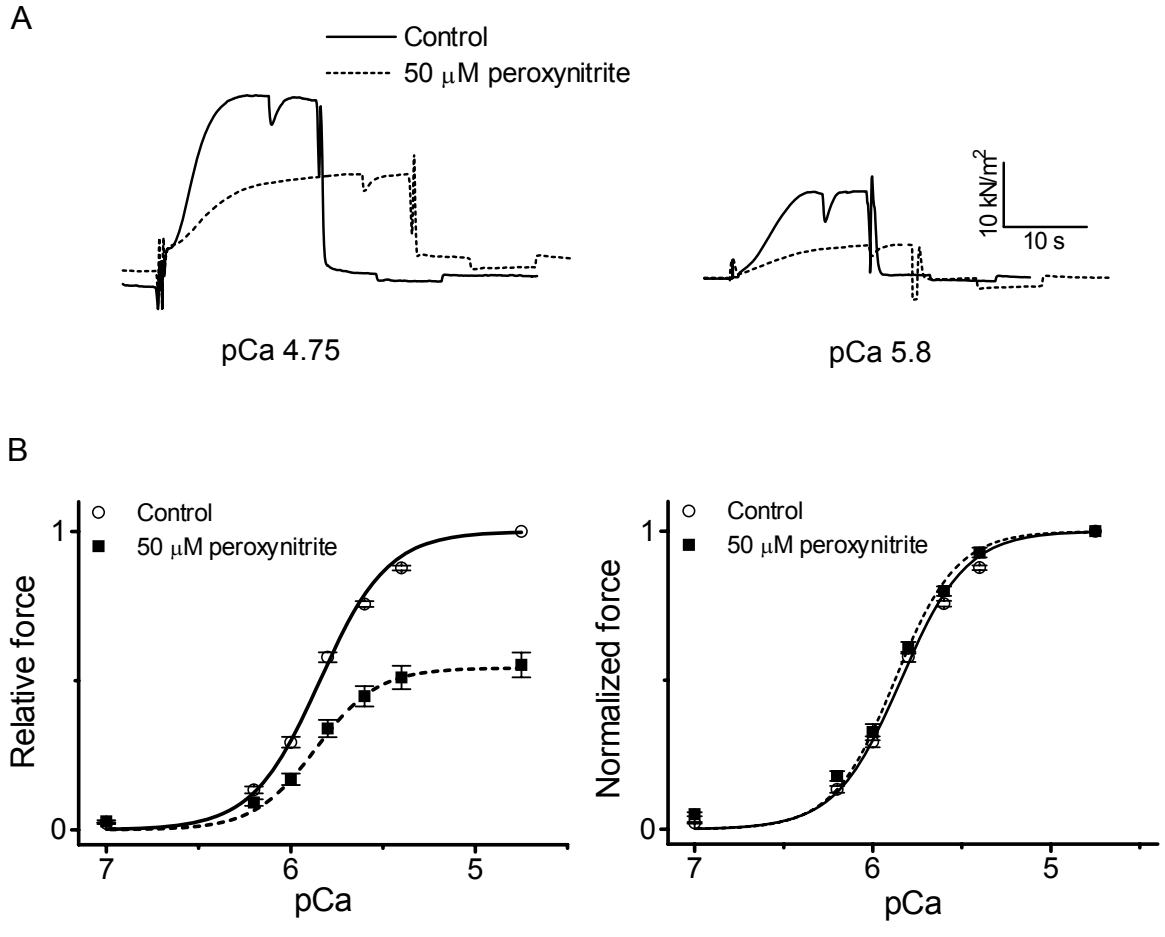


Figure 6. A. Original force recordings in different isolated permeabilized human cardiomyocytes during maximal Ca^{2+}activation (pCa = 4.75) before (solid lines) and after the application (dotted lines) of increasing concentrations of peroxynitrite. Control measurements were performed in the presence of degraded peroxynitrite (top left panel, 250 μM decomposed peroxynitrite). The protocol of length and [Ca^{2+}] changes during force measurements is given schematically in the upper left panel. B. Dot blot assays with poly- and monoclonal nitrotyrosine-specific (NT) primary antibodies illustrated increasing levels of nitrotyrosine formation in response to increasing concentrations of peroxynitrite in myocyte homogenates. (Identical protein loads were verified by monoclonal anti-α-actinin antibodies. Dot blot assays were performed in triplicate.) C. The dose–effect relations of peroxynitrite on the maximal isometric Ca^{2+}-activated force and on protein nitrotyrosine formation (means±S.E.M.) exhibited antiparallel concentration dependences in overlapping peroxynitrite concentrations. (Nitrotyrosine levels were expressed relative to the maximal staining intensities with poly- and monoclonal anti-nitrotyrosine antibodies respectively, during the dot blot assays.)

To elucidate the mechanistic background of peroxynitrite-induced contractile alterations, the [Ca^{2+}]-dependences of force and of the rate of force redevelopment following unloaded shortening and restretch (k_{tr}) were determined before and after 50 μM peroxynitrite exposure (*Figure 7*). Peroxynitrite decreased isometric force at all Ca^{2+} concentrations studied (*Figure 7A and 7B, left panel*). However, following force normalization to the respective maximum (*Figure 7B, right panel*), the Ca^{2+}-sensitivity curve before peroxynitrite treatment did not differ from that obtained after peroxynitrite application (pCa_{50}: 5.89±0.02 and 5.86±0.04; nHill: 2.22±0.11 and 2.42±0.25; before and after 50 μM peroxynitrite, respectively (P>0.05)). Additionally, the cross-bridge specific kinetic parameter k_{tr} did not change either at pCa 4.75 ($k_{tr,max}$: 1.14±0.03 1/s and 1.05±0.07 1/s before and after 50 μM peroxynitrite) or at submaximal Ca^{2+} concentrations (*Figure 7C*, P>0.05). Nevertheless, the cross-striation pattern of the myocyte preparations deteriorated after 50 μM peroxynitrite treatment (*Figure 7E*), and the $F_{passive}$ increased from 2.1±0.1 kN/m^2 to 2.5±0.2 kN/m^2 (n=57 cells; P<0.05), suggesting ultrastructural damage. Control force measurements (*Figure 7D*) verified that the observed mechanical changes were the direct consequence of 50 μM peroxynitrite exposure and not to contaminating hydrogen peroxide or the by-products of peroxynitrite (i.e. nitrite and nitrate).



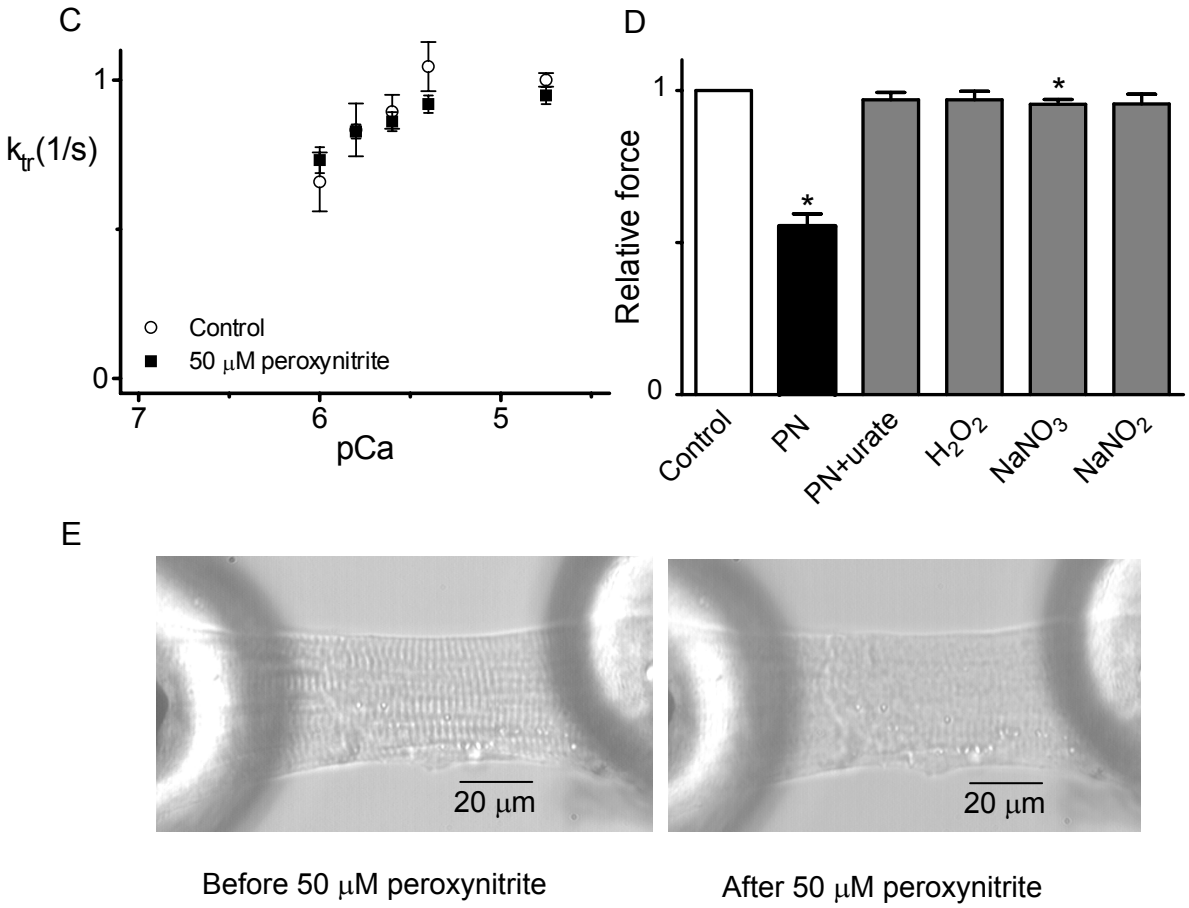


Figure 7. A. Original force recordings measured before (solid lines) and after 50 μM peroxynitrite exposure (dotted lines) at maximal (pCa 4.75) and submaximal (pCa 5.8) levels of Ca^{2+}activation in the same cardiomyocyte. B. The pCa-relative force relationship constructed from the force recordings indicated that 50 μM peroxynitrite decreased the maximal Ca^{2+}-activated force by 45% (left panel) (data points are means±S.E.M.; n=32-65 myocytes). When force values measured at submaximal Ca^{2+} concentrations before and after 50 μM peroxynitrite treatment were normalized to their respective maxima (pCa-normalized force relationship; right panel), no significant differences could be observed between the Ca^{2+}-sensitivity curves of isometric force production. C. 50 μM peroxynitrite did not alter the [Ca^{2+}]-dependence of the rate constant of tension redevelopment following unloaded shortening and restretch (k_{tr}) (n = 25 myocytes). D. The effects of 1-minute long incubations (from left to right, respectively) with 50 μM peroxynitrite (PN), 50 μM peroxynitrite plus 1 mM urate, 1 mM hydrogen peroxide, 1mM $NaNO_3$ or 1mM $NaNO_2$ on relative F_{max} in relaxing solution (n = 5-7 myocytes). Asterisks indicate significant differences vs. control. E. The cross-striation pattern of the myocytes was damaged by peroxynitrite. The representative example illustrates the cross-striation as viewed under the microscope before (left panel) and after (right panel) the application of 50 μM peroxynitrite in an isolated human myocyte preparation.

Next we attempted to identify the contractile proteins affected by nitrotyrosine formation and hence responsible for the decreased Ca^{2+}-activated force production in peroxynitrite-treated human myocytes. SDS-polyacrylamide gel electrophoreses followed by Western immunoblot assays were employed to identify the molecular masses of proteins with nitrotyrosine residues (*Figure 8*). Lower concentrations of peroxynitrite (25-100 μM) induced the nitration of a single protein at a molecular mass of about 100 kDa. At higher concentrations (250-500 μM) of peroxynitrite, additional proteins also underwent nitration as

indicated by the intense immunoreactivity in a wide range of protein molecular masses above and below 100 kDa. Although identical amounts of proteins were applied in the assays (as verified by Western immunoblotting with anti-α-actinin following stripping (*Figure 8, right panel*)), these latter proteins were not stained in the presence of 50 μM peroxynitrite.

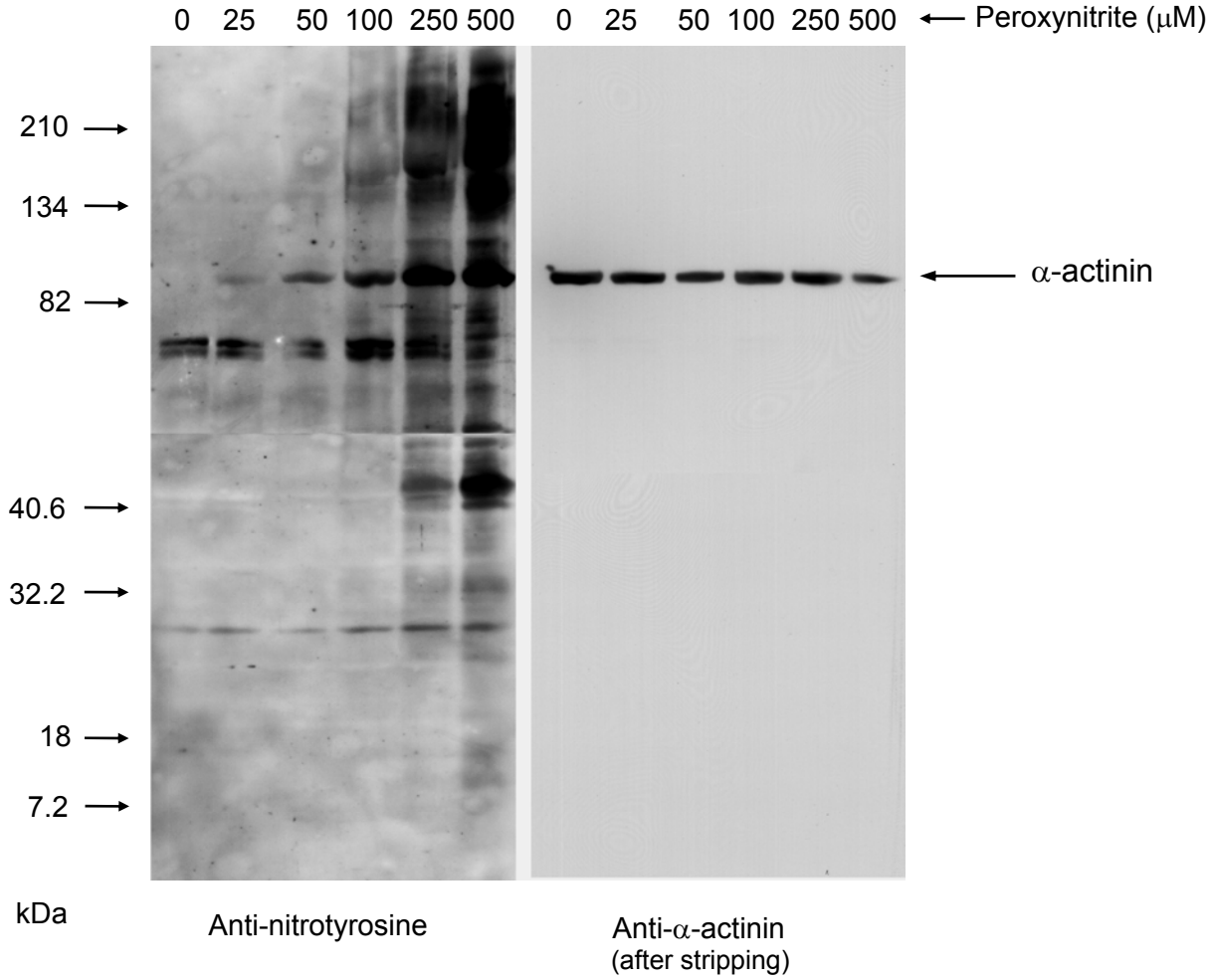


Figure 8. Sequential Western immunoblot analyses of peroxynitrite-treated human myocardial proteins. Peroxynitrite concentrations are depicted on the top, and molecular weights on the left. Left panel. Membranes were first developed with anti-nitrotyrosine antibody and subsequently with anti-α-actinin antibody (right panel) following stripping. A clear increase in the nitrotyrosine staining between peroxynitrite concentrations of 25 μM and 100 μM was apparent only at the 100 kDa protein level. The bands below 82 kDa and 32.2 kDa did not exhibit any peroxynitrite concentration-dependence and are therefore not considered specific for peroxynitrite treatment. High peroxynitrite concentrations (250-500 μM) induced nitrotyrosine formation in several myofibrillar proteins in a wide range of molecular weights. Equal intensities of α-actinin staining were present at 100 kDa (3 independent assays provided identical results).

The molecular mass of the nitrated 100 kDa protein was similar to that of α-actinin. To verify that the nitrated protein was indeed α-actinin, an immunoprecipitation study was performed (*Figure 9*). Myocardial protein homogenates were first incubated with active or decomposed peroxynitrite (500 μM). The samples were then divided into parts for α-actinin immunoprecipitation (with added α-actinin-specific antibody) and for the control (with the

same amount of IgG from the same species). The efficiency of the immunoprecipitation was tested with α-actinin-selective antibody (*Figure 9A*). In contrast with the control, the appearance of the specific immunostained bands at the level of α-actinin indicated that α-actinin was well separated from the other myocardial proteins both in the peroxynitrite-treated and in the peroxynitrite-untreated homogenates. Next the immunocomplexes were removed from the nitrocellulose membranes and the same membranes were stained with an antibody specific for nitrotyrosine. This procedure clearly identified the nitrated 100 kDa protein as α-actinin following peroxynitrite exposure (*Figure 9B*).

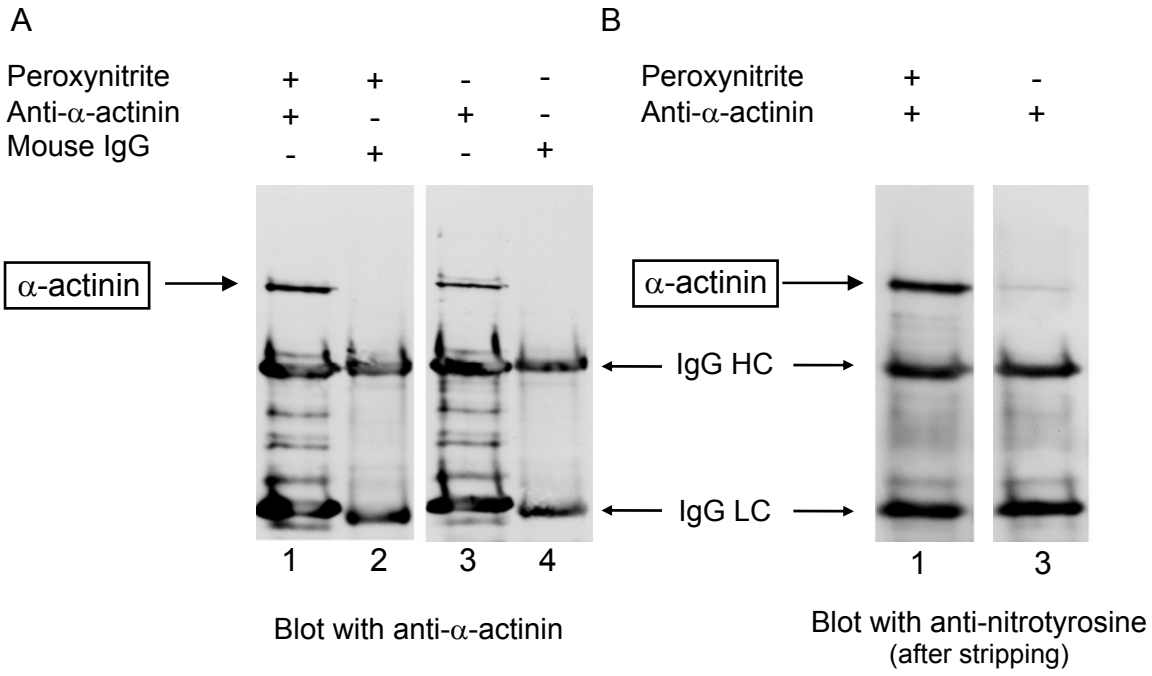


Figure 9. Identification of the 100 kDa nitrated protein as α-actinin by immunoprecipitation. A. The nitrocellulose membranes were developed with an α-actinin-selective antibody following immunoprecipitation with anti-α-actinin. Incubations with the same amount of IgG as for the immunoprecipitation from mouse served as controls. The α-actinin-specific staining at 100 kDa illustrated the effective separation of α-actinin from other myocardial proteins in both peroxynitrite-treated (500 μM peroxynitrite) and peroxynitrite-untreated myocardial preparations. B. After stripping, the same membranes as in A were developed with an antibody selective to nitrotyrosine, and the staining at 100 kDa in the peroxynitrite-treated sample identified α-actinin as a target protein for nitration. (Protocols for incubations are given schematically above the registrations. IgG HC: heavy chain of IgG; IgG LC: light chain of IgG. Immunoprecipitation assays were performed in triplicate.)

DISCUSSION

The results of this investigation revealed a close inverse relationship between the extents of α-actinin nitration and Ca^{2+}-activated force production in human myocyte-sized preparations. The nitration of α-actinin may therefore contribute to the cardiac dysfunction observed under conditions evoking increased peroxynitrite production (Beckman & Koppenol, 1996; Ferdinandy *et al.*, 2000; Finkel *et al.*, 1992; Gupte & Okada, 2001; Mihm *et al.*, 2001b; Paulus & Bronzwaer, 2002; Wang & Zweier, 1996; Weinstein *et al.*, 2000) in the human heart.

Peroxynitrite induced structural, rather than regulatory alterations in the contractile apparatus, because the Ca^{2+}-sensitivity curve of force production (described by pCa_{50} and nHill) and the cross-bridge cycling rates (k_{tr}) were not affected up to the IC_{50} value. Moreover, the reduction in maximal isometric force was tightly coupled to the deterioration in the cross-striation pattern and to a modest increase in the $F_{passive}$. This implies that the peroxynitrite-induced contractile alterations can be explained by a reduction in the number of force-generating cross-bridges due to the diminished longitudinal transmission of force along the sarcomeres (Horowits *et al.*, 1986; Papp *et al.*, 2000). The human α-actinin molecule is a relatively tyrosine-rich (2.9% tyrosine) structural protein that is essential for maintenance of the Z-line and for the integrity of the sarcomeres (Luther & Squire, 2002; Young & Gautel, 2000). It is fully conceivable, therefore, that the alterations caused in the conformation of α-actinin by its nitration are involved in the structural and consequently the functional alterations upon peroxynitrite exposure in these human myocardial preparations.

It should be noted that the concentrations of the peroxynitrite mixtures applied in this study were in all probability higher than those expected to occur under pathophysiological conditions. Accordingly, exposure to lower concentrations of peroxynitrite for a prolonged period of time would have mimicked *in vivo* conditions better, but this is hampered by the short lifetime of peroxynitrite at physiological pH. Interpretation of the results of *in vitro* peroxynitrite treatments might be complicated by inadvertent hydrogen peroxide contamination and by unspecific effects of peroxynitrite by-products. Our control force measurements in the presence hydrogen peroxide, $NaNO_3$, $NaNO_2$, and the prevention of peroxynitirite-evoked force reduction by the peroxynitrite scavenger urate, however, excluded these possibilities.

Peroxynitrite-induced cardiac protein nitration, myofibrillar thinning and irregular striation patterns have already been documented in doxorubicin-treated mice (Mihm *et al.*,

2002) and in the cardiac trabeculae of the rat following peroxynitrite exposure (Mihm *et al.*, 2003). Interestingly, during immunogold electron microscopy, longitudinal sections from the ventricular wall tissue of the doxorubicin-treated animals demonstrated high gold particle densities indicative of nitrotyrosine staining around the Z-lines (Mihm *et al.*, 2002). Moreover, similarly to our results, peroxynitrite incubation decreased the maximal Ca^{2+}-activated force without giving rise to alterations in the Ca^{2+}-sensitivity of force production in the peroxynitrite-treated permeabilized cardiac trabeculae of the rat (Mihm *et al.*, 2003). Although nitration of a number of myofibrillar proteins was observed in these animal models, the high levels of nitrotyrosine in the 40 kDa myofibrillar creatine kinase suggested that this was responsible for the peroxynitrite-elicited myofibrillar changes in Ca^{2+}-activated contractile function (Mihm *et al.*, 2001a; Mihm *et al.*, 2002; Mihm *et al.*, 2003). The mechanical alterations observed in the human myocyte preparations in our study extend previous experimental findings on the peroxynitrite-modulated myofibrillar function in animal hearts. However, our mechanical and biochemical data led us to propose an alternative explanation for the mechanical dysfunction.

In the range of peroxynitrite concentrations at which the isometric force was diminished, only the nitration of α-actinin was observed. Similarly to others (Kanski *et al.*, 2005; Mihm *et al.*, 2001b; Mihm *et al.*, 2003), we could additionally detect protein nitrotyrosine formation in several abundant proteins, though only after exposure to very high concentrations of peroxynitrite (250-500 μM). The molecular weights of these other proteins indicated possible nitration of the myosin heavy chain and of the myofibrillar isoform of creatine kinase. Nitration of these proteins and possibly others, may therefore also contribute to the disappearance of force in response to nominal concentrations of peroxynitrite. At lower concentrations (i.e. around the IC_{50} of peroxynitrite on the Ca^{2+}-activated force), however, any significant inactivation of creatine kinase was ruled out by the mechanical observations on our human myocyte preparations. Inhibition of the myofibrillar creatine kinase would disturb the regeneration of MgADP to MgATP and hence slow down cross-bridge cycling (Ogut & Brozovich, 2003; Papp *et al.*, 2002; Ventura-Clapier *et al.*, 1995). However, following exposure to 50 μM peroxynitrite, no alteration in k_{tr} or its Ca^{2+}-sensitivity was observed. Hence, it is concluded that the contractile dysfunction seen at this peroxynitrite concentration is a consequence of structural alterations leading to a deteriorated cross-striation pattern, most probably through the nitration of α-actinin.

The extent of protein nitration upon exposure to peroxynitrite correlated poorly with the levels of expression of certain myocardial proteins or with their tyrosine content in the rat

cardiac trabeculae. This leads to tentative explanations based on the tertiary structure of proteins, and their microenvironment and accessibility, which could modulate the susceptibility of the tyrosine residues to non-enzymatic nitration (Mihm *et al.*, 2003). This line of reasoning prompts us to suggest that, within the complex geometry of the myofibrillar system, the nitration of α-actinin might be favored over that of other relatively tyrosine-rich molecules in the human heart. Thus, α-actinin may be a principal target in cardiac pathologies involving increased peroxynitrite production. Our data further suggest that species differences could explain the apparently distinct sequences in the peroxynitrite-sensitivities of the myofibrillar proteins in human and animal hearts.

We have outlined here the mechanism by which peroxynitrite impairs Ca^{2+}-dependent myofibrillar force generation in the human heart. However, the peroxynitrite-evoked cardiac dysfunction may also depend on those additional peroxynitrite-sensitive processes that converge to the contractile function of the myocardium. Besides contractile protein nitration, these may include myofilament phosphorylation, Ca^{2+} transport systems and the energetic balance of the myocytes (Brunner & Wolkart, 2003; Gutierrez-Martin *et al.*, 2004; Ishida *et al.*, 1996; Lokuta *et al.*, 2005; Murray *et al.*, 2003; Stachowiak *et al.*, 1998; Walford *et al.*, 2004; Wendt *et al.*, 2003; Ziolo *et al.*, 2001). Further studies are therefore required to elucidate the relative contributions of the affected regulators to the overall pump function during peroxynitrite-induced human cardiac pathologies.

Chapter 4

CARDIOMYOCYTE STIFFNESS IN DIASTOLIC HEART FAILURE

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RESULTS

Patient classification

Diastolic heart failure (DHF) patients were referred for cardiac catheterization and endomyocardial biopsy procurement because of clinical suspicion of restrictive cardiomyopathy (n=7) or cardiac allograft rejection (n=5). They had all been admitted to hospital because of worsening heart failure. Left ventricular (LV) endomyocardial biopsies were obtained in patients suspected of restrictive cardiomyopathy and right ventricular (RV) endomyocardial biopsies in the transplant recipients. Subsequent histological examination ruled out infiltrative myocardial disease or rejection in all patients. Coronary angiography showed absence of significant coronary artery stenoses or graft vasculopathy. All patients satisfied the criteria as proposed by the European study group on DHF (European study group, 1998) i.e. signs and symptoms of congestive heart failure, LVEF>45% and LVEDP>16 mmHg. They all had one or more predisposing risk factors for diastolic LV dysfunction (*Table 2*) (Fischer *et al.*, 2003). LV muscle mass (119±16 g/m^2) was larger than normal (92±10 g/m^2; P<0.05) and 5 patients had significant LV hypertrophy (>125 g/m^2).

The control group consisted of 6 transplant recipients undergoing routine annual coronary angiography and biopsy procurement and of 2 patients referred for cardiac catheterization and endomyocardial biopsy procurement because of clinical suspicion of myocarditis. LV endomyocardial biopsies were obtained in the patients suspected of myocarditis and RV endomyocardial biopsies in the transplant recipients. Histological examination ruled out presence of myocarditis or rejection in all patients. They had no signs or symptoms of heart failure, a LVEF≥50% and a LVEDP≤16 mmHg.

Mean hemodynamic data of DHF patients and controls are listed in *Table 3*. Hemodynamic data were derived from LV angiograms, 2D echocardiograms and high fidelity LV catheter pressure measurements. The study protocol was approved by the local ethical committee. Written informed consent was obtained from all patients and there were no complications related to catheteriation or biopsy procurement.

Hemodynamic characteristics of the DHF patients

The mean hemodynamic data of the DHF group are compared to the control group in *Table 3*. Heart rate, LVEF, LVEDVI and CI in the DHF group were similar to the values measured in the control group. LVPSP, LVEDP, σ and E were significantly higher in the DHF group. The

higher LVEDP and σ at comparable LVEDVI implied reduced LV diastolic distensibility and the higher E increased myocardial stiffness (European study group, 1998).

Patient #	Age	Sex	Diagnosis	Medication
1	77	F	HT	ACEI; Amio; CCB; Diu; Stat
2	66	F	HT	Amio; ARB; ß; Dig; Diu
3	67	M	HT, DM, Ob	ACEI; Dig; Diu; Nit
4	82	F	HT	ACEI; ß; Dig; Diu; Stat
5	71	F	HT, DM	ACEI; Amio; CCB; Diu
6	71	M	HT, DM, Ob	ACEI; CCB; Nit; Diu
7	74	F	HT	ACEI; ARB; CCB; ß; Diu
8	74	M	HT, TX	ACEI; Amio; Diu; Imm
9	68	M	HT, DM, TX	ARB; Diu; Fibr; Imm
10	55	M	HT, DM, TX	Diu; Imm; Stat;
11	64	M	HT, TX	ACEI; Diu; Imm; Stat
12	61	M	HT, DM, TX	ARB; Diu; Imm; Stat

Table 2. Clinical characteristics of patients with diastolic heart failure
Diagnosis: DM: diabetes mellitus; HT: arterial hypertension; Ob: obesity; TX: patients after heart transplantation. Medication: ACE I, angiotensin converting enzyme inhibitors; Amio: Amiodarone; AR: antiarrhythmics; ARB: angiotensin II receptor blockers; ß: beta-blockers; CCB: calcium channel blockers; Dig: digitalis; Diu: diuretics; Fibr: Fibrates; Imm: immunosupressive therapy; Nit: nitrates; Stat: Statins.

	HR bpm	LVPSP mmHg	LVEDP mmHg	LVEDVI ml/m^2	LVEF %	CI l/min/m^2	σ kN/m^2	E kN/m^2
Control	80±16	135±15	13±4	78±23	71±12	2.7±0.6	1.6±0.3	2.2±0.7
DHF	84±11	186±35*	28±4*	74±16	71±11	2.8±0.6	5.1±1.1*	4.6±1.2*

Table 3. LV systolic and diastolic function of control and DHF groups (mean±SD).
HR: heart rate; LVPSP: left ventricular peak-systolic pressure; LVEDP: left ventricular end-diastolic pressure; LVEDVI: left ventricular end-diastolic volume index; LVEF: left ventricular ejection fraction; CI: cardiac index; σ: left ventricular end-diastolic wall stress; E: myocardial stiffness modulus. * P<0.05 DHF vs. control.

Force measurements in single cardiomyocytes

The average force-pCa relationships obtained in 6 control and 9 DHF patients are shown in *Figure 10A*. F_{total} at pCa 4.5 did not significantly differ between the DHF (20.3±7.5 kN/m^2, number of myocytes: n=23) and the control group (24.2±12.4 kN/m^2, n=15). However, $F_{passive}$ was significantly higher in the DHF (6.6±3.0 kN/m^2) than in the control group (3.5±1.7 kN/m^2; P<0.001). Higher $F_{passive}$ than in the control group was observed both in the DHF patients, who were transplant recipients (5.4±1.1 kN/m^2; P<0.05), and in the other DHF patients (7.2±2.9 kN/m^2; P<0.01). $F_{passive}$ of the control group was comparable to previously

reported values of cardiomyocytes isolated from non-failing donor hearts (van der Velden *et al.*, 2001; van der Velden *et al.*, 2003b).

No significant differences were found between the two groups in the Ca^{2+}-sensitivity of the contractile apparatus (pCa_{50}) and the steepness of the force-pCa curves (nHill) (*Table 4*).

	n	F_{total}	$F_{passive}$	nHill	pCa_{50}
	Before PKA				
Control	15	24.2±12.4	3.5±1.7	2.24±0.33	5.82±0.08
DHF	23	20.3±7.5	6.6±3.0*	2.43±0.53	5.86±0.07
	After PKA				
Control	11	24.3±14.1	2.6±1.4	2.67±0.47†	5.69±0.10†
DHF	16	22.2±6.8	3.4±1.0†	2.99±0.59†	5.70±0.08†

Table 4. Measurements of cardiomyocyte force and Ca^{2+}-sensitivity before and after PKA treatment.
n, number of cardiomyocytes; F_{total} and $F_{passive}$ (in kN/m^2) are forces measured at pCa 4.5 and pCa 9.0, respectively; *P<0.05 DHF vs. Control; †P<0.05 after vs. before PKA.

Myocardial tissue properties

DHF patients had higher collagen volume fraction (CVF) than controls (7.5±4.0% vs. 3.8±2.0%; P<0.05). CVF of the DHF patients, who were transplant recipients (7.5±3.0%) was similar to CVF of the other DHF patients (7.5±3.0%). DHF patients were equally distributed over the three classes of CVF (*Figure 11, left panel*) and one third of the patients therefore had low interstitial fibrosis. The higher values of LVEDP, σ and E in these patients compared to those of controls with low interstitial fibrosis indicates that CVF is not the sole contributor to diastolic LV dysfunction. No conspicuous differences in expression of myosin heavy chain, desmin, actin, TnT, tropomyosin, TnI, MLC-1 and MLC-2 were found between DHF and control myocardium. Western immunoblot analysis did not reveal any degradation product for desmin, TnT, TnI, MLC-1 and MLC-2 in either group. Moreover, the MLC-1/MLC-2 ratio did not differ between DHF (0.39±0.15) and controls (0.44±0.11). Phosphorylation status of TnI was determined in endomyocardial biopsies retrieved from 7 DHF patients and 7 controls. The ratio of dephosphorylated to total TnI was comparable in both groups (0.58±0.17 vs. 0.53±0.17). Furthermore, there was no correlation between this ratio and $F_{passive}$.

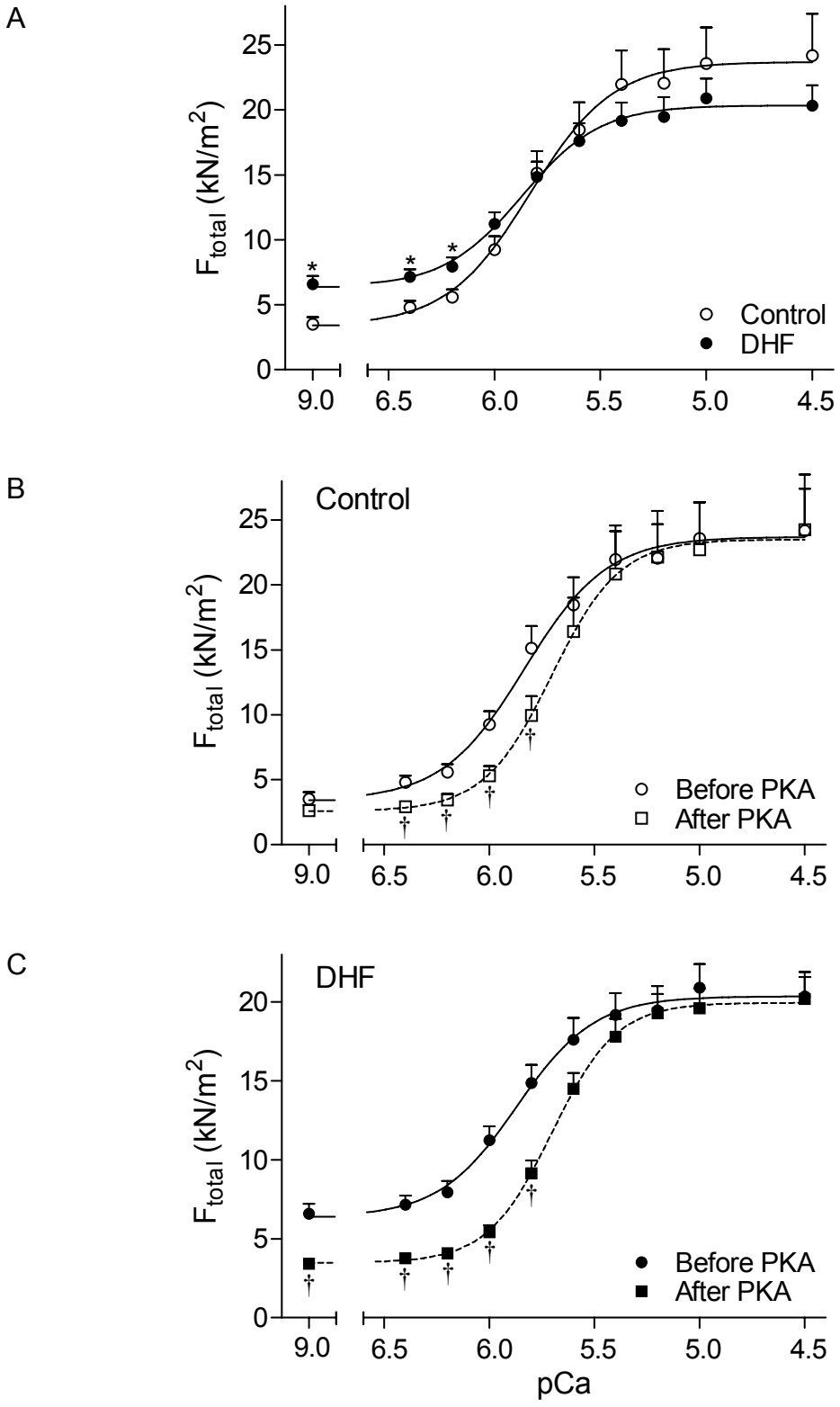

Figure 10. A. Average force-pCa relationship for the pooled cardiomyocytes of the control and DHF groups. F_{total} at pCa 4.5 and Ca^{2+}-sensitivity were similar in both groups but $F_{passive}$ was higher in the DHF group (*P<0.05). B. Average force-pCa relationship for the pooled cardiomyocytes of the control group before and after PKA. PKA decreased Ca^{2+}-sensitivity and left $F_{passive}$ unaltered. (†P<0.05 after vs. before PKA) C. Average force-pCa relationship for the pooled cardiomyocytes of the DHF group before and after PKA. PKA decreased Ca^{2+}-sensitivity and reduced $F_{passive.}$ (†P<0.05 after vs. before PKA)

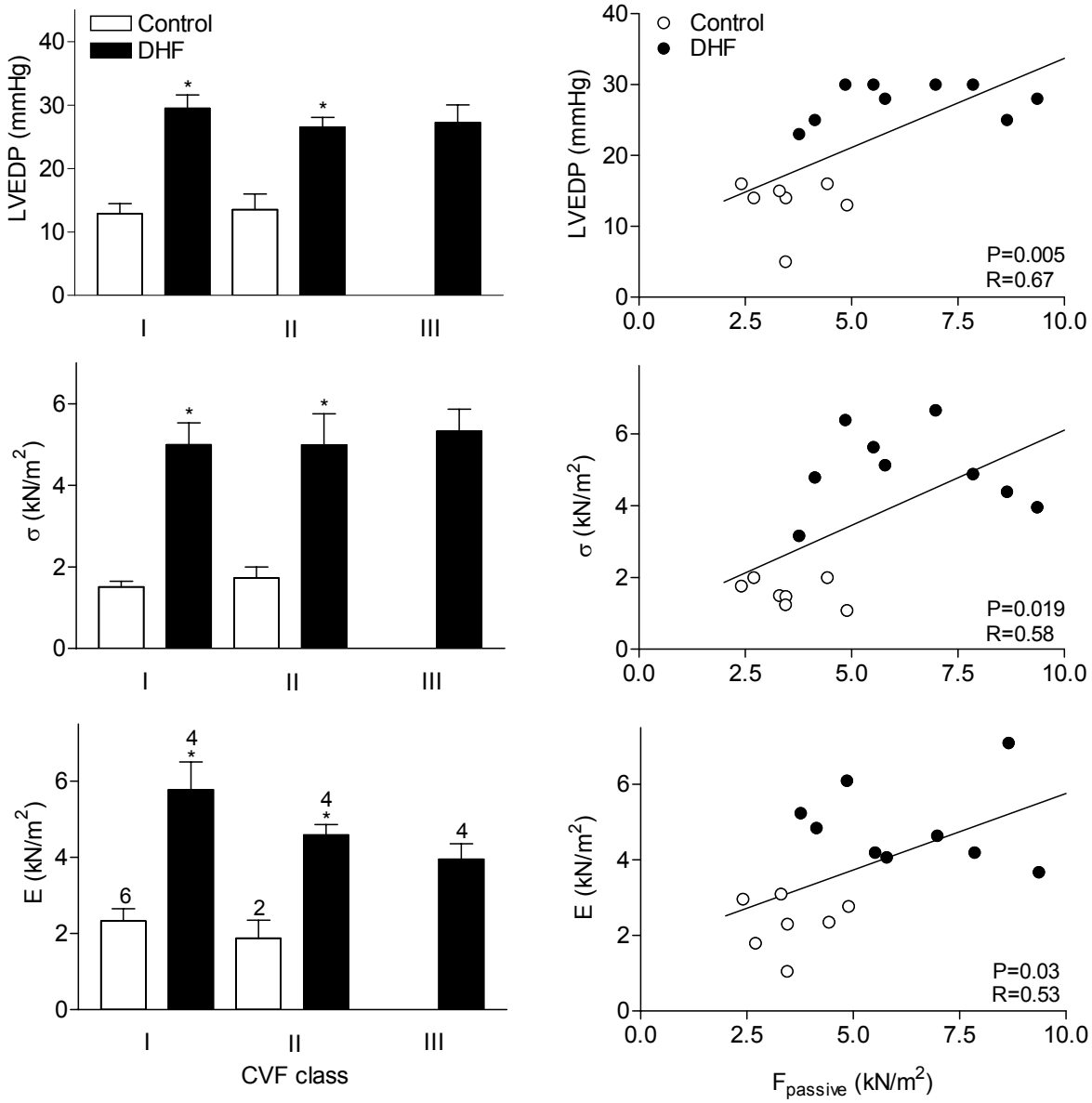

Figure 11. Left panel. At low (I) and intermediate (II) CVF class, DHF patients had higher LVEDP, σ and E than controls. Numbers above bars indicate number of individuals in each group. (* P<0.05 control vs. DHF within each CVF class) Right panel. Correlations between $F_{passive}$ averaged for all the cardiomyocytes of each individual and LVEDP, σ and E measured at the time of cardiac catheterization.

Correlation between *in vivo* hemodynamics and *in vitro* force

When the DHF and control groups were combined, a monovariate linear regression analysis revealed significant correlations between the average $F_{passive}$ of all the cardiomyocytes of each individual and LVEDP, σ or E measured in the same individual at the time of cardiac catheterization and biopsy retrieval (*Figure 11, right panel*). Note the quantitative agreement between the individual values of *in vivo* circumferential LV end-diastolic wall stress (σ) and $F_{passive}$ obtained in the isolated cardiomyocytes. These correlations were especially evident for

values of $F_{passive}$ up to 5.0 kN/m^2 and seemed to level off at higher values. A monovariate linear regression analysis also revealed significant correlations between CVF and LVEDP (R=0.63; P=0.009) or σ (R=0.68; P=0.004). In a bivariate linear regression analysis, the combination of $F_{passive}$ and CVF yielded stronger correlations with LVEDP (R=0.80; P=0.001) or σ (R=0.78; P=0.002) than $F_{passive}$ and CVF alone in monovariate analysis. $F_{passive}$ and CVF were unrelated (P=0.26).

PKA and cardiomyocyte force development

After PKA treatment, a second force-pCa relationship could be constructed in 11 cardiomyocytes isolated from biopsies of 4 control patients and in 16 cardiomyocytes isolated from biopsies of 7 DHF patients. *Figure 12* shows representative force recordings of a single cardiomyocyte retrieved from a DHF patient before and after PKA treatment at maximal (pCa 4.5) and intermediate (pCa 5.8) activation. At pCa 4.5 F_{total} remained the same, while at pCa 5.8 F_{total} was reduced after PKA. In addition, *Figure 12* illustrates the reduced $F_{passive}$ (pCa 9.0) after PKA. For control and DHF groups, F_{total} at pCa 4.5 was similar before and after PKA (*Table 4, Figures 10B, 10C*). At intermediate pCa (e.g. pCa 5.8), F_{total} was reduced after PKA because of PKA-induced myofilamentary desensitization (*Figures 10B, 10C*). The latter was also evident from the reduced pCa_{50} value observed in both DHF and control groups (*Table 4*). After PKA treatment $F_{passive}$ of DHF patients dropped to values observed in the control group both at baseline and after PKA treatment (*Figures 10B, 10C, 13A*). In addition, in DHF patients the PKA-induced fall in $F_{passive}$ was larger when baseline $F_{passive}$ was higher (*Figure 13B*).

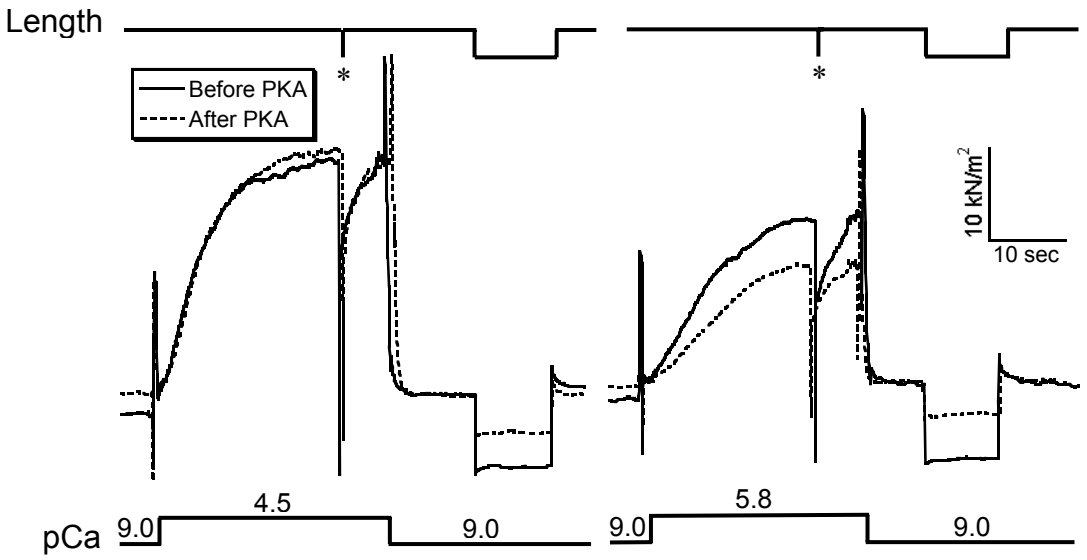

Figure 12. Contraction relaxation sequence recorded in a single cardiomyocyte before and after PKA treatment during maximal (pCa 4.5) and submaximal (pCa 5.8) activation. * Slack test (see text)

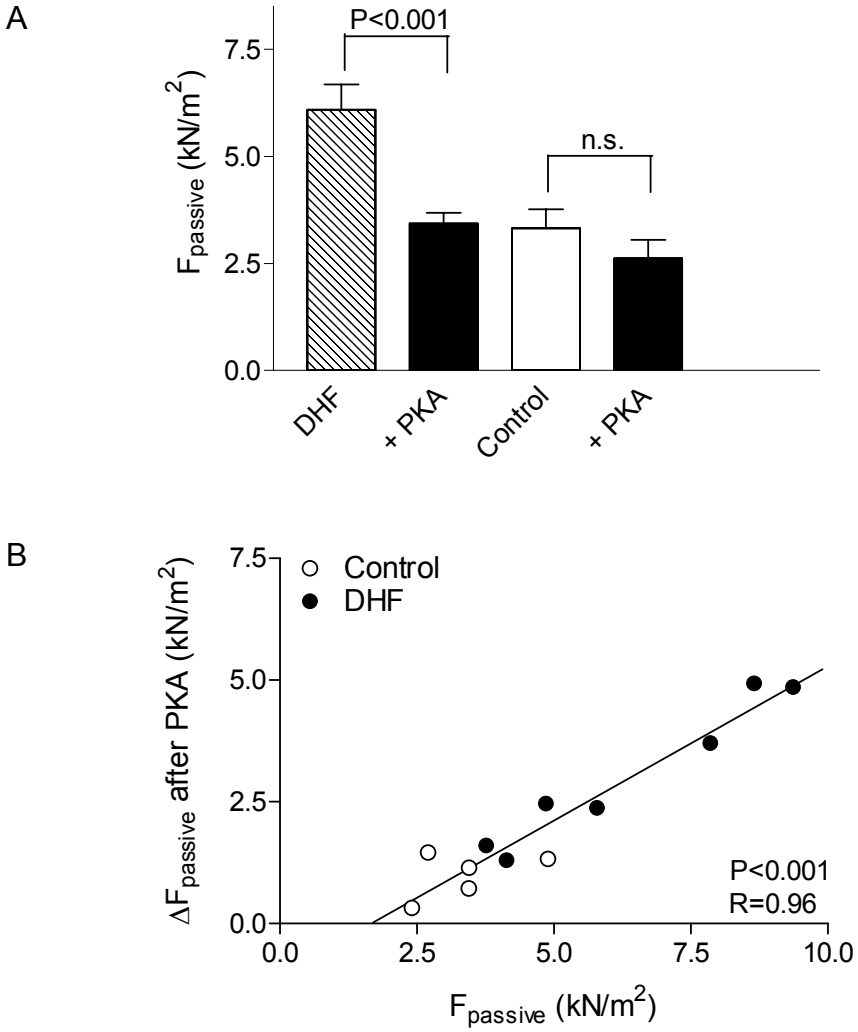

Figure 13. A. PKA treatment reduces $F_{passive}$ of DHF patients to values observed in the control group at baseline and after PKA treatment. B. Correlation between PKA-induced fall in $F_{passive}$ and baseline value of $F_{passive}$.

DISCUSSION

The present study analyzed endomyocardial biopsies obtained from patients with DHF and yielded the following: 1) When cardiomyocytes isolated from these biopsies were stretched to a sarcomere length of 2.2 μm, F_{total} at maximal [Ca^{2+}] was comparable to that of control cardiomyocytes but their $F_{passive}$ was twice as high; 2) The increase in $F_{passive}$ was reversible because administration of PKA lowered $F_{passive}$ to a level observed in control cardiomyocytes; 3) In-vivo hemodynamic measures of diastolic LV function such as LVEDP, LV end-diastolic wall stress (σ) and myocardial stiffness modulus (E) correlated with in-vitro measurements of both $F_{passive}$ and CVF.

High $F_{passive}$

Since the mechanical isolation procedure removed endomysial collagen structures, the high $F_{passive}$ of cardiomyocytes retrieved from DHF patients can only result from deranged diastolic stiffness of the cardiomyocytes themselves. Since cardiomyocytes were incubated in solution supplemented with 0.2% Triton X-100 prior to the experiments, integrity of sarcolemmal and sarcoplasmic membranes was disrupted and the cardiomyocytes became dependent on externally supplied calcium for active force development. Under these conditions, disturbed calcium handling because of modified expression and/or phosphorylation of sarcoplasmic reticular Ca^{2+}–ATPase (Frank *et al.*, 2002) , PLB (Hasenfuss & Pieske, 2002; MacLennan & Kranias, 2003), sarcoplasmic calcium release channel (Marks *et al.*, 2002) and sodium/calcium exchanger (Weber *et al.*, 2003) is effectively ruled out as a cause of the observed elevation of $F_{passive}$, which therefore needs to be attributed to alterations of myofilamentary or cytoskeletal proteins.

The present study revealed no difference between the DHF and control groups in the expression of cardiac sarcomeric proteins such as myosin heavy chain, actin, TnT, TnI, desmin and tropomyosin. Protein composition may also alter as result of enhanced proteolysis. This is especially evident for TnI, whose calpain-mediated breakdown is accelerated by a high LVEDP (Feng *et al.*, 2001). Western immunoblot analysis ruled out degradation of several contractile proteins, including TnI in both the control and DHF groups. Therefore, it is unlikely that a change in isoform composition or protein degradation accounts for the high $F_{passive}$ of cardiomyocytes observed in the DHF group.

Moreover, the correction by PKA treatment provides evidence that the high $F_{passive}$ results from phosphorylation of its sarcomeric target proteins: TnI, myosin binding protein-C

and/or titin. Treatment with PKA induced a large drop in $F_{passive}$ in cardiomyocytes of DHF patients, while neither $F_{passive}$ in control cardiomyocytes nor F_{total} at pCa 4.5 in cardiomyocytes from both groups were altered (*Table 4, Figure 10*). Such an isolated drop in $F_{passive}$, unaccompanied by a fall in F_{total}, is more easily reconciled with an action of PKA on a myofilamentary rather than on a cytoskeletal phosphorylation site because the parallel alignment of the cytoskeleton with the myofilaments would predict a fall in $F_{passive}$ generated by the cytoskeleton to also lower F_{total}.

The present study determined phosphorylation status of TnI but found no difference in the ratio of dephosphorylated to total TnI between control and DHF groups. It has recently been demonstrated in animal studies that phosphorylation of myosin binding protein-C and titin modifies diastolic properties. Phosphorylation (Flashman et al., 2004) or expression of a mutant isoform (Palmer *et al.*, 2004) releases the "braking" action of myosin binding protein-C on cross-bridge cycling thereby decreasing $F_{passive}$ in skinned mouse myocardial strips. Similarly, PKA-mediated phosphorylation of the elastic N2B spring element of titin reduces diastolic stiffness in isolated rat cardiomyocytes (Yamasaki *et al.*, 2002). Because of limited procurement of myocardial tissue by endomyocardial biopsy technique, phosphorylation of both proteins could not be addressed in the present study. Future studies using myocardial tissue of DHF patients should focus on the phosphorylation level of both proteins in order to detect the sarcomeric protein responsible for the high $F_{passive}$ of cardiomyocytes isolated from DHF patients.

In vitro versus *in vivo*

When $F_{passive}$ of control and DHF cardiomyocytes were pooled, in-vitro measurement of $F_{passive}$ correlated with in-vivo indices of diastolic LV dysfunction such as LVEDP, σ and E (*Figure 11*). The quantitative agreement between circumferential wall stress (σ) and $F_{passive}$ indicates that diastolic LV dysfunction is determined to an important extent by the rise in $F_{passive}$ of the cardiomyocytes. The relations between $F_{passive}$ and indices of diastolic LV function all leveled off at higher values of LVEDP, σ and E. This could have resulted from diuretic therapy to recompensate the patients prior to catheterization or from more intense interstitial fibrosis at the top end of diastolic LV dysfunction. In the in-vitro setting, all cardiomyocytes were stretched to the same sarcomere length of 2.2 μm. In the in-vivo setting, LV preload was uncontrolled and especially the DHF patients, who underwent more intense diuretic therapy in the interval between admission in pulmonary oedema and diagnostic

cardiac catheterization could have been operating at LV filling pressures lower than needed to achieve optimal sarcomere length. More intense interstitial fibrosis also provides an explanation for the relations between $F_{passive}$ and indices of diastolic LV function to level off at higher values of LVEDP, σ and E. Endomyocardial biopsies of patients with DHF had higher CVF than controls and in a bivariate linear regression analysis, both $F_{passive}$ and CVF significantly correlated with LVEDP and σ. A predominant effect of interstitial fibrosis at the top-end of diastolic LV dysfunction is in line with previous experimental studies, which showed diastolic muscle stiffness to originate from structures within the sarcomere for sarcomere lengths <2.2μm (Linke *et al.*, 1994)and from perimysial fibers once filling pressures exceeded 30 mmHg (Factor *et al.*, 1988).

Degradation of collagen in pressure-overloaded hypertrophied papillary muscles with plasmin did not reduce muscle stiffness to levels observed in normal muscles (Stroud *et al.*, 2002). Similarly, in the present study patients with DHF and low collagen volume fraction still had higher LVEDP, σ and E than controls (*Figure 11*). Therefore our data support the concept that diastolic LV dysfunction in the presence of a low collagen volume fraction is explained by higher $F_{passive}$ of the cardiomyocytes. However, as half of the DHF patients suffered from diabetes mellitus, collagen cross-links formed by advanced glycation end products could also explain impairment of diastolic LV function at low collagen volume fraction (Herrmann *et al.*, 2003).

The present study observed low CVF and high $F_{passive}$ in some patients with DHF but failed to detect DHF patients with high CVF and low $F_{passive}$. This suggests diastolic LV dysfunction to result from a sequence of events, which starts off with a rise in cardiomyocyte $F_{passive}$ subsequently followed by development of interstitial fibrosis. A similar sequence of events has also been reported in experimental tachypacing-induced heart failure models. In these models, elevation of diastolic LV muscle stiffness was paralleled by expression of shorter and stiffer N2B isoform of titin (Wu *et al.*, 2002) and not by interstitial fibrosis, which only developed if angiotensin II infusion was superimposed on the pacing stress (Senzaki *et al.*, 2000). Similar coordination between titin isoform shift and extracellular matrix deposition has also been reported in other experimental models (Wu *et al.*, 2000). The evidence provided by the present study that DHF patients can have low CVF also explains why angiotensin II receptor blockers and ACE inhibitors, which reduce interstitial fibrosis (Brilla *et al.*, 2000; Yamamoto *et al.*, 2002), have not been uniformly successful in large clinical trials on DHF patients (Yusuf *et al.*, 2003).

Study limitations

Five of the 12 DHF patients and six of the 8 controls had undergone cardiac transplantation. Transplant recipients were included in the study because they frequently suffer of DHF (European study group, 1998) and because their myocardial biopsy material is readily available. The pathogenetic mechanisms responsible for their DHF could differ from other DHF patients because of ongoing rejection and use of immunosupressant drugs. Force recordings of isolated cardiomyocytes and extent of interstitial fibrosis of the transplanted subgroup of DHF patients were however similar to the measurements obtained in the other DHF patients and both data sets were therefore merged into a single DHF group. The same also applied to the control group.

Isolation of cardiomyocytes and assessment of myocardial tissue properties was performed on a limited number of right or left ventricular biopsies and could potentially have overlooked tissue heterogeneity. The extent of tissue heterogeneity was addressed in previous studies using explanted hearts (van der Velden *et al.*, 2001; van der Velden *et al.*, 2003b) or surgically procured biopsies (van der Velden *et al.*, 1999). In these studies the variability of force measurements of cardiomyocytes isolated from different portions of the heart was always less than 5%. To validate the use of defrosted biopsy samples, force recordings of cardiomyocytes isolated from a biopsy sample immediately after procurement were compared to force recordings of cardiomyocytes isolated from a defrosted biopsy of the same patient. These force recordings yielded identical results.

Conclusions

Cardiomyocytes isolated from endomyocardial biopsies of DHF patients had elevated $F_{passive}$, which together with CVF, determined in-vivo diastolic LV dysfunction. Administration of PKA to these cardiomyocytes normalized $F_{passive}$. Since integrity of sarcolemmal and sarcoplasmic membranes was disrupted by prior Triton incubation and since expression of sarcomeric proteins and the phosphorylation level of TnI were unaltered, the PKA-induced fall of $F_{passive}$ probably resulted from correction of a phosphorylation deficit of myosin binding protein-C or titin. This hypophosphorylated sarcomeric protein could, together with extracellular matrix modification, be a specific myocardial target for drug therapy of DHF.

Chapter 5

EFFECTS OF Ca^{2+}-SENSITIZERS IN PERMEABILIZED CARDIAC MYOCYTES FROM DONOR AND END-STAGE FAILING HUMAN HEARTS

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RESULTS

Patient characteristics

Left ventricular biopsies were obtained during heart transplantation surgery from 3 explanted end-stage failing (New York Heart Association class IV) hearts (3 males, age range 45-65 years) and 3 non-failing donor hearts (2 males, 1 female; age range 23-52 years). The heart failure resulted from ischaemic (*n*=1) or dilated (*n*=2) cardiomyopathy. Patients received angiotensin converting enzyme inhibitors and diuretics before heart transplantation. In addition, some patients with heart failure received anticoagulants, digitoxin and/or nitrates. The cardiac tissue was transported in cardioplegic solution and upon arrival in the laboratory was stored in liquid nitrogen. Samples from failing hearts were obtained after informed consent and with the approval of the local Ethical Committees. All procedures followed were in accordance with institutional guidelines.

Cardiomyocyte force measurements

F_{max} of the failing and non-failing myocytes (38±4 kN/m^2 and 36±3 kN/m^2, respectively) and $F_{passive}$ of the failing and non-failing myocytes (1.5±0.3 kN/m^2 and 1.2±0.2 kN/m^2, respectively) did not differ significantly under control conditions (pH 7.2; 0 mM P_i). In agreement with previous studies, however, the Ca^{2+}-sensitivity of the isometric force of the failing myocytes was significantly higher (ΔpCa_{50}=0.15) than that observed in the non-failing myocytes. Under mimicked ischaemic conditions (pH 6.5; 10 mM P_i), the maximum force development declined to 22±1% of the control value, and a marked decrease in Ca^{2+}-sensitivity was also observed ($\Delta pCa_{50}\approx$1.4) in both the failing and the non-failing myocytes. Surprisingly, the initial difference in the [Ca^{2+}] - force relations of the failing and non-failing myocytes disappeared in the presence of the ischaemic metabolites (*Figure 14*).

The Ca^{2+}-sensitizer EMD 53998 (10 μM) increased the force effectively in a wide range of [Ca^{2+}] under all conditions studied (*Figure 15*) and induced a pronounced leftward shift in the Ca^{2+}-sensitivity relations under the control conditions. This shift, however, was smaller in the failing (ΔpCa_{50}=0.24) than in the non-failing (ΔpCa_{50}=0.48) myocytes (Table 1). EMD 53998 enhanced the maximum force-generating capacity (F_{max}) and the force in the virtual absence (pCa 9) of Ca^{2+} ($F_{passive}$) (*Table 5*).

The Ca^{2+}-sensitizing effect of 10 μM OR-1896 was less than that of 10 μM EMD 53998, but it was comparable ($\Delta pCa_{50} \approx 0.1$) in the failing and non-failing myocytes (*Figure 16, Table 5*). Ca^{2+}-sensitization due to OR-1896 could not be observed under mimicked ischaemic conditions and, in contrast with EMD 53998, OR-1896 did not modulate F_{max} or $F_{passive}$.

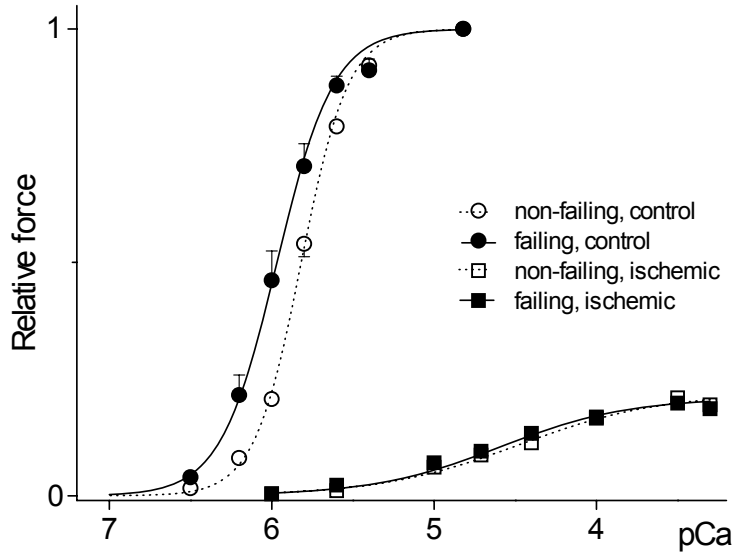

Figure 14. The Ca^{2+}-dependence of isometric force production under control conditions (pH 7.2; no added inorganic phosphate (Pi)) and under mimicked ischemic conditions (pH 6.5; 10 mM Pi) in isolated Triton-skinned myocytes. Continuous and dashed lines show results of Hill fits to the relative force values of failing and non-failing hearts, respectively. Error bars indicate SEM when larger than symbol size.

Conditions	Failing pCa_{50}	Non-failing pCa_{50}	Failing nHill	Non-failing nHill	Failing F_{max}	Non-failing F_{max}	Failing $F_{passive}$	Non-failing $F_{passive}$
pH 7.2, P_i 0 mM (Control)	5.97±0.04 *	5.82±0.01	2.49±0.17 *	2.99±0.13	1	1	0	0
Control EMD 53998	6.21±0.10 †	6.30±0.05 †	2.32±0.53	1.65±0.21 †	1.08±0.03 *	1.21±0.03 †	0.20±0.05 *†	0.11±0.02 †
Control OR-1896	6.11±0.04 *†	5.90±0.03 †	2.73±0.21	3.07±0.23	0.93±0.03	0.97±0.02	0	0
pH 6.5, P_i 10 mM (Ischaemic)	4.59±0.19 ‡	4.47±0.08 ‡	1.10±0.15 ‡	1.00±0.07 ‡	0.21±0.02 ‡	0.22±0.01 ‡	0	0
Ischaemic EMD 53998	4.71±0.12 ‡	4.86±0.10 †‡	0.61±0.06 †‡	1.21±0.21 ‡	0.44±0.04 †‡	0.40±0.02 †‡	0.01±0.01	0.02±0.01 †‡
Ischaemic OR-1896	4.51±0.11 ‡	4.37±0.07 ‡	1.39±0.32 ‡	1.20±0.17 ‡	0.18±0.02 ‡	0.23±0.02 ‡	0	0

Table 5. Parameters of Ca^{2+}-sensitivity curves.
EMD 53998 and OR-1896 were employed in 10 μM concentration. The data for each group were calculated from 4-17 cells. *$p<0.05$ vs non-failing myocytes; † $p<0.05$ vs drug-free under the same conditions; ‡$p<0.05$ vs drug-free control conditions (pH 7.2, P_i 0 mM).

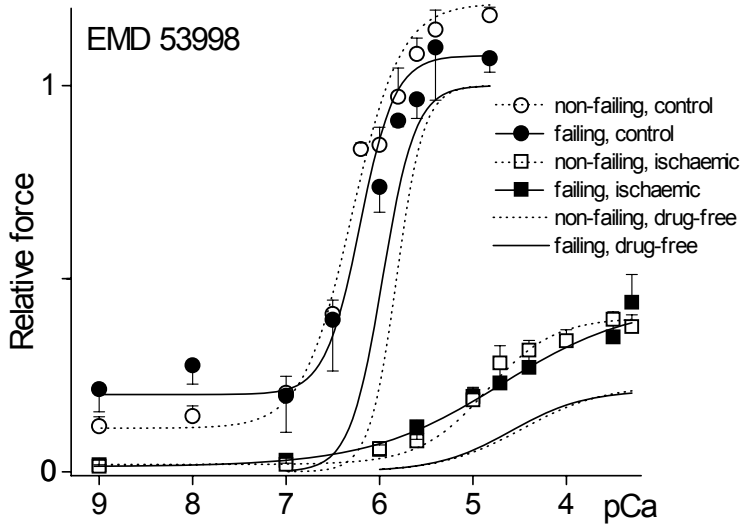

Figure 15. The influence of 10 μM EMD 53998 on the Ca^{2+}-dependences of isometric force production under control conditions and under mimicked ischemic conditions. Continuous and dashed lines show results of Hill fits to the relative force values of failing and non-failing hearts, respectively. The continuous and dashed lines without symbols on the right of the data represent results of Hill fits under drug-free conditions (also shown in *Figure 14*) and serve for comparative purposes.

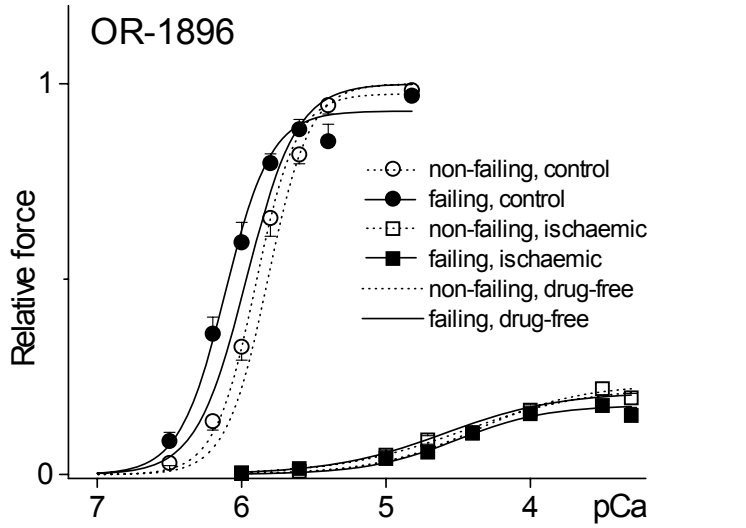

Figure 16. The influence of 10 μM OR-1896 on the Ca^{2+}-dependences of isometric force production under control conditions and under mimicked ischemic conditions. Continuous and dashed lines show results of Hill fits to the relative force values of failing and non-failing hearts, respectively. The continuous and dashed lines without symbols on the right of the data represent results of Hill fits under drug-free conditions (also shown in *Figure 14*) and serve for comparative purposes.

DISCUSSION

In this study, a comparison was made between the effects of two Ca^{2+}-sensitizers (EMD 53998 and OR-1896) under normal physiological conditions and under mimicked ischaemic conditions in human myocytes. In line with previous animal studies (Palmer *et al.*, 1995; Soergel *et al.*, 2004; Szilagyi *et al.*, 2004; Vannier *et al.*, 1997), it was found that at equal concentrations the Ca^{2+}-sensitizing effect of EMD 53998 was higher than that of OR-1896 but, in contrast with OR-1896, EMD 53998 also increased the force in the absence of Ca^{2+}.

The (+) enantiomer of EMD 53998 (EMD 57033) promotes force-generating cross-bridge transitions through a probable interaction with the signalling between troponin I and troponin C, and its action is modulated by the regulatory proteins of the thin filament (Kogler *et al.*, 1998; Soergel *et al.*, 2004). Considerable research efforts have been conducted to elucidate the mechanistic consequences of EMD 57033 on the actin-myosin cycle. Nevertheless, no consensus has been reached on whether it increases the apparent rate of cross-bridge attachment f_{app}, decreases the apparent rate of cross-bridge detachment g_{app}, and/or increases the force generated per cross-bridge (Lee & Allen, 1997; Palmer *et al.*, 1995; Solaro *et al.*, 1993; Vannier *et al.*, 1997). EMD 53998 increased both P_o and P_{rest} (under control conditions). Therefore, our results are consistent with the suggestion that EMD 53998 increases the force generated per cross-bridge (Kraft & Brenner, 1997), with an additional effect on the cooperativity between cross-bridges (Palmer *et al.*, 1995) in human myocytes. EMD 53998 affected pCa_{50} to a lesser extent in the failing as compared with the non-failing myocytes.

This study and our previous data indicate that failing myocytes display a higher Ca^{2+}-sensitivity than that of myocytes from non-failing donor hearts, mainly because troponin I is less phosphorylated (van der Velden *et al.*, 2003b). In this respect, it is of interest that an augmented systolic response to EMD 57033 was recently observed in a transgenic animal model with troponin I truncation (Soergel *et al.*, 2004). A decrease in phosphorylation of myosin light chain 2 (van der Velden *et al.*, 2003a) might also explain the decreased efficacy of EMD 57033 in failing hearts, because myosin light chain 2 dephosphorylation moves the myosin head toward the backbone of the thick filament away from the thin filament, and this may decrease the actin - myosin interaction (Levine *et al.*, 1996).

The extent of Ca^{2+}-sensitization by OR-1896 was similar to that previously reported for levosimendan and OR-1896 in guinea pig myocyte preparations (Szilagyi *et al.*, 2004). Our

results therefore support the Ca^{2+}-sensitizing function of OR-1896 in the human heart via a mechanism similar to that for its parent molecule, levosimendan (Edes *et al.*, 1995; Takahashi *et al.*, 2000b). In addition, OR-1896 induced a small, but comparable leftward shift in the $[Ca^{2+}]$-force relation of both the failing and the non-failing myocytes under the control conditions. Hence, Ca^{2+}-sensitization by OR-1896, which has a long half-life (≈ 80 hours), may potentially contribute to the improved survival rate of patients with advanced heart failure following a single infusion of levosimendan (Follath *et al.*, 2002).

Both in the failing and in the non-failing myocytes, the Ca^{2+}-sensitivity and the steepness of the $[Ca^{2+}]$-force relationship were strongly reduced under mimicked ischaemic conditions (pH 6.5; P_i 10 mM). Decrease of the pH has a major depressant influence on the Ca^{2+}-binding function of troponin C (Palmer & Kentish, 1994) and P_i reverses the P_i-release step of the cross-bridge cycle by mass action, thereby decreasing the proportion of cross-bridges in the high-force conformation (Kentish, 1991; Papp *et al.*, 2002; Regnier *et al.*, 1995). Hence, the more pronounced effects of ischaemic metabolites in failing myocytes than in non-failing myocytes extends the distinctions between the myofilament Ca^{2+}-regulation of failing and non-failing myocytes (van der Velden *et al.*, 2001; van der Velden *et al.*, 2003b). The antagonistic effect of P_i on EMD 57033-evoked force enhancement (Strauss *et al.*, 1992) supports the previously suggested intimate relationship between EMD 57033 and the force-generating actin-myosin interactions (Solaro *et al.*, 1993). In addition, in the presence of 10 mM P_i at pH 6.5 (and also at 10 mM P_i at pH 7.2; data not shown), the EMD 53998-evoked Ca^{2+}-independent resting force component was eliminated. However, ischaemic metabolites did not abolish the force-enhancing effect of EMD 53998 in the presence of Ca^{2+}. This suggests different modalities of action for EMD 53998 in the absence and presence of Ca^{2+}. In contrast with EMD 53998, no Ca^{2+}-sensitization could be resolved by OR-1896 when ischaemic metabolites were present.

In summary, the increased Ca^{2+}-sensitivity of the failing myocytes did not prevent further pharmacological Ca^{2+}-sensitization under normal metabolic conditions pertinent to the myocardium with a preserved energy status. The Ca^{2+}-responsivenes of the contractile apparatus, however, is also a function of the ischaemic metabolites, which may modulate the apparent Ca^{2+}-sensitivity and the efficacy of Ca^{2+}-sensitizing agents.

CONCLUSIONS and GENERAL DISCUSSION

In animal models of heart failure, the impairment of cardiac contractility has been associated with several functional, biochemical and molecular alterations at the level of the myocardial contractile proteins. However, the functional consequences of these changes during the development of human heart failure are still unclear (Chapter 1).

In this thesis mechanical properties of isolated cardiomyocytes and the contractile proteins were studied of donor and failing human hearts (Chapter 2). Isometric force development and its Ca^{2+}-sensitivity were measured in permeabilized single cardiomyocytes under control conditions and after the application of: authentic peroxynitrite (Chapter 3), the active subunit of protein kinase A (Chapter 4), or the Ca^{2+}-sensitizers OR-1896 and EMD 53998 (Chapter 5). Furthermore, these measurements were combined with biochemical methods for the analysis of myocardial proteins and protein nitration, to get a further insight in the relation between contractile protein composition and the mechanical properties of the human heart.

In Chapter 3 an attempt was made to elucidate the molecular background of peroxynitrite-evoked alterations in the human myocardium. The maximal Ca^{2+}-activated isometric force decreased to zero with increasing concentrations of peroxynitrite, in a concentration-dependent manner. However, there were no differences before and after the application of 50 μM peroxynitrite in the Ca^{2+}-sensitivity of force production, in the steepness of the Ca^{2+}-force relation and in the actin-myosin turnover kinetics. Nevertheless, 50 μM peroxynitrite greatly deteriorated the cross-striation pattern and induced a slight, but significant increase in the passive force component ($F_{passive}$), reflecting ultrastructural alterations. Western immunoblots revealed that 50 μM peroxynitrite selectively induced the nitration of a protein with an apparent molecular mass of about 100 kDa. Subsequent immunoprecipitation assays identified this nitrated protein as α-actinin, a major Z-line protein. These results suggest α-actinin as a novel target for peroxynitrite in the human myocardium; its nitration induces a contractile dysfunction, presumably by decreasing the longitudinal transmission of force between adjacent sarcomeres.

Heart failure with preserved left ventricular ejection fraction is increasingly recognized and usually referred to as diastolic heart failure. Its pathogenetic mechanism remains unclear partly because of lack of myocardial biopsy material.

In Chapter 4 endomyocardial biopsies obtained in diastolic heart failure (DHF) patients were therefore analyzed for collagen volume fraction (CVF) and sarcomeric protein composition and compared to control biopsies. Single cardiomyocytes were isolated from these biopsies in order to assess cellular contractile performance. DHF patients had a normal left ventricular ejection fraction, an elevated end-diastolic pressure and no significant coronary artery stenoses. DHF patients had higher CVF than controls and no conspicuous changes in sarcomeric protein composition were detected. Compared to cardiomyocytes of controls, cardiomyocytes of DHF patients developed similar total isometric force at maximal Ca^{2+}concentration but their $F_{passive}$ in the absence of Ca^{2+} was almost twice as high. $F_{passive}$ and CVF combined yielded stronger correlations with LVEDP than either of them alone. Administration of PKA to DHF cardiomyocytes lowered $F_{passive}$ to control value. In conclusion, DHF patients had stiffer cardiomyocytes, as evident from a higher $F_{passive}$ at the same sarcomere length. Together with CVF, $F_{passive}$ determined in-vivo diastolic LV dysfunction. Correction of this high $F_{passive}$ by PKA suggests that reduced phosphorylation of sarcomeric proteins is involved in DHF. These hypophosphorylated sarcomeric proteins could, together with extracellular matrix modification, be specific myocardial targets for drug therapy of DHF.

In Chapter 5 an attempt was made to quantify the magnitude of the effects of Ca^{2+}-sensitizers in cardiomyocytes from end-stage failing and non-failing donor hearts under control conditions (pH 7.2; no added inorganic phosphate (P_i)) and under mimicked ischaemic conditions (pH 6.5; 10 mM P_i). Two different Ca^{2+}-sensitizers were used: EMD 53998 (10 μM), which exerts its influence through the actin-myosin interaction, and OR-1896 (10 μM) (the active metabolite of levosimendan), which affects the Ca^{2+}-sensory function of the thin filaments. F_{max} measured at saturating Ca^{2+} concentration and $F_{passive}$ determined in the virtual absence of Ca^{2+} did not differ between the failing and non-failing myocytes, but the Ca^{2+} concentration required to induce the half-maximal force under control conditions was significantly lower in the failing than in the non-failing myocytes (ΔpCa_{50}=0.15). This difference in Ca^{2+}-sensitivity, however, was abolished during mimicked ischaemia. EMD 53998 increased F_{max} and $F_{passive}$ by approximately 15% of F_{max} and greatly enhanced the Ca^{2+}-sensitivity (ΔpCa_{50}>0.25) of force production. OR 1896 did not affect F_{max} and $F_{passive}$, and provoked a small, but significant Ca^{2+}-sensitization ($\Delta pCa_{50}\approx$0.1). All of these effects were comparable in the donor and failing myocytes, but, in contrast with OR-1896, EMD 53998 considerably diminished the difference in the Ca^{2+}-sensitivities between the failing and

non-failing myocytes. The action of Ca^{2+}-sensitizers under mimicked ischaemic conditions was impaired to a similar degree in the donor and the failing myocytes. Our results indicate that the Ca^{2+}-activation of the myofibrillar system is altered in end-stage human heart failure. This modulates the effects of Ca^{2+}-sensitizers both under control and under mimicked ischaemic conditions.

Taken together, the measurement of force generation in isolated cardiomyocytes in combination with biochemical assays to determine myocardial protein alterations are appropriate, reliable and valuable methods for the characterization of human heart failure.

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2. Borbély, A., van der Velden, J., Papp, Z., Bronzwaer, J.G.F., Édes, I., Stienen, G.J.M., Paulus, W.J. (2005) Cardiomyocyte stiffness in diastolic heart failure. *Circulation.* 111, 774-781. IF: 11,164
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4. Papp, Z., van der Velden, J., Borbély, A., Édes, I., Stienen, G.J.M. Altered myocardial force generation in end-stage human heart failure. (in preparation)

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1. Papp, Z., van der Velden, J., de Jong, JW., Vaszily, M., Borbély, A., Édes, I., Stienen, G.J.M. (2002) Specific alterations of the actin-myosin cycle during heart failure. *Cardiol. Hung*. Suppl.1., 89. IF: 0
2. Papp, Z., van der Velden, J., de Jong, J.W., Borbély, A., Édes, I., Stienen, G.J.M. (2002) Altered P_i-release step of the actin-myosin cycle during human heart failure J. *Mol. Cell. Cardiol.* 34, A49. IF: 4,091
3. Papp, Z., van der Velden, J., de Jong, J.W., Borbély, A., Édes, I., Stienen, G.J.M. (2002) Heart failure specific alterations in the Ca^{2+}-dependencies of force generation and actin-myosin turnover rate in isolated human myocardial cells *J. Muscle Res. Cell Motil.* 23, 32. IF: 1,318

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