

SUMMARY OF DOCTORAL (Ph.D.) THESIS

ALTERATIONS IN MYOCARDIAL FUNCTION AND CONTRACTILE
PROTEINS DURING HUMAN HEART FAILURE

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Alterations in Myocardial Function and Contractile Proteins during Human
Heart Failure

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GENERAL 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. 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.

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.

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. 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, altered hemodynamics, neurohumoral activation, cytokine overexpression and endothelial dysfunction.

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. 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. Controversy exists regarding myofilament Ca^{2+} -sensitivity in end-stage heart failure, which was suggested to be unchanged, decreased or increased. Moreover, alterations primarily in the myofilamentary proteins have been implicated in myocardial injuries that develop during reperfusion following ischaemia, or as a consequence of exposure to inflammatory cytokines. During end-stage human heart failure, a number of alterations have been reported that affect the expression and phosphorylation of the contractile proteins. These changes may contribute to both the systolic and the diastolic dysfunction observed in end-stage failing hearts.

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. It was previously demonstrated in different animal preparations, that peroxynitrite modulated myocardial proteins via the formation of nitrotyrosine, and the amount of nitrated proteins correlated with the reduction in cardiac pump function. 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, 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. 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. 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. 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, as evident from a population-based survey, in which diastolic LV dysfunction was observed five times more often than systolic LV dysfunction.

In contrast 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. 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.

DHF is currently diagnosed in as much as 49% of heart failure patients. 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.

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 or from subtle systolic LV dysfunction, unappreciated by a routine LVEF measurement and possibly exacerbated by high arterial impedance, is still a matter of debate. Furthermore, explanations proposed for diastolic LV dysfunction are divergent ranging from high LV myocardial stiffness to pericardial or right ventricular constraint. Moreover, the relative importance of myocardial fibrosis and of high cardiomyocyte resting tension for LV myocardial stiffness remains undefined.

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

Ca²⁺-sensitizers in heart failure

Ca²⁺-sensitizers represent a new class of inotropic drugs. They improve myocardial performance by directly acting on contractile proteins without increasing intracellular Ca²⁺ load. Thus, they avoid the undesired effects of an increased intracellular Ca²⁺ load. Ca²⁺-sensitizers may enhance myocardial performance without increasing myocardial oxygen consumption and without provoking fatal arrhythmias. The therapeutic consequences, however, are not understood in detail.

Isometric force production and its Ca²⁺-sensitivity are determined by the cooperative interplay between the Ca²⁺ regulation on the thin filaments and strongly bound force-generating cross-bridges. Ca²⁺ regulation and force generation, on the other hand, are impaired by accumulating intracellular metabolites (i.e. H⁺ and inorganic phosphate (P_i)) during ischaemia. Hence, ischaemic metabolites may also influence pharmacological Ca²⁺-sensitization.

EMD 57033, the (+) enantiomer of 5-[1-(3,4-dimethoxybenzoyl)-1,2,3,4-tetrahydro-6-quinolyl]-6-methyl-3,6-dihydro-2H-1,3,4-thiadiazin-2-one), EMD 53998, interferes with the force-generating actin-myosin interactions and antagonises the effects of P_i. In multicellular preparations of failing human hearts, the positive inotropy of EMD 57033 was accompanied by a pronounced negative lusitropic effect. This negative lusitropy was associated with an EMD 57033-evoked Ca²⁺-independent force component in porcine skinned cardiac trabeculae. However, the development of this Ca²⁺-independent force was not consistently observed in human myocardial preparations.

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 cardiostimulant 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 Ca²⁺-sensitizing property in intact rabbit papillary muscles, permeabilized myocyte-sized preparations of the guinea pig, and canine ventricular intact trabeculae both at the normal and at acidic pH. Furthermore, it has been suggested that, due to its structural homology to levosimendan, OR-1896 facilitates force production by stabilising the Ca²⁺-

bound conformation of TnC. 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 defrosted and mechanically disrupted. A demembrated single cardiomyocyte was mounted between two thin insect needles with silicone adhesive while viewed under an inverted microscope.

2.1.2. Solutions

The compositions of the relaxing and activating solutions used during force measurements were calculated as described by Fabiato (1979).

2.1.3. Experimental protocol

Isometric force was measured after the myocyte had been transferred from the relaxing solution to a Ca^{2+} -containing solution. Peak isometric force (F_{total}) and the rate constant of force redevelopment (k_{tr}) at various $[\text{Ca}^{2+}]$ levels were measured. 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}).

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

EMD 53998 and OR-1896 were kindly provided by Orion Pharma and peroxynitrite with low H_2O_2 content was obtained from Calbiochem. The catalytic subunit of protein kinase A was purchased from SIGMA.

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). For visualization, biotinylated secondary antibodies and enhanced chemiluminescence were used. Some of the assays were combined with the removal of the bound antibody complexes (stripping).

2.2.2. Dot blot

Samples treated with 0-500 μM peroxynitrite were dotted onto nitrocellulose membranes. Dot intensities were quantified from unsaturated recordings by densitometry, using custom-prepared software.

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.

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 and expressed as collagen volume fraction (CVF). 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.

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. 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:

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

where F_{total} is the steady-state force at a given $[\text{Ca}^{2+}]$; F_{max} is the steady Ca^{2+} -activated force component at saturating $[\text{Ca}^{2+}]$; and F_{passive} is the Ca^{2+} -independent force production at pCa 9. Ca_{50} (or pCa₅₀) corresponds to the $[\text{Ca}^{2+}]$ at which $F_{\text{total}} - F_{\text{passive}} = F_{\text{max}} / 2$ 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.

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.

Values are given as mean \pm 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).

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

RESULTS

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. 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. The maximal isometric Ca^{2+} -activated force (F_{\max} : $28 \pm 2 \text{ kN/m}^2$) decreased to zero in a range of peroxynitrite concentrations (IC_{50} : $55 \pm 4 \text{ }\mu\text{M}$) in which the protein nitration level exhibited a dramatic increase.

To elucidate the mechanistic background of peroxynitrite-induced contractile alterations, the $[\text{Ca}^{2+}]$ -dependences of force and of the rate of force redevelopment following unloaded shortening and restretch (k_{tr}) were determined before and after $50 \text{ }\mu\text{M}$ peroxynitrite exposure. Peroxynitrite decreased isometric force at all Ca^{2+} concentrations studied. However, following force normalization to the respective maximum, the Ca^{2+} -sensitivity curve before peroxynitrite treatment did not differ from that obtained after peroxynitrite application (pCa₅₀: 5.89 ± 0.02 and 5.86 ± 0.04 ; nHill: 2.22 ± 0.11 and 2.42 ± 0.25 ; before and after $50 \text{ }\mu\text{M}$ peroxynitrite, respectively ($P > 0.05$)). Additionally, the cross-bridge specific kinetic parameter k_{tr} did not change either at pCa 4.75 ($k_{\text{tr,max}}$: $1.14 \pm 0.03 \text{ 1/s}$ and $1.05 \pm 0.07 \text{ 1/s}$ before and after $50 \text{ }\mu\text{M}$ peroxynitrite) or at submaximal Ca^{2+} concentrations. Nevertheless, the cross-striation pattern of the myocyte preparations deteriorated after $50 \text{ }\mu\text{M}$ peroxynitrite treatment, and the F_{passive} increased from $2.1 \pm 0.1 \text{ kN/m}^2$ to $2.5 \pm 0.2 \text{ kN/m}^2$ ($n=57$ cells; $P < 0.05$), suggesting ultrastructural damage. Control force measurements verified that the observed mechanical changes were the direct consequence of $50 \text{ }\mu\text{M}$ peroxynitrite exposure and not to contaminating hydrogen peroxide or the by-products of peroxynitrite (i.e. nitrite and nitrate).

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. 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), these latter proteins were not stained in the presence of 50 μM peroxynitrite.

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. 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. 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.

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 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 n_{Hill}) 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 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. 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. 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, NaNO₃, NaNO₂, and the prevention of peroxynitrite-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 and in the cardiac trabeculae of the rat following peroxynitrite exposure. 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. Moreover, similarly to our results, peroxynitrite incubation decreased the maximal Ca²⁺-activated force without giving rise to alterations in the Ca²⁺-sensitivity of force production in the peroxynitrite-treated permeabilized cardiac trabeculae of the rat. 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 Ca²⁺-activated contractile function. 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, we could additionally detect protein nitrotyrosine formation in several abundant proteins, though only after exposure to very high concentrations of peroxynitrite (250-500 μ M). 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. However, following exposure to 50 μ M 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. 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. 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.

CARDIOMYOCYTE STIFFNESS IN DIASTOLIC HEART FAILURE

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 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. LV muscle mass (119 ± 16 g/m²) was larger than normal (92 ± 10 g/m²; P<0.05) and 5 patients had significant LV hypertrophy (>125 g/m²).

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. 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.

Hemodynamic characteristics of the DHF patients

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.

Force measurements in single cardiomyocytes

F_{total} at pCa 4.5 did not significantly differ between the DHF (20.3 ± 7.5 kN/m², number of myocytes: n=23) and the control group (24.2 ± 12.4 kN/m², n=15). However, F_{passive} was significantly higher in the DHF (6.6 ± 3.0 kN/m²) than in the control group (3.5 ± 1.7 kN/m²; 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/m²; $P < 0.05$), and in the other DHF patients (7.2 ± 2.9 kN/m²; $P < 0.01$). F_{passive} of the control group was comparable to previously reported values of cardiomyocytes isolated from non-failing donor hearts.

No significant differences were found between the two groups in the Ca^{2+} -sensitivity of the contractile apparatus ($p\text{Ca}_{50}$) and the steepness of the force- $p\text{Ca}$ curves (nHill).

Myocardial tissue properties

DHF patients had higher collagen volume fraction (CVF) than controls ($7.5 \pm 4.0\%$ vs. $3.8 \pm 2.0\%$; $P < 0.05$). CVF of the DHF patients, who were transplant recipients ($7.5 \pm 3.0\%$) was similar to CVF of the other DHF patients ($7.5 \pm 3.0\%$). DHF patients were equally distributed over the three classes of CVF 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} .

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

When the DHF and control groups were combined, a monivariate 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. 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/m² and seemed to level off at higher values. A monivariate 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 monivariate 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. For control and DHF groups, F_{total} at pCa 4.5 was similar before and after PKA. At intermediate pCa (e.g. pCa 5.8), F_{total} was reduced after PKA because of PKA-induced myofilamentary desensitization. The latter was also evident from the reduced pCa₅₀ value observed in both DHF and control groups. After PKA treatment F_{passive} of DHF patients dropped to values observed in the control group both at baseline and after PKA treatment. In addition, in DHF patients the PKA-induced fall in F_{passive} was larger when baseline F_{passive} was higher.

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 $[\text{Ca}^{2+}]$ 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 endomyocardial 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, phospholamban, sarcoplasmic calcium release channel and sodium/calcium exchanger 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. 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. 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 or expression of a mutant isoform 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. 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. 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 edema 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 $<2.2\mu\text{m}$ and from perimysial fibers once filling pressures exceeded 30 mmHg.

Degradation of collagen in pressure-overloaded hypertrophied papillary muscles with plasmin did not reduce muscle stiffness to levels observed in normal muscles. Similarly, in the present study patients with DHF and low collagen volume fraction still had higher LVEDP, σ and E than controls. 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.

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 and not by interstitial fibrosis, which only developed if angiotensin II infusion was superimposed on the pacing stress. Similar coordination between titin isoform shift and extracellular matrix deposition has also been reported in other experimental models. 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, have not been uniformly successful in large clinical trials on DHF patients.

Study limitations

Five of the 12 DHF patients and 6 of the 8 controls had undergone cardiac transplantation. Transplant recipients were included in the study because they frequently suffer of DHF 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 immunosuppressant 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 or surgically procured biopsies. 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 CVP, 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.

EFFECTS OF Ca²⁺-SENSITIZERS IN PERMEABILIZED CARDIAC MYOCYTES FROM DONOR AND END-STAGE FAILING HUMAN HEARTS

RESULTS

F_{\max} of the failing and non-failing myocytes (38 ± 4 kN/m² and 36 ± 3 kN/m², respectively) and F_{passive} of the failing and non-failing myocytes (1.5 ± 0.3 kN/m² and 1.2 ± 0.2 kN/m², respectively) did not differ significantly under control conditions (pH 7.2; 0 mM P_i). In agreement with previous studies, however, the Ca²⁺-sensitivity of the isometric force of the failing myocytes was significantly higher ($\Delta pCa_{50}=0.15$) 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 $22\pm 1\%$ of the control value, and a marked decrease in Ca²⁺-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 [Ca²⁺]-force relations of the failing and non-failing myocytes disappeared in the presence of the ischaemic metabolites.

The Ca²⁺-sensitizer EMD 53998 (10 μ M) increased the force effectively in a wide range of [Ca²⁺] under all conditions studied and induced a pronounced leftward shift in the Ca²⁺-sensitivity relations under the control conditions. This shift, however, was smaller in the failing ($\Delta pCa_{50}=0.24$) than in the non-failing ($\Delta pCa_{50}=0.48$) myocytes. EMD 53998 enhanced the maximum force-generating capacity (F_{\max}) and the force in the virtual absence (pCa 9) of Ca²⁺ (F_{passive}).

The Ca²⁺-sensitizing effect of 10 μ M OR-1896 was less than that of 10 μ M EMD 53998, but it was comparable ($\Delta pCa_{50}\approx 0.1$) in the failing and non-failing myocytes. Ca²⁺-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} .

DISCUSSION

In this study, a comparison was made between the effects of two Ca²⁺-sensitizers (EMD 53998 and OR-1896) under normal physiological conditions and under mimicked ischaemic conditions in human myocytes. In line with previous animal studies, it was found that at equal concentrations the Ca²⁺-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 Ca²⁺.

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. 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. EMD 53998 increased both F_{max} and $F_{passive}$ (under control conditions). Therefore, our results are consistent with the suggestion that EMD 53998 increases the force generated per cross-bridge, with an additional effect on the cooperativity between cross-bridges in human myocytes. EMD 53998 affected $p\text{Ca}_{50}$ 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. 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. A decrease in phosphorylation of myosin light chain 2 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.

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. 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. In addition, OR-1896 induced a small, but comparable leftward shift in the $[\text{Ca}^{2+}]$ -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.

Both in the failing and in the non-failing myocytes, the Ca^{2+} -sensitivity and the steepness of the $[\text{Ca}^{2+}]$ -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 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. 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. The antagonistic effect of P_i on

EMD 57033-evoked force enhancement supports the previously suggested intimate relationship between EMD 57033 and the force-generating actin-myosin interactions. 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 Ca²⁺-independent resting force component was eliminated. However, ischaemic metabolites did not abolish the force-enhancing effect of EMD 53998 in the presence of Ca²⁺. This suggests different modalities of action for EMD 53998 in the absence and presence of Ca²⁺. In contrast with EMD 53998, no Ca²⁺-sensitization could be resolved by OR-1896 when ischaemic metabolites were present.

In summary, the increased Ca²⁺-sensitivity of the failing myocytes did not prevent further pharmacological Ca²⁺-sensitization under normal metabolic conditions pertinent to the myocardium with a preserved energy status. The Ca²⁺-responsiveness of the contractile apparatus, however, is also a function of the ischaemic metabolites, which may modulate the apparent Ca²⁺-sensitivity and the efficacy of Ca²⁺-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 analysed 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 the 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 ($\Delta\text{pCa}_{50}=0.15$). 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 ($\Delta\text{pCa}_{50}>0.25$) of force production. OR 1896 did not affect F_{max} and F_{passive} , and provoked a small, but significant Ca^{2+} -sensitization ($\Delta\text{pCa}_{50}\approx 0.1$). 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.

LIST of PUBLICATIONS

In extenso publications related to the thesis:

1. Borbély, A., Tóth, A., Édes, I., Virág, L., Papp, J.G., Varró, A., Paulus, W.J., van der Velden, J., Stienen, G.J.M., Papp, Z. (2005) Peroxynitrite-induced α -actinin nitration and contractile alterations in isolated human myocardial cells *Cardiovasc. Res.* (in press) IF: 5,164
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
3. Papp, Z., van der Velden, J., Borbély, A., Édes, I., Stienen, G.J.M. (2004) Effects of Ca^{2+} -sensitisers in permeabilized cardiac myocytes from donor and end-stage failing human hearts. *J. Muscle Res. Cell Motil.* 25, 219-224. IF: 1,297

Other in extenso publications:

1. Szilágyi, Sz., Pollesello, P., Levojkó, J., Haikala, H., Bak, I., Tószaki Á., Borbély, A., Édes, I., Papp, Z. (2005) Two positive inotropes with different mechanisms of action: contractile, PDE-inhibitory and direct myofibrillar effects of levosimendan and enoximone. *J. Cardiovasc. Pharmacol.* (in press) IF: 1,905
2. van der Velden, J., Narolska, N.A., Boontje, N.M., Borbély, A., Zaremba, R., Bronzwaer, J.G.F., Papp, Z., Jaquet, K., Paulus, W.J., Stienen G.J.M. (2005) Positive effect of PKC-mediated phosphorylation at the myofilament level for contractile function in human myocardium. (submitted)
3. Kaheinen, P., Pollesello, P., Hertelendi, Z., Borbély, A., Édes, I., Levijoki, J., Haikala, H., Papp, Z. (2005) The positive inotropic effect of levosimendan is correlated to its stereoselective Ca^{2+} -sensitizing effect but not to stereoselective phosphodiesterase inhibition. (submitted)
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)

Published abstracts:

1. Papp, Z., van der Velden, J., de Jong, J.W., 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

4. Borbély, A., Barta, J., Édes, I., Vaszily, M., Virág, L., Varró, A., Papp, Z. (2003) Peroxynitrite induced functional alterations in isolated human myocardial cells. *Cardiol. Hung.* 33, A64. IF: 0
5. Borbély, A., Barta, J., Édes, I., Vaszily, M., Virág, L., Varró, A., Papp, Z. (2003) Peroxynitrite induced mechanical alteration of the actin-myosin interaction in isolated human myocardial cells. *Eur. J. Heart Failure Suppl.* 2/1, A132. IF: 2,134
6. Borbély, A., Barta, J., Édes, I., Vaszily, M., Virág, L., Varró, A., Papp, Z. (2003) Peroxynitrite induced mechanical alterations of the actin-myosin interaction in isolated human myocardial cells. *Exp. Clin. Cardiol.* 8: A7. IF: 0
7. Borbély, A., Tóth, A., Édes, I., Vaszily, M., Virág, L., Papp, J.Gy., Varró, A., Papp, Z. (2004) Peroxynitrite induced alpha-actinin nitration and contractile alterations in isolated human myocardial cells. *J. Muscle Res. Cell Motil.* 25, 260. IF: 1,297
8. Borbély, A., van der Velden, J., Papp, Z., Bronzwaer, J.G.F., Stienen, G.J.M., Paulus, W.J. (2004) Cardiomyocyte stiffening in diastolic heart failure. *Circulation Suppl.* III, Vol 110, No 17, 636. IF: 11,164
9. Borbély, A., van der Velden, J., Papp, Z., Bronzwaer, J.G.F., Stienen, G.J.M., Paulus, W.J. (2004) Protein kinase A corrects high resting tension of cardiomyocytes from patients with diastolic heart failure. *Circulation Suppl.* III, Vol 110, No 17, 445. IF: 11,164
10. Borbély, A., van der Velden, J., Papp, Z., Édes, I., Bronzwaer, J.G.F., Stienen, G.J.M., Paulus, W.J. (2005) Increased cardiomyocyte stiffness in diastolic heart failure. *Cardiol. Hung.* 35, A16. IF: 0

Oral presentations:

1. Borbély, A., Barta, J., Édes, I., Vaszily, M., Virág, L., Varró, A., Papp, Z. (2003) Peroxynitrite induced functional alterations in isolated human myocardial cells. Annual Meeting of the Hungarian Society of Cardiologists, Balatonfüred.
2. Borbély, A., Barta, J., Édes, I., Vaszily, M., Virág, L., Varró, A., Papp, Z. (2003) Peroxynitrite impairs Ca²⁺-regulated force production in isolated human myocardial cells. 11th Alpe Adria Cardiology Meeting, Balatonfüred
3. Borbély, A., Barta, J., Édes, I., Vaszily, M., Virág, L., Varró, A., Papp, Z. (2003) Peroxynitrite induced functional alterations in isolated human myocardial cells. IV. International Symposium of Myocardial Cytoprotection, Pécs.
4. Borbély, A., Tóth, A., Édes, I., Vaszily, M., Virág, L., Papp, J. Gy., Varró, A. and Papp, Z. (2004) Peroxynitrite induced α -actinin nitration and contractile alterations in isolated human myocardial cells. XXXIII. European Muscle Conference, Isola d'Elba.
5. Borbély, A., Tóth, A., Édes, I., Vaszily, M., Virág, L., Papp, J. Gy., Varró, A. and Papp, Z. (2004) Peroxynitrite induced α -actinin nitration and contractile alterations in isolated human myocardial cells. 6th Meeting France-New CEE members, La Grande-Motte, France.
6. Borbély, A., van der Velden, J., Papp, Z., Bronzwaer, J.G.F., Stienen, G.J.M., Paulus, W.J. (2004) Cardiomyocyte stiffening in diastolic heart failure. American Heart Association, Scientific Sessions, New Orleans, USA.
7. Borbély, A., van der Velden, J., Papp, Z., Bronzwaer, J.G.F., Stienen, G.J.M., Paulus, W.J. (2005) Increased cardiomyocyte stiffness in diastolic heart failure. Annual Meeting of the Hungarian Society of Cardiologists, Balatonfüred.

Poster presentations:

1. Borbély, A., Barta, J., Édes, I., Vaszily, M., Virág, L., Varró, A., Papp, Z.(2003) Peroxynitrite induced mechanical alterations of the actin-myosin interaction in isolated human myocardial cells. Heart Failure 2003/ISHR-ES, Strasbourg, France.
2. Borbély, A., Tóth, A., Édes, I., Vaszily, M., Virág, L., Papp, J.Gy., Varró, A., Papp Z. (2004) Peroxynitrite induced α -actinin nitration and contractile alterations in isolated human myocardial cells. XXXIII. European Muscle Conference, Isola d'Elba.
3. Borbély, A., Tóth, A., Édes, I., Vaszily, M., Virág, L., Papp, J.Gy., Varró, A., Papp Z. (2004) Peroxynitrite induced α -actinin nitration and contractile alterations in isolated human myocardial cells. 6th Meeting France-New CEE members, La Grande-Motte, France.
4. Borbély, A., van der Velden, J., Papp, Z., Bronzwaer, J.G.F., Stienen, G.J.M., Paulus, W.J. (2004) Protein kinase A corrects high resting tension of cardiomyocytes from patients with diastolic heart failure. American Heart Association, Scientific Sessions, New Orleans, USA.