

Cardiomyocyte Stiffness in Diastolic Heart Failure

Attila Borbély, MD; Jolanda van der Velden, PhD; Zoltán Papp, MD, PhD;

Jean G.F. Bronzwaer, MD, PhD; Istvan Edes, MD, PhD;

Ger J.M. Stienen, PhD; Walter J. Paulus, MD, PhD

Background—Heart failure with preserved left ventricular (LV) ejection fraction (EF) is increasingly recognized and usually referred to as diastolic heart failure (DHF). Its pathogenetic mechanism remains unclear, partly because of a lack of myocardial biopsy material. Endomyocardial biopsy samples obtained from DHF patients were therefore analyzed for collagen volume fraction (CVF) and sarcomeric protein composition and compared with control samples. Single cardiomyocytes were isolated from these biopsy samples to assess cellular contractile performance.

Methods and Results—DHF patients ($n=12$) had an LVEF of $71\pm 11\%$, an LV end-diastolic pressure (LVEDP) of 28 ± 4 mm Hg, and no significant coronary artery stenoses. DHF patients had higher CVFs ($7.5\pm 4.0\%$, $P<0.05$) than did controls ($n=8$, $3.8\pm 2.0\%$), and no conspicuous changes in sarcomeric protein composition were detected. Cardiomyocytes, mechanically isolated and treated with Triton X-100 to remove all membranes, were stretched to a sarcomere length of $2.2\ \mu\text{m}$ and activated with solutions containing varying $[\text{Ca}^{2+}]$. Compared with cardiomyocytes of controls, cardiomyocytes of DHF patients developed a similar total isometric force at maximal $[\text{Ca}^{2+}]$, but their resting tension (F_{passive}) in the absence of Ca^{2+} was almost twice as high (6.6 ± 3.0 versus 3.5 ± 1.7 kN/m², $P<0.001$). F_{passive} and CVF combined yielded stronger correlations with LVEDP than did either alone. Administration of protein kinase A (PKA) to DHF cardiomyocytes lowered F_{passive} to control values.

Conclusions—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. (*Circulation*. 2005;111:774-781.)

Key Words: diastole ■ heart failure ■ myocytes ■ contractility ■ collagen

Heart failure with preserved left ventricular (LV) ejection fraction (EF) is frequently referred to as diastolic heart failure (DHF) in contrast to systolic heart failure, which is characterized by HF with reduced LVEF.¹⁻⁴ DHF is currently diagnosed in as much as 49% of HF patients.⁵ Diastolic LV dysfunction is also increasingly recognized,⁶ as evident from a recent population-based survey in which diastolic LV dysfunction was observed 5 times more often than systolic LV dysfunction.⁷

Despite the increased recognition of both DHF and diastolic LV dysfunction, their pathophysiology remains incompletely understood. Whether HF with preserved LVEF results from diastolic LV dysfunction⁸ or from subtle systolic LV dysfunction, unappreciated by a routine LVEF measurement⁹ and possibly exacerbated by high arterial impedance,¹⁰ is still a matter of debate. Furthermore, explanations proposed for diastolic LV dysfunction are divergent, ranging from high LV myocardial stiffness^{1,8} to pericardial or right ventricular constraint.^{11,12} Moreover, the relative importance of myocar-

dial fibrosis and of high cardiomyocyte resting tension for LV myocardial stiffness remains undefined.¹³

Failure to resolve these controversies about DHF and diastolic LV dysfunction could arise from a lack of myocardial biopsy or necropsy material,^{4,13} which would allow clinical and hemodynamic features to be matched with cellular and molecular myocardial properties. We therefore studied endomyocardial biopsy samples from patients with DHF in whom sample procurement was requested because of clinical suspicion of restrictive cardiomyopathy or, in the case of transplant recipients, because of clinical suspicion of rejection and in whom subsequent histological examination revealed no signs of infiltrative myocardial disease or rejection. Apart from routine histological examination, biopsies were used for determination of collagen volume fraction (CVF), sarcomeric protein expression, and isolation of single cardiomyocytes, whose developed and resting tensions were measured to detect systolic and/or diastolic dysfunction at the cellular level.^{14,15} To evaluate the relative contributions to

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From the Laboratory for Physiology (A.B., J.v.d.V., J.G.F.B., G.J.M.S., W.J.P.), Institute for Cardiovascular Research, VUMC, Amsterdam, the Netherlands, and the Division of Clinical Physiology (A.B., Z.P., I.E.), Institute of Cardiology, UDMHSC, Debrecen, Hungary.

The online-only Data Supplement, which contains an additional table, can be found with this article at <http://www.circulationaha.org>.

Correspondence to Prof Dr Walter J. Paulus, Laboratory for Physiology, Institute for Cardiovascular Research, VU University Medical Center, Van der Boerhorststraat 7, 1081 BT Amsterdam, The Netherlands. E-mail wj.paulus@vumc.nl

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TABLE 1. Clinical Characteristics of DHF Patients

Patient No.	Age, y	Sex	Diagnoses	Medications
1	77	F	HT	ACEI, Amio, CCB, Diu, Stat
2	66	F	HT	Amio, ARB, β , Dig, Diu
3	67	M	HT, DM, Ob	ACEI, Dig, Diu, Nit
4	82	F	HT	ACEI, β , 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, β , 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

HT indicates arterial hypertension; DM, diabetes mellitus; Ob, obesity; TX, transplant recipients; ACEI, angiotensin-converting-enzyme inhibitors; Amio, amiodarone; CCB, calcium channel blockers; Diu, diuretics; Stat, statins; ARB, angiotensin II receptor blockers; β , β -blockers; Dig, digitalis; Nit, nitrates; Imm, immunosuppressants; and Fibr, fibrates.

diastolic LV dysfunction of myocardial fibrosis and of cardiomyocyte resting tension, in vitro data were subsequently correlated with in vivo hemodynamic indices of diastolic LV function.

Methods

Patients

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 HF. LV endomyocardial biopsy samples were obtained from patients suspected of restrictive cardiomyopathy, and RV endomyocardial samples were obtained from the transplant recipients. Subsequent histological examination ruled out infiltrative myocardial disease or rejection in all patients. Coronary angiography showed the absence of significant coronary artery stenoses or graft vasculopathy. All patients satisfied the criteria as proposed by the European study group on DHF,¹⁶ ie, signs and symptoms of congestive HF, LVEF >45%, and LVEDP >16 mm Hg. They all had 1 or more predisposing risk factors for diastolic LV dysfunction (Table 1).⁷ 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. LV endomyocardial biopsy samples were obtained from the patients suspected of myocarditis, and RV endomyocardial samples were

obtained from the transplant recipients. Histological examination ruled out the presence of myocarditis or rejection in all patients. They had no signs or symptoms of HF, an LVEF \geq 50%, and an LVEDP \leq 16 mm Hg.

Mean hemodynamic data of DHF patients and controls are listed in Table 2; individual hemodynamic data can be found in the Data Supplement Table. Hemodynamic data were derived from LV angiograms, 2D echocardiograms, and high-fidelity LV catheter pressure measurements. The local ethics committee approved the study protocol. Written, informed consent was obtained from all patients, and there were no complications related to catheterization or biopsy sample procurement.

Force Measurements in Isolated Cardiomyocytes

Force measurements were performed in mechanically isolated, single cardiomyocytes at 15°C as described previously.¹⁴ In brief, frozen biopsy samples (5 mg wet weight) were defrosted in cold relaxing solution (in mmol/L: free Mg 1, KCl 100, EGTA 2, Mg-ATP 4, and imidazole 10; pH 7.0), mechanically disrupted, and incubated for 5 minutes in relaxing solution supplemented with 0.2% Triton X-100 to remove all membranes. Thereafter, cells were washed twice in relaxing solution, and a single cardiomyocyte was attached with silicone adhesive between a force transducer and a piezoelectric motor (Figure 1A).

Resting sarcomere length in isolated cardiomyocytes was adjusted to 2.2 μ m. Mean length between the attachments, width, and depth of control cardiomyocytes were 51±19, 24±8, and 18±5 μ m, respectively. Corresponding values of DHF cardiomyocytes were 69±25, 28±10, and 21±6 μ m. The composition of the relaxing and activating solutions was described previously.¹⁷ Their pCa ($-\log_{10}[\text{Ca}^{2+}]$) ranged from 9.0 (relaxing) to 4.5 (maximal activation). Maximal activation at pCa 4.5 was used to calculate maximal calcium-activated isometric force (F_{max}). Subsequently, exposure to a series of solutions with intermediate pCa yielded the force-pCa relation. All force values were normalized for myocyte cross-sectional area. A typical contraction-relaxation sequence is shown in Figure 1B. On transfer of the myocyte from relaxing to activating solution, isometric force started to develop. Once a steady-state force level was reached, the cell was shortened within 1 ms to 80% of its original length (slack test) to determine the baseline of the force transducer. The distance between the baseline and the steady force level is the total force (F_{total}). After 20 ms, the cell was restretched and returned to the relaxing solution, in which a second slack test of 10-second duration was performed to determine resting or passive force (F_{passive}).

After establishing the baseline force-pCa relation, some of the myocytes were incubated for 40 minutes at 22°C in relaxing solution containing the catalytic subunit of protein kinase A (PKA, 100 U/mL; Sigma, batch 12K7495) and 6 mmol/L dithiothreitol (MP Biochemicals). Subsequently, a second force-pCa relation was determined.

Myocardial Tissue Properties

The extent of interstitial fibrosis was determined on elastica-von Gieson's-stained sections of tissue placed in 5% formalin with an automated image analyzer (Prodit) and was expressed as low (0% to 5%, class I), intermediate (5% to 10%, class II), or high (10% to 15%, class III) CVF.¹⁸

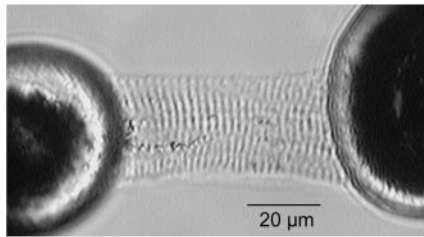
Expression of myosin heavy chain, desmin, actin, troponin T (TnT), tropomyosin, troponin I (TnI), and myosin light-chain 1 and -2 (MLC-1 and MLC-2) was analyzed by 1D sodium dodecyl

TABLE 2. LV Systolic and Diastolic Function of Control and DHF Groups (Mean±SD)

	HR, bpm	LVPSP, mm Hg	LVEDP, mm Hg	LVEDVI, mL/m ²	LVEF, %	CI, L/min per 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*

HR indicates heart rate; LVPSP, left ventricular peak systolic pressure; LVEDVI, left ventricular end-diastolic volume index; and CI, cardiac index. *P<0.05, DHF vs controls.

A



B

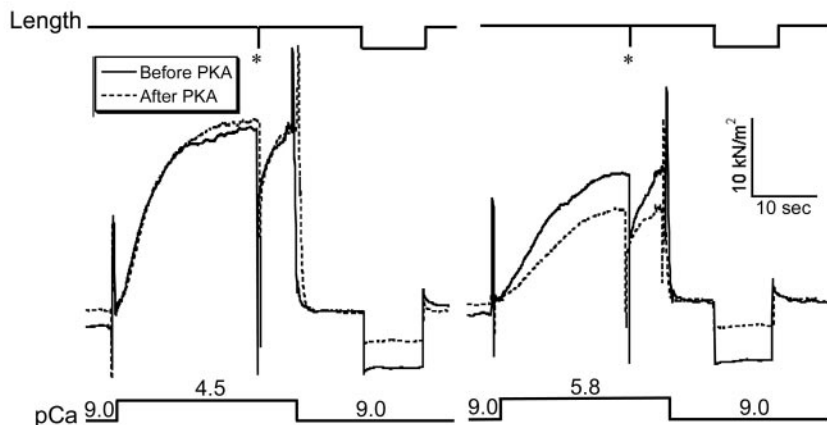


Figure 1. A, Single cardiomyocyte, isolated from endomyocardial biopsy sample from DHF patient, mounted between force transducer and piezoelectric motor. B, Contraction-relaxation sequence recorded in single cardiomyocyte before and after PKA treatment during maximal (pCa 4.5) and submaximal (pCa 5.8) activation. *Slack test (see text).

sulfate-polyacrylamide gel electrophoresis as described previously.¹⁵ To detect degradation products of myofilament proteins, Western immunoblot analysis was performed with specific monoclonal antibodies against desmin (clone DE-U-10, Sigma), TnT (clone 2G3, Spectral Diagnostics), TnI (clone 8I-7, Spectral Diagnostics), and MLC-1 and MLC-2 (clones F109.16A12 and F109.3E1, Alexis). Signals were visualized with a secondary horseradish peroxidase-labeled goat-anti-mouse antibody and enhanced chemiluminescence (Amersham Biosciences).

An ELISA was used to determine the phosphorylation status of TnI with specific monoclonal antibodies against whole TnI (clone 16A11) and dephosphorylated TnI (clone 22B11).¹⁹ Biopsy samples were treated with 10% trichloroacetic acid to preserve the phosphorylation status of proteins.

Data Analysis

Circumferential LV end-diastolic wall stress (σ) was computed by using a thick-wall ellipsoid model of the LV: $\sigma = PD/2h \times [1 - (h/D) - (D^2/2L^2)]$, where P is LVEDP, h is echocardiographically determined LV wall thickness, and D and L are LV short-axis diameter and long-axis length at the midwall, respectively.²⁰

The radial myocardial stiffness modulus (E) was calculated to assess myocardial material properties from the formula: $E = \Delta\sigma_r / \Delta\epsilon_r = \Delta P / (\Delta h/h) = -\Delta P / \Delta \ln h$ 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 ($\Delta\epsilon_r$) 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 versus $\ln h$ plot.^{18,21} 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.²²

The Hill constant (nHill), a measure of the steepness of the sigmoidal force-pCa relation, was derived from a modified Hill equation fitted to the individual force-pCa data points of each myocyte: $F_{\text{total}} = F_{\text{max}} \times [\text{Ca}^{2+}]^{\text{nHill}} / (\text{Ca}_{50}^{\text{nHill}} + [\text{Ca}^{2+}]^{\text{nHill}}) + F_{\text{passive}}$, where Ca_{50} (or pCa_{50}) corresponds to the $[\text{Ca}^{2+}]$ at which

$F_{\text{total}} - F_{\text{passive}} = F_{\text{max}}/2$. pCa_{50} is a measure of Ca^{2+} sensitivity of the contractile apparatus.

Values are given as mean \pm SD. Statistical significance was set at $P < 0.05$ and was obtained for multiple comparisons between groups by ANOVA followed by a Bonferroni test and for single comparisons by an unpaired Student t test. Monovariate and bivariate linear regression analyses were performed with SPSS (version 9.0).

Results

Hemodynamic Characteristics of the DHF Patients

The mean hemodynamic data of the DHF group are compared with that of the control group in Table 2. Heart rate, LVEF, LV end-diastolic volume index, and cardiac index in the DHF group were similar to the values measured in the control group. LV peak systolic pressure, LV end-diastolic volume index, σ , and E were significantly higher in the DHF group. The higher LVEDP and σ at comparable LVEDVI implied a reduced LV diastolic distensibility and the higher E , increased myocardial stiffness.¹⁶

Force Measurements in Single Cardiomyocytes

The average force-pCa relations obtained for 6 control and 9 DHF patients are shown in Figure 2A. 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 (24.2 ± 12.4 kN/m², $n=15$) groups. However, F_{passive} was significantly higher in the DHF (6.6 ± 3.0 kN/m²) than in the control (3.5 ± 1.7 kN/m², $P < 0.001$) group. A higher F_{passive} than in the control group was observed in both types of DHF patients: those who were transplant recipients (5.4 ± 1.1 kN/m², $P < 0.05$) and those who were not (7.2 ± 2.9 kN/m², $P < 0.01$). The F_{passive} of the control group was comparable to previously reported values of cardiomyocytes isolated from nonfailing

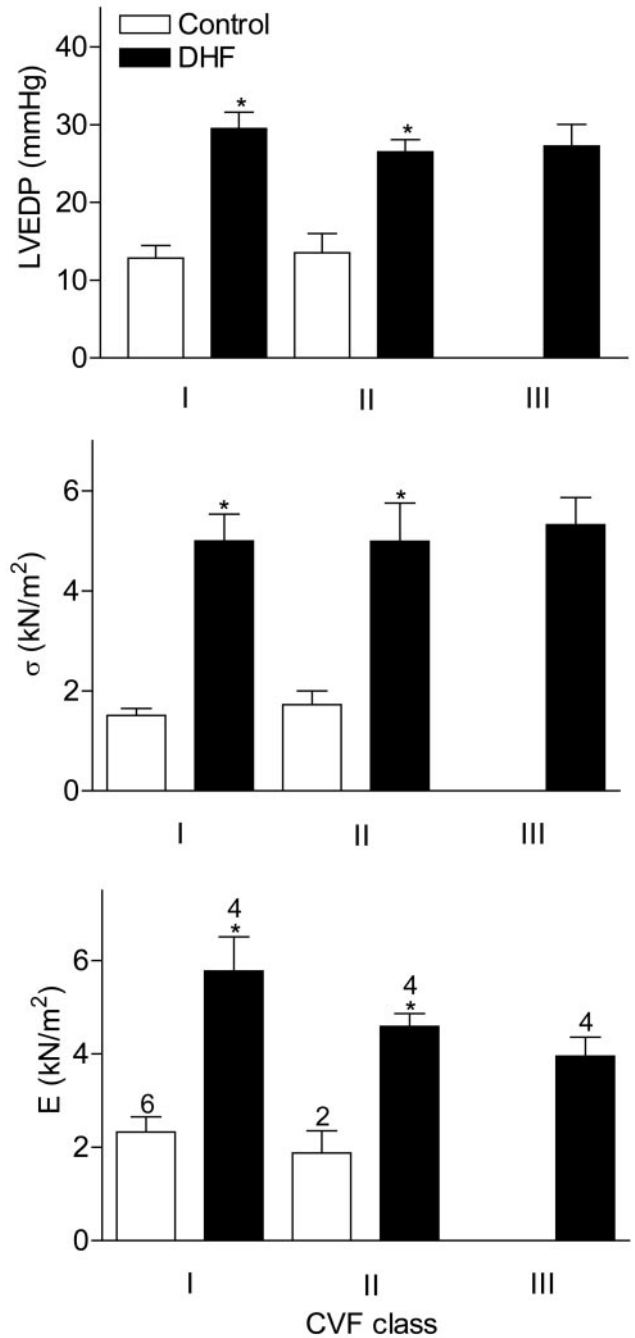
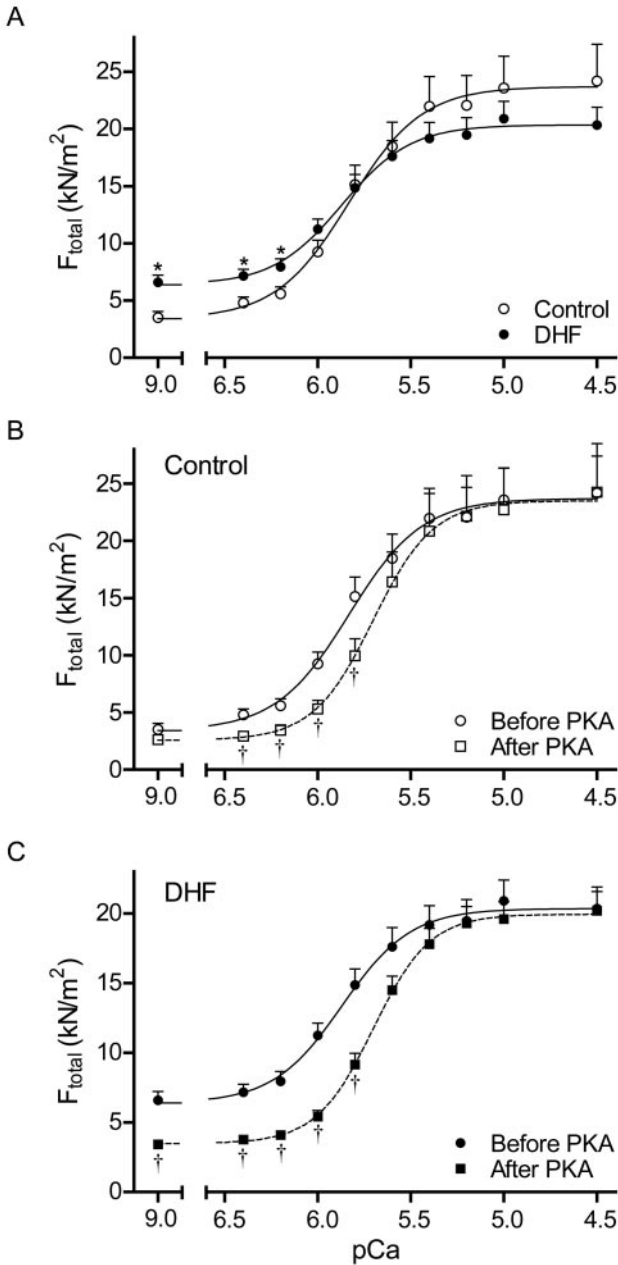


Figure 2. A, Average force-pCa relation for pooled cardiomyocytes of control and DHF groups. F_{total} at pCa 4.5 and Ca^{2+} sensitivity were similar in both groups, but $F_{passive}$ was higher in DHF group ($*P < 0.05$). B, Average force-pCa relation for pooled cardiomyocytes of control group before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

Figure 3. At low (I) and intermediate (II) CVF class, DHF patients had higher LVEDP, σ , and E than did controls. Numbers above bars indicate number of individuals in each group. $*P < 0.05$, control vs DHF within each CVF class.

donor hearts.^{14,15} No significant differences were found between the 2 groups in terms of Ca^{2+} sensitivity of the contractile apparatus (pCa_{50}) and the steepness of the force-pCa curves (ie, nHill; Table 3).

Myocardial Tissue Properties

DHF patients had a higher CVF than did controls ($7.5 \pm 4.0\%$ versus $3.8 \pm 2.0\%$, $P < 0.05$). CVF of the DHF patients who

were transplant recipients ($7.5 \pm 3.0\%$) was similar to the CVF of the other DHF patients ($7.5 \pm 3.0\%$). DHF patients were equally distributed over the 3 classes of CVF (Figure 3), and one third of patients therefore had low interstitial fibrosis. The higher values of LVEDP, σ , and E in these patients compared with 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, or MLC-1 and MLC-2 were found between DHF and control

TABLE 3. Measures of Cardiomyocyte Force and Ca²⁺ Sensitivity Before and After PKA Treatment

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

n indicates number of cardiomyocytes.

* $P<0.05$, DHF vs controls.

† $P<0.05$, after vs before PKA.

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 patients (0.39 ± 0.15) and controls (0.44 ± 0.11). Phosphorylation status of TnI was determined in endomyocardial biopsy samples retrieved from 7 DHF patients and 7 controls. The ratio of dephosphorylated to total TnI was comparable in both groups (0.58 ± 0.17 versus 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 monovariate linear regression analysis revealed significant correlations between the average $F_{passive}$ of all cardiomyocytes of each individual and the LVEDP, σ , or E measured in the same individual at the time of cardiac catheterization and biopsy sample retrieval (Figure 4). Note the quantitative agreement between the individual values of in vivo circumferential 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 relation could be constructed for 11 cardiomyocytes isolated from biopsy samples of 4 control patients and for 16 cardiomyocytes isolated from biopsy samples of 7 DHF patients. Figure 1 (bottom) 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, whereas at pCa 5.8, F_{total} was reduced after PKA. In addition, Figure 1 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 3; Figure 2B and 2C). At intermediate pCa (eg, pCa 5.8), F_{total} was reduced after PKA because of PKA-induced myofilament desensitization (Figure 2B and 2C). The latter was also evident from the reduced pCa₅₀ value observed in both DHF and control groups (Table 3). After PKA

treatment, $F_{passive}$ of cardiomyocytes from DHF patients dropped to values observed in the control group both at baseline and after PKA treatment (Figures 2B, 2C, and 5A). In addition, in DHF patients, the PKA-induced fall in $F_{passive}$ was larger when baseline $F_{passive}$ was higher (Figure 5B).

Discussion

The present study analyzed endomyocardial biopsy samples obtained from patients with DHF and yielded the following information: (1) When cardiomyocytes isolated from these samples were stretched to a sarcomere length of $2.2\ \mu\text{m}$, 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, σ , and E were correlated with in vitro measurements of both $F_{passive}$ and CVF.

High $F_{passive}$

Because 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. Because cardiomyocytes were incubated in solution supplemented with 0.2% Triton X-100 before the experiments, the 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 Ca²⁺-ATPase,²³ phospholamban,^{24,25} sarcoplasmic calcium release channel,²⁶ and sodium/calcium exchanger²⁷ is effectively ruled out as a cause of the observed elevation of $F_{passive}$, which therefore needs to be attributed to alterations of myofilament 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 change as a result of enhanced proteolysis. This is especially evident for TnI, whose calpain-mediated breakdown is accelerated by a high LVEDP.²⁸ 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.

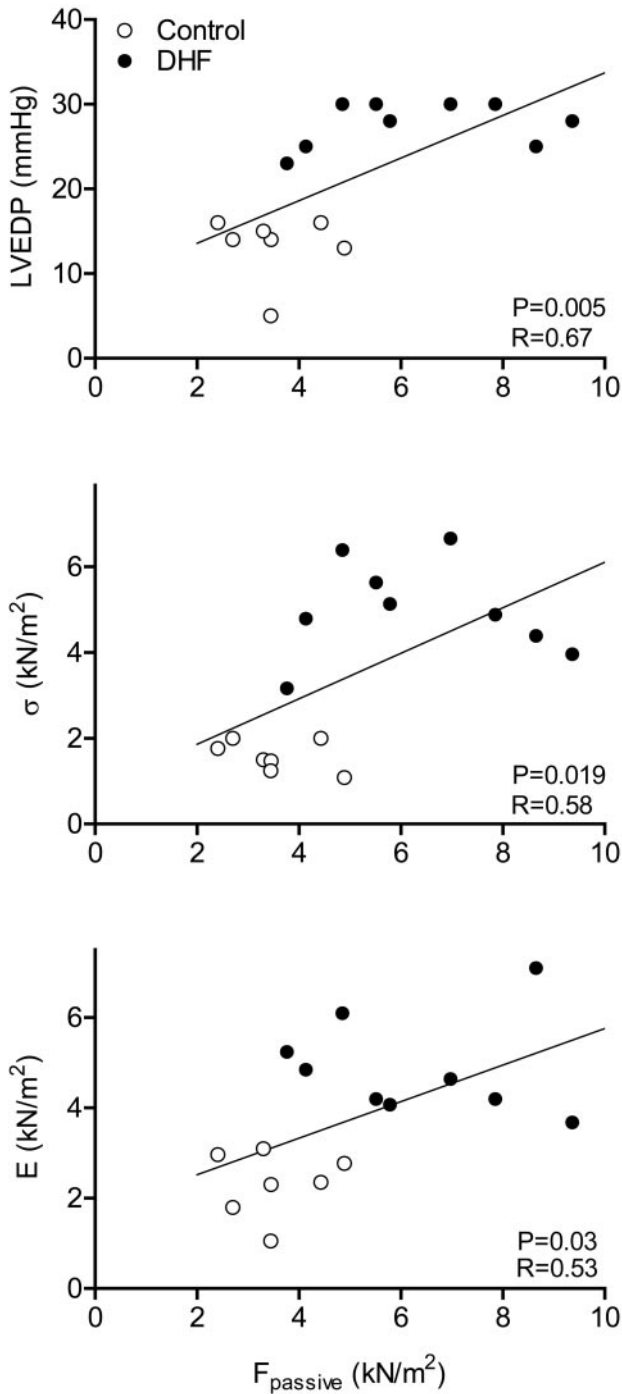


Figure 4. Correlations between F_{passive} averaged for all cardiomyocytes of each individual and LVEDP, σ , and E measured at time of cardiac catheterization.

Moreover, the correction by PKA treatment provides evidence that the high F_{passive} resulted 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, whereas neither F_{passive} in control cardiomyocytes nor F_{total} at pCa 4.5 in cardiomyocytes from both groups was altered (Table 3 and Figure 2). Such an isolated drop in F_{passive} , unaccompanied by a fall in F_{total} , is more easily reconciled with an action of PKA

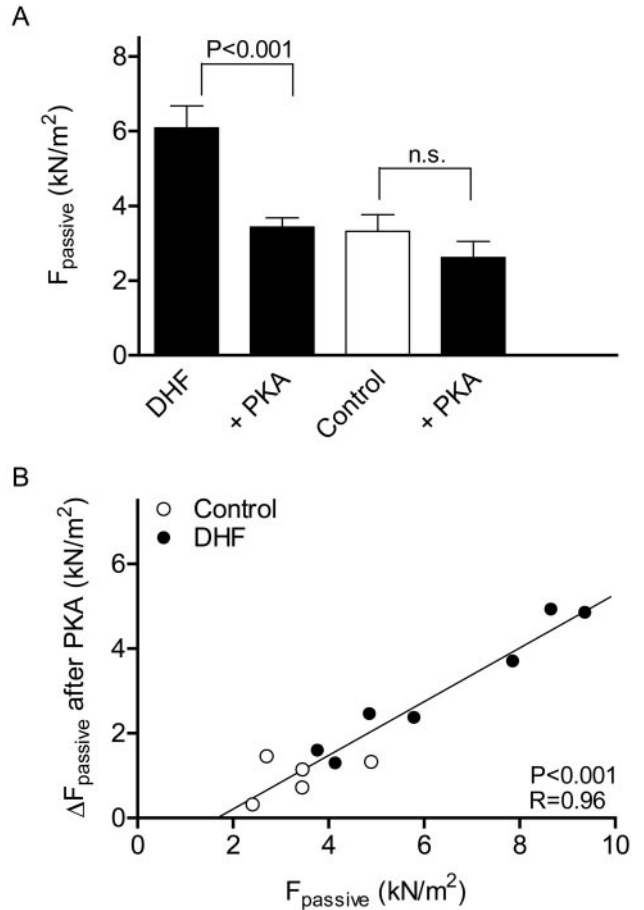


Figure 5. A, PKA treatment reduces F_{passive} of DHF patients to values observed in control group at baseline and after PKA treatment. B, Correlation between PKA-induced fall in F_{passive} and baseline value of F_{passive} .

on a myofilament 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 also determined the 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. Phosphorylation²⁹ or expression of a mutant isoform³⁰ 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.³¹ Because of limited procurement of myocardial tissue by endomyocardial biopsy techniques, phosphorylation of both proteins could not be assessed in the present study. Future studies on myocardial tissue from DHF patients should focus on the phosphorylation level of both proteins 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} values of control and DHF cardiomyocytes were pooled, in vitro measurement of F_{passive} was correlated with in vivo indices of diastolic LV dysfunction, such as LVEDP, σ , and E (Figure 4). The quantitative agreement between 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 before 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 for 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 biopsy samples from patients with DHF had higher CVF than did controls, and in a bivariate linear regression analysis, both F_{passive} and CVF were 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 $<2.2 \mu\text{m}$ ³² and from perimysial fibers once filling pressures exceeded 30 mm Hg.³³

Degradation of collagen in pressure-overloaded, hypertrophied papillary muscles with plasmin did not reduce muscle stiffness to levels observed in normal muscles.³⁴ Similarly, in the present study, patients with DHF and low CVFs still had higher LVEDP, σ , and E than did controls (Figure 3). Therefore, our data support the concept that diastolic LV dysfunction in the presence of a low CVF is explained by the higher F_{passive} of the cardiomyocytes. However, because half of the DHF patients suffered from diabetes mellitus, collagen cross-links formed by advanced glycation end products could also explain the impairment of diastolic LV function at low CVF.³⁵

The present study observed low CVF and high F_{passive} values in some patients with DHF but failed to detect DHF patients with high CVF and low F_{passive} values. This suggests diastolic LV dysfunction to result from a sequence of events, which start with a rise in cardiomyocyte F_{passive} followed by the development of interstitial fibrosis. A similar sequence of events has also been reported in experimental tachypacing-induced HF models. In those models, elevation of diastolic LV muscle stiffness was paralleled by expression of the shorter and stiffer N2B isoform of titin³⁶ and not by interstitial fibrosis, which only developed when an angiotensin II infusion was superimposed on the pacing stress.³⁷ Similar coordination between titin isoform shift and extracellular matrix deposition has also been reported in other experimental models.³⁸ The evidence provided by the present study that

DHF patients can have low CVF also explains why angiotensin II receptor blockers and angiotensin-converting enzyme inhibitors, which reduce interstitial fibrosis,^{39,40} have not been uniformly successful in large clinical trials in DHF patients.⁴¹

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 DHF¹⁶ and because their myocardial biopsy material is readily available. The pathogenetic mechanisms responsible for their DHF could differ from that of other DHF patients because of ongoing rejection and use of immunosuppressant drugs. Force recordings of isolated cardiomyocytes and the 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 RV or LV samples and could potentially have overlooked tissue heterogeneity. The extent of tissue heterogeneity was addressed in previous studies on explanted hearts^{14,15} or surgically procured biopsy samples.⁴² In those studies, the variability of force measurements in cardiomyocytes isolated from different portions of the heart was always $<5\%$. To validate the use of defrosted biopsy samples, force recordings of cardiomyocytes isolated from a biopsy sample immediately after procurement were compared with force recordings of cardiomyocytes isolated from a defrosted biopsy sample from the same patient. These force recordings yielded identical results.

Conclusions

Cardiomyocytes isolated from endomyocardial biopsy samples 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} . Because the integrity of sarcolemmal and sarcoplasmic membranes was disrupted by prior Triton incubation and because expression of sarcomeric proteins and the phosphorylation level of TnI were unaltered, the PKA-induced fall in 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.

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