Depressed cardiac myofilament function in human diabetes mellitus

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Abstract

Diabetes mellitus is associated with depressed cardiac myofilament function. Depressed cardiac myofilament function may underlie the decreased ventricular function characteristic of human diabetic cardiomyopathy.

Heart Failure is a Highly Prevalent Disease Syndrome in Industrialized Countries

It is estimated that 5,000,000 people in the United States have chronic heart failure, with 550,000 new cases diagnosed each year, resulting in a total mortality of 287,000 deaths per year (4). Diabetes mellitus has been identified as an etiologic factor in the development of heart failure, in particular diastolic heart failure, and the incidence of diabetes mellitus in patients with ischemic cardiomyopathy is markedly increased (18). Additionally, diabetes mellitus is an added risk factor for morbidity and mortality in heart failure. The prevalence of diabetes mellitus has increased in recent years from 2–5% to 4–7% of the population in the United States. Thus the incidence of patients with heart failure and diabetes is expected to increase (26).

All medical strategies used to treat heart failure in diabetic patients, including angiotensin-converting enzyme inhibitors, β-blockers, and aldosterone blockers, aim to counteract the neurohormonal mechanisms thought to drive the progression of heart failure. Recently, however, there have been attempts to construct treatment strategies that affect intracellular mechanisms, such as Ca2+ homeostasis and myofilament function. In diabetes mellitus, this is potentially quite relevant, inasmuch as multiple lines of evidence suggest that PKC activity and associated downstream signaling processes are upregulated (1, 24). Furthermore, activation of PKC and the subsequent phosphorylation of contractile proteins, notably troponin I (TnI) (12, 23, 29, 35) and troponin T (TnT) (29, 41), have been shown experimentally to reduce Ca2+ responsiveness of the cardiac sarcomere and reduce maximum tension development (37). Accordingly, the purpose of this study was to determine whether human diabetes mellitus is associated with depressed cardiac myofilament function.

Materials and Methods

Tissue acquisition and preservation. All human tissue was acquired from patients who had consented to coronary artery bypass surgery. A separate informed consent to enroll in the study was obtained in accordance with and with approval of the University of Illinois Hospital Institutional Review Board.

Patients were divided into two groups: diabetic and nondiabetic. Diabetes was defined clinically by virtue of ongoing therapy with insulin or oral hypoglycemic agents or a fasting serum glucose ≥126 mg/dl. Patients with end-stage organ disease or other complex intercurrent illness were excluded. Left ventricular ejection fraction was determined by cardiac catheterization and ventriculography or by echocardiography. Diastolic function was assessed by Doppler echocardiography, according to standard echocardiographic criteria. Blood samples were analyzed for total glycated hemoglobin to determine the level of glucose control in the diabetic cohort.

Left ventricular tissue was obtained after the patient was placed on cardiopulmonary bypass, and the heart was arrested with cold, high-potassium cardioplegia solution. A ∼10-mg biopsy of left ventricular tissue was cut from an area of the epicardium that was least affected by coronary artery disease as best determined from the angiogram and visual inspection by the surgeon. The biopsy was flash frozen in liquid nitrogen and stored at −80°C. In a subset of the study, half of the biopsy sample was not flash frozen but, instead, transported on ice to the laboratory, where mechanical experiments were conducted immediately. The purpose of these preliminary studies was to determine the impact of flash freezing and storage on myofilament Ca2+ responsiveness.

Tissue preparation, experimental apparatus, and protocol. Skinned myocytes were obtained by mechanical isolation as described previously (21). After the cells were washed twice with the relaxing solution, they were resuspended in standard relaxing solution and stored on ice for <10 h before data collection.

The experiments were performed on the stage of an inverted microscope (Nikon; ×40, 0.7 NA) that allowed visualization of the cell (17, 21). Cell width and thickness thus determined were used to normalize force to myocyte cross-sectional area to allow for compar-

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Graphically by measuring transmitral and pulmonary vein flow using standard echocardiography (†). Diastolic dysfunction was determined echocardiographically by measuring transmural and pulmonary vein flow using standard techniques.

Isochron between the control and the diabetic cohort (i.e., developed force is expressed as mN/mm²).

The myocyte was suspended above a movable stage that contained several solution wells; temperature was controlled at 15°C. Rapid solution changes were made by translating the stage laterally via stage manipulators such that another solution-containing well was brought into contact with the myocyte. The myocyte was suspended above a movable stage that contained several solution wells; temperature was controlled at 15°C. Rapid solution changes were made by translating the stage laterally via stage manipulators such that another solution-containing well was brought into contact with the myocyte. The myocyte was suspended above a movable stage that contained several solution wells; temperature was controlled at 15°C. Rapid solution changes were made by translating the stage laterally via stage manipulators such that another solution-containing well was brought into contact with the myocyte. 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**RESULTS**

Table 1 summarizes clinical data from seven diabetic and five nondiabetic subjects. Both groups were similar in age (64 ± 12 and 68 ± 8 yr for controls and diabetic patients, respectively), but there were more women in the diabetic cohort. All diabetic patients were diagnosed with Type 2 diabetes and had elevated hemoglobin A1c, in the range of 6–10%, consistent with adequate, but not optimal, glucose control. Nevertheless, the overall ejection fraction was higher in the diabetic than in the nondiabetic group: 47 ± 7 vs. 63 ± 8% (P < 0.05). As expected, the incidence of diastolic dysfunction as detected by echocardiography was higher in the diabetic group. All tissue samples were taken from normal-appearing myocardium (as determined by prior review of the angiogram and direct surgical inspection).

An example of a myocyte from a flash-frozen and stored human left ventricular epicardial biopsy is shown in Fig. 1. The images showing the attached cell as viewed from the bottom and side illustrate that clear sarcomere striations could be resolved in both optical projections.

![Image](http://ajpheart.physiology.org/)

**Table 1. Clinical data**

<table>
<thead>
<tr>
<th>Gender, M/F</th>
<th>Hg A1C, %</th>
<th>EF, %</th>
<th>Diastolic Dysfunction</th>
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<tbody>
<tr>
<td>DM</td>
<td>66 M</td>
<td>6.5</td>
<td>65†</td>
</tr>
<tr>
<td>DM</td>
<td>64 M</td>
<td>10.2</td>
<td>56†</td>
</tr>
<tr>
<td>DM</td>
<td>74 M</td>
<td>6.6</td>
<td>55†</td>
</tr>
<tr>
<td>DM</td>
<td>77 M</td>
<td>8.0</td>
<td>58*</td>
</tr>
<tr>
<td>DM</td>
<td>61 F</td>
<td>5.9</td>
<td>60*</td>
</tr>
<tr>
<td>DM</td>
<td>58 F</td>
<td>6.9</td>
<td>70†</td>
</tr>
<tr>
<td>DM</td>
<td>56 F</td>
<td>7.1</td>
<td>75*</td>
</tr>
<tr>
<td>Mean ± SE</td>
<td>68 ± 8</td>
<td>7.3 ± 1.4</td>
<td>63 ± 8</td>
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</table>

Con: control; EF, ejection fraction, determined at the time of cardiac catheterization by standard angiographic techniques (*) or via echocardiographically by measuring transmitral and pulmonary vein flow using standard techniques.

DM, diabetes mellitus; Con, control; EF, ejection fraction, determined at the time of cardiac catheterization by standard angiographic techniques (*) or via echocardiographically by measuring transmitral and pulmonary vein flow using standard techniques.

DM, diabetes mellitus; Con, control; EF, ejection fraction, determined at the time of cardiac catheterization by standard angiographic techniques (*) or via echocardiographically by measuring transmitral and pulmonary vein flow using standard techniques.
Before analysis and comparison of muscle specimens from patients with and without cardiac disease, it was necessary to critically evaluate our tissue-handling protocols. Specifically, we were concerned whether harvesting of muscle in the operating room and subsequent flash freezing and storage at −80°C would alter muscle mechanics. In Fig. 2, myofilament function of samples obtained from six human biopsies processed immediately after surgical excision was compared with the function of these same samples after flash freezing and storage at −80°C for ~1 mo. The pooled data from both groups clustered closely over the entire range of the force-[Ca2+] relation. Paired Student’s t-tests revealed no significant differences for \( F_{\text{max}} \), \( EC_{50} \), or \( n_H \) between freshly processed and flash-frozen-and-stored biopsy samples. Hence, these data ensured that flash freezing and subsequent storage of human myocardial biopsy samples in the operating room did not adversely affect the results.

Figure 3 contrasts the pooled force-[Ca2+] relations between the nondiabetic and the diabetic cohort. The average Hill fit parameters obtained in biopsy samples from individual patients are summarized in Table 2. Type 2 diabetes was associated with a significant reduction in myofilament Ca2+ sensitivity, as evidenced by a 29% increase in EC50 (\( P < 0.05 \)). Similarly, \( F_{\text{max}} \) showed a trend toward depression in the diabetic cohort (\( F_{\text{max}} = 29\% \), \( P = 0.08 \), borderline significance). There was no difference in cooperative activation (\( n_H \)) between the two groups (\( P = 0.2 \)). Finally, there was no clear correlation between any of the clinical parameters (e.g., age, gender, hemoglobin A1c, or systolic or diastolic function) and myofilament function beyond the presence of diabetes.

Figure 4 shows the phosphorylation profile of myofilament proteins in adult rat cardiomyocytes from nondiabetic and diabetic rats. Diabetes was associated with a significant increase in TnI and TnT phosphorylation.

**DISCUSSION**

There continues to be a debate as to whether the intrinsic function of cardiac myofilaments is altered in heart failure (15, 32, 33). Several theories regarding myofilament alterations have been proposed. The first of these theories is a change in myofilament isoform expression. This area of research has not yielded insights into the pathophysiology of human heart failure, with the exception of familial hypertrophic cardiomyopathy (10, 28, 38, 42) and, possibly, expression of the fetal isoform of TnT (5–7, 9, 19, 36). The second theory is the development of proteolysis, specifically cardiac troponin (8, 13, 27). This appears to be most relevant in the setting of acute myocardial injury, such as acute myocardial infarction, but is likely not germane in chronic heart failure. Although these two areas of research have provided much useful information, the area of myofilament protein research that appears most relevant in heart failure is posttranslational modification of contractile proteins by phosphorylation and dephosphorylation (14, 15, 39, 40).

It is well known that contractility is modulated by phosphorylation of myofilament proteins. For example, when TnI is phosphorylated by PKA (16, 39) or by PKC at Ser23 and Ser24 (20, 29), Ca2+ responsiveness decreases. Additionally, when Ser13 and Ser17 in TnI are phosphorylated by any PKC isoform, maximal activity of Ca2+-stimulated actomyosin Mg2+ ATPase is reduced (29, 31, 35). Similarly, phosphorylation of TnT at PKC target site Thr206 is associated with marked depression of myofilament function in skinned myocardium (41).

PKC is activated in diabetes (22, 24). This is known to occur through several pathways. First, increased serum levels of ANG II, as commonly seen in diabetes, activate PKC via the ANG II receptor. Additionally, hyperglycemia is thought to cause de novo synthesis of diacylglycerol, a cofactor that is

![Fig. 2. Average pooled force-Ca2+ concentration ([Ca2+]i) relations (\( n = 6 \)) in myocytes from human ventricular biopsies that were immediately processed (○) and the same biopsies after flash freezing in liquid nitrogen and storage for 1 mo (●). Force data were normalized to maximum Ca2+-saturated developed force (\( F_{\text{max}} \)) measured in the freshly processed cells. Flash freezing and subsequent storage did not significantly affect any of the Hill fit parameters: \( F_{\text{max}} = 100\%, \ EC_{50} = 2.50 ± 0.32 \mu M \), and Hill coefficient = 2.9 ± 0.4 for fresh samples; \( F_{\text{max}} = 103 ± 20 \), \( EC_{50} = 2.96 ± 0.19 \mu M \), and Hill coefficient = 3.0 ± 0.2 for flash-frozen samples.

![Fig. 3. Average pooled force-[Ca2+] relations in myocytes from human ventricular biopsies from control (○) and diabetic (●) cohorts. Force is normalized to myocyte cross-sectional area and fit in each individual myocyte to a modified Hill equation; average Hill fit parameters are summarized in Table 2. Diabetes was associated with depressed myofilament function.](http://ajpheart.physiology.org/)

<table>
<thead>
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<th>Table 2. Average Hill fit parameters</th>
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<tr>
<td>Control (( n = 5 ))</td>
</tr>
<tr>
<td>DM (( n = 7 ))</td>
</tr>
<tr>
<td>( EC_{50} ) [Ca2+] ( \mu M )</td>
</tr>
<tr>
<td>( F_{\text{max}} ) mN/mm²</td>
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<td>( n_H )</td>
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Values are means ± SE; \( n \), number of subjects/patients. Diabetes was associated with a significant (\( P < 0.05 \)) decrease in myofilament Ca2+ sensitivity (increased \( EC_{50} \)) and decreased maximum Ca2+ saturated-force development (\( F_{\text{max}} \), \( P = 0.08 \), \( n_H \), Hill coefficient).
rate limiting in the activation of PKC, in cardiac tissue. Increased PKC activation and cardiac troponin phosphorylation in cardiomyopathy have been shown to occur in vitro and in vivo in diabetic rat models (3, 22–24). Furthermore, losartan, an ANG II (type 1) receptor blocker, has been shown to block these effects (24). Consistent with this notion, direct measurement of contractile protein phosphorylation in diabetic rat myocardium by the Pro-Q Diamond phosphostaining technique revealed significant increases in TnI and TnT phosphorylation levels (Fig. 4). Hence, these data indicate that cardiac TnI and TnT may be constitutively phosphorylated in the diabetic heart, and, as a consequence, myofilament function and Ca\textsuperscript{2+} responsiveness might be expected to be depressed.

The data from the present study support this hypothesis. Myofilament Ca\textsuperscript{2+} sensitivity was significantly depressed, whereas maximum Ca\textsuperscript{2+}-saturated developed force also tended to decrease. The level of myofilament cooperative activation, however, was not affected by diabetes. These functional results are consistent with the impact of PKC-mediated phosphorylation on myofilament function in vitro (16, 20, 29–31, 35, 41). Whether troponin phosphorylation at the PKC sites is indeed elevated in the diabetic human heart samples cannot be established, as much as we did not have sufficient tissue to study protein phosphorylation levels. Nonetheless, depressed myofilament function in human biopsy material, as seen here, strongly supports this conclusion.

Several groups that have investigated the level of troponin phosphorylation in end-stage human heart failure (explanted hearts at the time of transplant) found a decrease in TnI phosphorylation concomitant with an increase in Ca\textsuperscript{2+} sensitivity compared with controls (43, 44), contrary to our findings in the human diabetic heart. However, studies of human myocardial myofilament function in the setting of cardiac transplantation have several shortcomings. The first general problem is related to the “control” group. In most studies, control heart samples are obtained from brain-dead organ donors, many of whom likely exhibited elevated serum levels of catecholamines, which most likely will have resulted in increased myocardial PKA/PKC activity. Second, it is well established that β-adrenergic receptors are downregulated in heart failure (11), and, consequently, myofilament proteins, specifically the PKA target sites on TnI, are expected to be phosphorylated to a lesser extent in the heart failure samples (2, 25, 45). This decreased basal PKA-dependent phosphorylation state may be a pathological change associated with and contributing to heart failure or an adaptive change to increase Ca\textsuperscript{2+} responsiveness in the face of impaired Ca\textsuperscript{2+} cycling. Regardless of the underlying mechanisms, the combination of these two factors likely biases the analysis of myofilament Ca\textsuperscript{2+} responsiveness and leads to the incorrect conclusion of an increase in myofilament Ca\textsuperscript{2+} responsiveness in heart failure. These factors did not affect the present study, because we specifically studied diabetic patients and not end-stage heart failure patients. Furthermore, our nondiabetic heart samples were obtained from patients undergoing surgery under the same general circumstances as our diabetic cohort. That is, our nondiabetic and diabetic tissue samples were obtained under similar conditions.

A weakness of our study is the heterogeneity of the clinical data of the patients studied as well as the relatively small sample size. Nevertheless, despite this heterogeneity, the data demonstrate that diabetes had an independent effect on myofilament Ca\textsuperscript{2+} responsiveness. Also, the small sample size excluded the possibility of any meaningful subgroup analysis of patient populations. Furthermore, all samples were obtained from patients with coronary artery disease severe enough to prompt coronary bypass surgery. Hence, whether our data can be extrapolated to human diabetes in general, that is, in the absence of coronary artery disease, cannot be determined from this study.

Although the mechanisms that underlie cardiac myofilament dysfunction in association with diabetes cannot be definitively determined from this study, it is likely that altered myofilament contractile protein phosphorylation plays a significant role in this process. Regardless of the mechanism, reduced myofilament Ca\textsuperscript{2+} sensitivity should affect the global function of the heart, and a greater amount of cytosolic Ca\textsuperscript{2+} would be needed to generate a normal amount of force in the diabetic cardiac myocyte. Increased cytosolic Ca\textsuperscript{2+} would provide one explanation for the diastolic dysfunction and arrhythmias commonly seen with diabetes (34). Viewed another way, the relatively preserved global systolic function seen in diabetic patients in general might be viewed as reduced myofilament Ca\textsuperscript{2+} sensitivity compensated for by a potentially maladaptive increase in

Fig. 4. Phosphorylation profile of myofilament proteins in adult rat cardiomyocytes. Cardiomyocytes were isolated from hearts of control or diabetic adult rats, and a crude myofilament fraction was isolated. Myofilament proteins were separated by SDS-PAGE, and Pro-Q Diamond staining was used to visualize phosphoproteins. Equal loading was confirmed by Coomassie blue staining. Troponin I and T (TnI and TnT) positions were confirmed by Western blot (data not shown). A: representative Pro-Q Diamond-stained samples. B: average relative phosphorylation level of TnI and TnT in control, nondiabetic (open bars; n = 8) and diabetic (solid bars; n = 9) animals. MWM, molecular weight marker. *P < 0.05 vs. control.
cytosolic Ca\textsuperscript{2+}. Clearly, further investigation is required in the area of cardiac myofilament protein phosphorylation as well as Ca\textsuperscript{2+} homeostasis in (human) diabetes. As our knowledge of these complex and finely controlled mechanisms grows, our ability to design new therapies to enhance cardiac performance and survival in diabetic patients with heart disease will follow.

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GRANTS

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