

Mechanical and Metabolic Rescue in a Type II Diabetes Model of Cardiomyopathy by Targeted Gene Transfer

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The Otsuka–Long–Evans Tokushima Fatty rat represents a model for spontaneous non-insulin-dependent type II diabetes mellitus (DM), characterized by diastolic dysfunction and associated with abnormal calcium handling and decrease in sarcoplasmic reticulum Ca²⁺-ATPase (SERCA2a) expression. The aim of this study was to examine whether SERCA2a gene transfer can restore the energetic deficiency and left ventricular (LV) function in this model. DM rats were randomized to receive adenovirus carrying either the SERCA2a gene (DM + Ad.SERCA2a) or the β -galactosidase gene (DM + Ad. β Gal) or saline (DM + saline). LV mechanoenergetic function was measured in cross-circulated heart preparations 3 days after infection. In DM, end-systolic pressure at 0.1 ml intraballoon water (ESP_{0.1}) was low and end-diastolic pressure at 0.1 ml intraballoon water (EDP_{0.1}) was high (22 mm Hg), compared with non-DM (EDP_{0.1} 12 mm Hg). In DM + Ad.SERCA2a, however, ESP_{0.1} was increased over 200 mm Hg and EDP_{0.1} was decreased to 7 mm Hg. LV relaxation rate was fast in DM + Ad.SERCA2a, but slow in the other DM groups. There was no difference in relation between cardiac oxygen consumption per beat and systolic pressure–volume area among all groups. Finally, the oxygen cost of LV contractility in DM was about three times as high as that of normal. In DM + Ad.SERCA2a, the oxygen cost decreased to control levels, but in DM + Ad. β Gal/DM + saline it remained high. In DM failing hearts, the high oxygen cost indicates energy wasting, which contributes to the contractile dysfunction observed in diabetic cardiomyopathy. SERCA2a gene transfer transforms this inefficient energy utilization into a more efficient state and restores systolic and diastolic function to normal.

Key Words: gene therapy, heart failure, SERCA2a, energetic function, diabetes mellitus

Abbreviations used: DM, diabetes mellitus; OLETf, Otsuka–Long–Evans Tokushima Fatty; LETO, Long–Evans Tokushima Otsuka; CHF, congestive heart failure; BW, body weight; BG, blood glucose; SR, sarcoplasmic reticulum; LV, left ventricle or ventricular; RV, right ventricle or ventricular; MHC, myosin heavy chain; CK, creatine kinase; RyR, ryanodine receptor; FKBP12.6, FK506-binding protein; NaCaX, Na⁺–Ca²⁺ exchanger; E-C, excitation–contraction; SERCA2a, sarcoplasmic reticulum Ca²⁺-ATPase pump; Ad.SERCA2a, adenovirus carrying SERCA2a gene; Ad. β Gal, adenovirus carrying β -galactosidase gene; LVP, left ventricular pressure; ESPVR, end-systolic pressure–volume relation; EDPVR, end-diastolic pressure–volume relation; ESP_{0.1}, end-systolic pressure at 0.1 ml of intraballoon water; EDP_{0.1}, end-diastolic pressure at 0.1 ml of intraballoon water; PVA, systolic pressure–volume area; VO₂, myocardial oxygen consumption per beat; eEmax, equivalent maximal elastance; mLVV, midrange LV volume; ESPmLVV, end-systolic pressure observed at mLVV; PVAmLVV, systolic pressure–volume area at mLVV; T_L, logistic time constant.

INTRODUCTION

Cardiovascular disease resulting from diabetes predisposes to left ventricular (LV) dysfunction via concomitant coronary artery disease, endothelial dysfunction, hypertension, LV hypertrophy, coronary microvascular disease, autonomic dysfunction, and metabolic abnormalities [1]. *Diabetic cardiomyopathy*, or LV dysfunction independent of these risk factors, has been the subject of debate; however, there are now substantive data demonstrating that diabetes impairs left ventricular function directly. This specific diabetic cardiomyopathy has been associated with both insulin-dependent and non-insulin-dependent diabetes mellitus (DM) and is characterized by both systolic and diastolic dysfunction. In clinical practice, it is often difficult to ascertain whether the influence of diabetes on left ventricular function results

from direct metabolic effects on the myocardium or is secondary to concomitant coronary artery disease and hypertension, which are common comorbid conditions associated with diabetes. For this reason, animal models of diabetes have proved invaluable to the understanding of the pathophysiological mechanisms that result in diabetic cardiomyopathy.

Systolic and diastolic function of cardiac myocytes is tightly associated with excitation–contraction (E–C) coupling in cardiac cells. The sarcoplasmic reticulum (SR), which releases Ca^{2+} during contraction by a trigger of Ca^{2+} entry via the L-type calcium channel and takes it up during relaxation by the SR-ATPase (SERCA2a) pump, plays a major role in controlling the synchronized movement of Ca^{2+} in myocardial cells [2]. In mammalian cardiomyocytes, the removal of Ca^{2+} from the cytoplasm is governed mainly by the SERCA2a pump, and the activity is regulated by phospholamban and to a lesser extent the Na^+ – Ca^{2+} exchanger (NaCaX). A decrease in SERCA2a protein/mRNA expression and activity has been reported in human heart failure and in a number of animal models of heart failure [3–5]. The abnormal Ca^{2+} handling by the SERCA2a reduction contributes to the systolic and diastolic dysfunction in failing hearts.

We have previously shown that adenoviral gene transfer of SERCA2a, phospholamban, or antisense of phospholamban modifies intracellular Ca^{2+} handling and modulates physiological mechanical performance both in isolated human failing and rat neonatal cardiomyocytes and in normal, senescent, aortic banding-induced failing hearts [6–9]. In addition, global cardiac gene transfer of SERCA2a improved survival and energetic state shown as phosphocreatine/ATP ratio in aortic-banded rats and reduced ventricular arrhythmias in the model rat of ischemia followed by reperfusion [6–9]. Thus cardiac gene transfer of Ca^{2+} handling proteins provides a potential therapeutic modality in heart failure. However, it is not clear how SERCA2a overexpression will influence the energy utilization, analyzed by myocardial oxygen consumption (VO_2), in failing hearts.

The Otsuka–Long–Evans Tokushima Fatty (OLETF) rat, which was established as a model of congenital DM by selective mating, was reported to manifest stable clinical and pathological features like human type II DM [10]. These DM rats showed LV diastolic dysfunction [11]. In the prestage of type II DM (15 weeks of age) with increased cardiac collagen content, Doppler echocardiography exhibited a prolongation in deceleration time and a decrease in peak velocity of early diastolic transmitral inflow [11]. Furthermore, we recently reported a significant slowing of isovolumic LV relaxation rate in cross-circulated hearts excised from the DM rats (62–66 weeks of age) with depressed expression of cardiac SERCA2a protein [12].

The goal of the present study was to test whether senescent OLETF DM rats would manifest both contrac-

tile dysfunction and inefficient energy utilization and further to test whether adenoviral gene transfer of SERCA2a to whole hearts *in vivo* could improve the LV mechanical and energetic functions, especially in terms of oxygen cost of LV contractility, in the excised hearts of these DM rats. The oxygen cost of LV contractility is a physiological index that describes the amount of VO_2 for Ca^{2+} handling in E–C coupling used per unit of LV contractility [13].

RESULTS

Characterization of Animals

Blood glucose (BG) levels, measured after 5 h of fasting, in four diabetic OLETF groups were three times as high as that of the non-DM LETO group, although there was no statistical difference in body weight (BW) among all five groups (Table 1). The BG levels were increased over 200 mg/dl in 50-week-old DM rats, and these high BG levels were maintained for 20–30 weeks until sacrifice. LV/BW and LV + RV/BW ratios in four DM groups were significantly higher than those of the non-DM group, while the RV/BW ratio showed no difference among the five groups (Table 1). LV mass in DM + Ad.SERCA2a was significantly smaller than that of DM and DM + saline. In addition, mean LV/BW ratio in DM + Ad.SERCA2a was considerably, but not significantly, decreased compared with the other three DM groups. Thus the diabetic rats had a significant increase in LV mass normalized to BW, and a cross section of the diabetic hearts showed apparent concentric hypertrophy (data not shown). The gene transfer of SERCA2a appears to have somewhat of an antihypertrophic effect.

LV Mechanics

Fig. 1A shows representative control end-systolic pressure–volume relations (ESPVRs) and end-diastolic pressure–volume relations (EDPVRs) without any inotropic interventions in non-DM, DM, and DM + Ad.SERCA2a hearts. Curvilinear ESPVRs and EDPVRs of DM + Ad.βGal and DM + saline hearts were similar to those of DM hearts (data not shown). The ESPVR of DM + Ad.SERCA2a hearts was shifted upward compared with the ESPVR of DM hearts. Summarized data of LV mechanics are shown in Table 2. There were no significant differences in the best fitting parameters (A , A' , B , and B') of ESPVR/EDPVR equations. However, the other fitting parameters, V_0 and V_u , of the four DM groups were significantly smaller than those of the non-DM group resulting from LV concentric hypertrophy. In DM hearts, the end-systolic pressure observed at 0.1 ml of intraballoon water volume ($\text{ESP}_{0.1}$), was low (131 ± 14 mm Hg), and end-diastolic pressure observed at 0.1 ml of intraballoon water volume ($\text{EDP}_{0.1}$), was high (21.9 ± 6.6 mm Hg), compared with non-DM hearts ($\text{ESP}_{0.1}$ 185 ± 35 mm Hg, $\text{EDP}_{0.1}$ 12.0 ± 5.4 mm Hg). In the DM + Ad.SERCA2a group, however, the $\text{ESP}_{0.1}$

TABLE 1: Blood glucose levels and morphometric analyses

| Group | n | BW (g) | Blood glucose (mg/dl) | LV (g) | RV (g) | LV/BW ($\times 10^{-3}$) | RV/BW ($\times 10^{-3}$) | LV + RV/BW ($\times 10^{-3}$) |
|----------------------|---|---------------|---------------------------|--------------------------------|-------------------|------------------------------|----------------------------|---------------------------------|
| Non-DM (LETO) | 6 | 594 \pm 24 | 111 \pm 17 | 1.083 \pm 0.061 | 0.307 \pm 0.043 | 1.83 \pm 0.09 | 0.52 \pm 0.06 | 2.34 \pm 0.14 |
| DM (OLETF) | 6 | 485 \pm 81 | 269 \pm 76 ^a | 1.176 \pm 0.037 | 0.322 \pm 0.018 | 2.49 \pm 0.38 ^a | 0.68 \pm 0.10 | 3.17 \pm 0.47 ^a |
| DM + Ad.SERCA2a | 4 | 483 \pm 35 | 275 \pm 50 ^a | 1.027 \pm 0.099 ^b | 0.347 \pm 0.046 | 2.13 \pm 0.11 ^a | 0.72 \pm 0.08 | 2.85 \pm 0.14 ^a |
| DM + Ad. β gal | 3 | 497 \pm 133 | 255 \pm 51 ^a | 1.152 \pm 0.065 | 0.334 \pm 0.024 | 2.44 \pm 0.45 ^a | 0.72 \pm 0.18 | 3.16 \pm 0.63 ^a |
| DM + saline | 3 | 539 \pm 104 | 278 \pm 53 ^a | 1.220 \pm 0.065 | 0.332 \pm 0.035 | 2.32 \pm 0.31 ^a | 0.63 \pm 0.11 | 2.96 \pm 0.40 ^a |

All data are shown as means \pm SD. BW, body weight; LV, left ventricle; RV, right ventricle; n, number of hearts.

^a $P < 0.05$ compared to non-DM group.

^b $P < 0.05$ compared to DM and DM + saline groups.

was increased over 200 mm Hg and the $EDP_{0.1}$ was decreased to 6.8 ± 6.6 mm Hg ($EDP_{0.1}$ in two of four rats was almost zero), although the $ESP_{0.1}$ in the DM + Ad. β Gal and DM + saline groups was as low as that of DM group. As shown in Fig. 1B, moreover, we analyzed logistic time constant (T_L) obtained from LV isovolumic relaxation pressure–time curves at mLVV in all groups. The T_L in the DM group was significantly longer than that of the non-DM LETO group ($P < 0.05$). In the DM + Ad.SERCA2a group, however, the T_L was decreased to the T_L level observed in the non-DM group, although the T_L in the DM + Ad. β Gal and DM + saline groups remained as long as that of the DM group.

LV Energetics: VO_2 –Systolic Pressure–Volume Area (PVA) Relations

Fig. 1C shows representative control VO_2 –PVA relations without any inotropic interventions in non-DM LETO, DM, and DM + Ad.SERCA2a hearts. We obtained similar linear relations of VO_2 –PVA in DM + Ad. β Gal and DM + saline hearts (data not shown). Summarized data of LV energetics are shown in Table 3. There was no significant difference in the slope and VO_2 intercept of VO_2 –PVA relation among all five groups. In the four DM groups, moreover, the minute VO_2 for basal metabolism and for Ca^{2+} handling in E-C coupling, which are both components of the VO_2 intercept of the VO_2 –PVA relation, were not significantly different from those of the non-DM group.

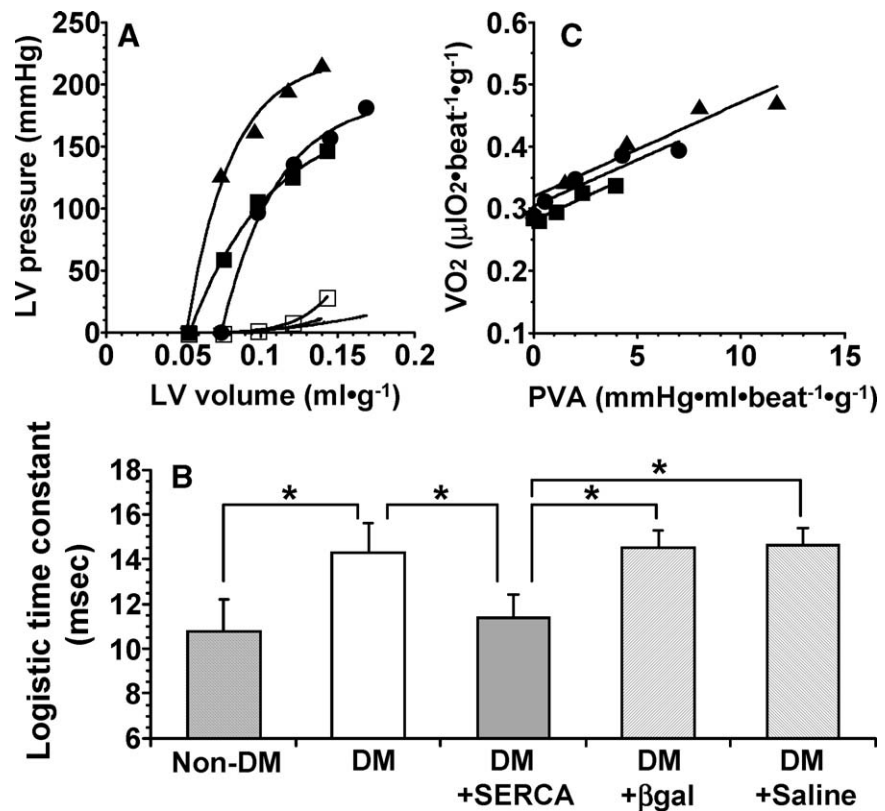
ESP_{mLVV} in Response to Calcium

Table 4 shows changes in end-systolic pressure observed at a mLVV (ESP_{mLVV}) in response to Ca^{2+} infusion. ESP_{mLVV} prior to Ca^{2+} infusion in DM + Ad.SERCA2a and non-DM LETO groups were significantly higher than those of DM and/or DM + saline groups. The ESP_{mLVV} was gradually increased as the infusion rate of Ca^{2+} solution was increased step-wise from 2 to 6 ml/h. In the DM + Ad.SERCA2a and normal groups, the maximal increases in ESP_{mLVV} in response to Ca^{2+} solution (6 ml/h) were almost the same and were also significantly higher than those of the DM and/or DM + saline groups. In some experiments, we infused dobutamine solution into the coronary perfusion tubing at 2–4 ml/h infusion rate after the Ca^{2+} infusion. The changes in ESP_{mLVV} in response to dobutamine were similar to those to Ca^{2+} in all groups (data not shown).

LV Energetics: Oxygen Cost of LV Contractility

The equivalent maximal elastance (eE_{max}) at mLVV, an index of LV contractility, in the DM + Ad.SERCA2a group was significantly higher than those of the other four groups (Fig. 2). The eE_{max} at mLVV in the DM group was not different from those of DM + Ad. β Gal and DM + saline, although lower than that of the non-DM group. Fig. 3A shows representative relations between VO_2 for Ca^{2+}

FIG. 1. (A) End-systolic pressure–volume relation (closed symbols) and end-diastolic pressure–volume relation (open symbols) in non-DM (● and ○), DM (■ and □), and DM + SERCA2a (▲ and △) hearts. Both relations were obtained by control volume-loading runs in which intraballoon water was changed from 0 to 0.1 ml by 0.025 ml. (B) Comparison of logistic time constants among all groups. Logistic time constants were obtained from a best fit logistic curve of pressure–time during isovolumic relaxation at midrange LV volume (0.05 ml of intraballoon water volume). * $P < 0.05$. $n = 6$ in non-DM, $n = 6$ in DM, $n = 4$ in DM + SERCA2a, $n = 3$ in DM + β Gal, $n = 3$ in DM + saline. (C) Linear relations between myocardial oxygen consumption per beat (VO_2) and systolic pressure–volume area (PVA) in non-DM (●), DM (■), and DM + SERCA2a (▲) hearts.



handling in the E-C coupling and eEmax at mLVV during Ca²⁺ inotropism run in non-DM, DM, and DM + Ad.SERCA2a hearts. These three distinct linear relations showed different slopes, which mean different oxygen costs of LV contractility. In some experiments, we performed intracoronary infusion of dobutamine, which followed the Ca²⁺ infusion, in the same heart preparation. As shown in Fig. 3B, there was a fairly good correlation between the oxygen costs of LV contractility in response to Ca²⁺ and those in response to dobutamine ($r = 0.96$). The oxygen cost of LV contractility for Ca²⁺ in the DM group was about three times as high as that of the non-DM group ($P < 0.05$) (Fig. 3C). In the DM + Ad.SERCA2a group, the oxygen cost of LV contractility was as low as that of the non-DM group ($P > 0.05$), but in the DM + Ad. β Gal and DM + saline groups the oxygen costs remained as high as that of the DM group ($P > 0.05$).

SERCA2a mRNA/Protein Expression

Finally we examined the mRNA and protein expression of SERCA2a in the heart preparations used for the analysis of mechanical and energetic function. As shown in Figs. 4A and 4B, the mRNA and protein expression of SERCA2a were significantly decreased in the DM group compared with that of the non-DM LETO group. *In vivo* adenoviral gene transfer of SERCA2a in DM failing hearts both increased expression of SERCA2a and restored it to the

levels observed in non-DM hearts, whereas gene transfer of a control vector (Ad. β Gal) had no effect on SERCA2a expression.

DISCUSSION

Therapies for congestive heart failure (CHF) have prolonged the lives of patients but have not reversed the systolic dysfunction of the failing hearts. Gene transfer either of calcium-handling proteins, including SERCA2a, the dominant form of phospholamban, antisense of phospholamban, parvalbumin, and S100A1, or of dominant negative β -adrenergic receptor kinase has been reported to restore the contractile function in human and experimental models of heart failure [14–17]. In all these studies, however, the functional analyses were restricted to mechanical function. In this study we report that SERCA2a gene transfer can restore the energetic cardiac function, especially the oxygen cost of LV contractility, in failing hearts.

DM Model of Cardiac Dysfunction

In our model of diabetes, the animals are characterized by (1) late onset of hyperglycemia (after 18 weeks of age), (2) a chronic course of DM, (3) mild obesity, (4) inheritance by males, (5) renal complication, and (6) conversion of DM from non-insulin-dependent DM to insulin-dependent

TABLE 2: Variables of LV mechanics

| Group | n | ESPVR | | | | EDPVR | | | |
|-----------------|---|-----------|-------------|----------------------------|----------------------------|-------------|-------------|----------------------------|----------------------------|
| | | A (mm Hg) | B (1/ml) | V ₀ (ml/g) | ESP _{0.1} (mm Hg) | A' (mm Hg) | B' (1/ml) | V ₀ (ml/g) | EDP _{0.1} (mm Hg) |
| Non-DM (LETO) | 6 | 225 ± 52 | 23.8 ± 11.1 | 0.074 ± 0.005 | 185 ± 35 | 1.94 ± 2.81 | 33.9 ± 14.0 | 0.074 ± 0.005 | 12.0 ± 5.4 |
| DM (OLETF) | 6 | 197 ± 51 | 15.0 ± 6.7 | 0.051 ± 0.002 ^a | 131 ± 14 | 1.79 ± 2.01 | 36.9 ± 12.8 | 0.051 ± 0.003 ^a | 21.9 ± 6.6 |
| DM + Ad.SERCA2a | 4 | 233 ± 39 | 49.2 ± 20.4 | 0.058 ± 0.006 ^a | 239 ± 35 ^b | 0.30 ± 0.24 | 36.7 ± 5.5 | 0.056 ± 0.007 ^a | 6.8 ± 6.6 ^c |
| DM + Ad.βgal | 3 | 149 ± 16 | 29.4 ± 11.2 | 0.052 ± 0.003 ^a | 135 ± 2 | 3.54 ± 3.32 | 22.2 ± 7.7 | 0.052 ± 0.003 ^a | 12.2 ± 3.5 |
| DM + saline | 3 | 144 ± 12 | 32.3 ± 15.0 | 0.049 ± 0.002 ^a | 133 ± 27 | 4.77 ± 2.58 | 19.6 ± 2.5 | 0.049 ± 0.002 ^a | 19.0 ± 11.9 |

All data are shown as means ± SD. ESPVR, end-systolic pressure–volume relation; A and B are parameters in the equation $ESP = A(1 - \exp[-B(V - V_0)])$; V₀, volume intercept of end-systolic pressure–volume relation; ESP_{0.1}, end-systolic pressure observed at maximum left ventricular volume (0.1 ml of balloon water volume); EDPVR, end-diastolic pressure–volume relation; A' and B' are parameters in the equation $EDP = A'[\exp\{B'(V - V_{0.1})\} - 1]$; V_{0.1}, volume intercept of end-diastolic pressure–volume relation; EDP_{0.1}, end-diastolic pressure observed at maximum left ventricular volume (0.1 ml of balloon water volume); n, number of hearts.

^a P < 0.05 compared to non-DM group.
^b P < 0.05 compared to DM, DM + Ad.βgal and DM + saline groups.
^c P = 0.06 compared to DM group.

DM after about 40 weeks of age [10]. In the present study, increased LV/BW ratio and decreased V₀ were found in the DM rats (70–80 weeks of age) compared with age-matched LETO rats, indicating that the hearts of our DM rats developed concentric hypertrophy. The global LV cardiac hypertrophy was due to both hypertrophied cardiomyocytes and increased extracellular matrix [11,18]. In addition, these DM rats showed increased myocardial collagen content, increased collagen [11], and cardiac interstitial/perivascular fibrosis. In a previous study in which the same OLETF rats were fed 30% sucrose between 20 and 28 weeks and studied at 60–64 weeks there was mild diastolic dysfunction but no overt systolic dysfunction [12]. In this study, in which we used the DM rats without sucrose feeding at 70–80 weeks there were significant downward shifts of ESPVR, decreased ESP_{0.1}, and decreased eE_{max} at mL_{LV} compared with non-DM LETO rats. In addition, increased time constant of isovolumic relaxation and increased EDP_{0.1} were evident in our DM rats. Thus the DM rats used in this study showed severe LV systolic and diastolic dysfunction. The systolic dysfunction of the DM hearts may be caused by the decreased SERCA2a expression demonstrated in the present and previous studies, the increased sarcolemmal NaCaX [20], and/or the switching of cardiac myosin components from V₁ (a homodimer of α-myosin heavy chain (MHC)) to V₃ (a homodimer of β-MHC) [12,19]. The reduced Ca²⁺ content in the SR, leading to the limited Ca²⁺ release from the SR, and thereby contractile dysfunction, can be caused by decreased SERCA2a and/or increased NaCaX, both of which compete for cytosolic Ca²⁺ removal during relaxation [20]. V₁, with higher Ca²⁺- and actin-activated ATPase activity, is associated with an increased shortening velocity of the cardiac fibers, while V₃, with lower ATPase activity, is associated with slower shortening velocity [21]. On the other hand, the LV diastolic dysfunction may be caused by decreased SERCA2a expression and/or the cardiac stiffness that is increased by the increased collagen and fibrosis [11,18,19]. However, the complete restoration of cardiac mechanical function (ESPVR, ESP_{0.1}, EDP_{0.1}, and time constant of isovolumic relaxation) by SERCA2a gene transfer, observed in the present DM, indicates that the decreased SERCA2a expression is a central cause of LV systolic and diastolic dysfunction. In addition, the SERCA2a gene transfer induced a prominent eE_{max} at mL_{LV}, which appears to be attributable to a hypertrophied LV wall, i.e., well-developed LV free wall muscle. In a streptozotocin-induced diabetic state of transgenic SERCA2a-overexpressing mice, furthermore, overexpression of SERCA2a protected diabetic hearts from severe contractile dysfunction [22]. Thus SERCA2a overexpression can improve myocardial contractile dysfunction in rodents with diabetic cardiomyopathy.

In the present DM hearts, the increase in ESP_{mLV} in response to Ca²⁺ infusion was significantly lower than

TABLE 3: Variables of LV energetics

| Group | n | VO ₂ -PVA relation | | VO ₂ per minute ($\mu\text{l O}_2 \text{ min}^{-1} \text{ g}^{-1}$) | |
|----------------------|---|--|---|---|-----------------|
| | | Slope ($\times 10^{-2} \mu\text{l O}_2 \text{ mm Hg}^{-1} \text{ ml}^{-1}$) | VO ₂ intercept ($\mu\text{l O}_2 \text{ beat}^{-1} \text{ g}^{-1}$) | Basal metabolism | E-C coupling |
| Non-DM (LETO) | 6 | 1.33 \pm 0.38 | 0.357 \pm 0.055 | 31.3 \pm 4.0 | 73.0 \pm 14.0 |
| DM (OLETF) | 6 | 1.39 \pm 0.42 | 0.327 \pm 0.079 | 32.8 \pm 5.1 | 64.2 \pm 20.7 |
| DM + Ad.SERCA2a | 4 | 1.13 \pm 0.27 | 0.372 \pm 0.049 | 29.1 \pm 1.7 | 80.1 \pm 15.7 |
| DM + Ad. β gal | 3 | 1.74 \pm 0.24 | 0.346 \pm 0.047 | 34.6 \pm 3.7 | 69.5 \pm 17.4 |
| DM + saline | 3 | 1.47 \pm 0.30 | 0.368 \pm 0.043 | 29.3 \pm 4.2 | 70.5 \pm 7.2 |

All data are shown as means \pm SD. VO₂, myocardial oxygen consumption per beat; PVA, systolic pressure–volume area; E-C, excitation–contraction; n, number of hearts. VO₂ per minute for basal metabolism was measured in the hearts under KCl-induced arrest. VO₂ per minute for E-C coupling was obtained by subtracting VO₂ per minute for basal metabolism from VO₂ per minute for mechanically unloaded (i.e., free of balloon water) contraction. There was no statistically significant difference in all variables among the five groups.

that of non-DM LETO hearts. This lowered inotropic response to Ca²⁺ may be induced by decreased SERCA2a expression, SERCA2a dysfunction by deficient production of cyclic AMP, Ca²⁺ handling impaired by decreased energy reserve via creatine kinase (CK) reaction, and decreased Ca²⁺ sensitivity of contractile myofilaments as reported in acidotic or stunned myocardium. The present SERCA2a gene transfer was capable of completely reversing the inotropic response to Ca²⁺ (i.e., contractile reserve) as well as the LV systolic and diastolic dysfunction, indicating that in the DM hearts the decreased contractile reserve is caused mainly by the decreased expression of SERCA2a. Furthermore, SERCA2a gene transfer improved the energy reserve via CK reaction in aorta banding-induced failing hearts [23].

LV Energetic Function and SERCA2a Gene Transfer

In the DM rat hearts, the VO₂ intercept (i.e., PVA-independent VO₂) and slope of the VO₂-PVA relation did not differ from those of non-DM hearts, as reported in previous studies [12]. In addition, adenoviral gene transfer did not cause any changes in these energetic variables. The PVA-independent VO₂ reflects VO₂ for nonmechanical works consisting of Ca²⁺ handling during E-C coupling and basal metabolism [24]. We found no differences in VO₂ per minute for Ca²⁺ handling and basal metabolism among all the groups. This finding indicates that neither cardiac hypertrophy nor adenoviral

gene transfer affects VO₂ for Ca²⁺ handling and basal metabolism in steady state of mechanically unloaded and Ca²⁺ unloaded DM hearts. Furthermore, this unchanged VO₂ per minute for Ca²⁺ handling in DM hearts suggests that SERCA2a, albeit down-regulated, can exert its function normally because of a lower level of intracellular free Ca²⁺ during relaxation and diastole. On the other hand, the unchanged slope of the VO₂-PVA relation in all the groups shows that neither cardiac hypertrophy nor adenoviral gene transfer affects the contractile efficiency, which is the reciprocal of slope of the VO₂-PVA relation, in DM hearts. The contractile efficiency, which reflects the chemomechanical energy transduction efficiency of the contractile machinery, is the product of the efficiency from VO₂ to ATP (mitochondrial oxidative phosphorylation) and the efficiency from ATP to PVA (cross-bridge cycling). Therefore, the efficiency of cross-bridge cycling appears to be unchanged in the DM hearts [12]. The unaltered contractile efficiency was also found in hypothyroid rat hearts, where the myosin isozyme was transformed from V₁ to V₃. Thus it seems unlikely that the efficiency of cross-bridge cycling is affected by a decrease in myosin isozyme V₁/V₃ ratio, i.e., decreased myosin ATPase activity. Further, this is supported by the unchanged contractile efficiency observed during cardiac cooling, which decreases myosin ATPase activity and cross-bridge cycling rate [24].

The most important finding of the present study is that the O₂ cost of LV contractility, defined as the slope

TABLE 4: ESP at midrange left ventricular volume in response to Ca²⁺ infusion

| Group | n | ESP _{mLVV} before Ca ²⁺ infusion (mm Hg) | ESP _{mLVV} during Ca ²⁺ infusion (mm Hg) | Increase in ESP _{mLVV} (mm Hg) |
|----------------------|---|--|--|---|
| Non-DM (LETO) | 6 | 130.4 \pm 30.2 ^a | 163.8 \pm 43.9 ^a | 33.4 \pm 16.9 ^a |
| DM (OLETF) | 6 | 82.8 \pm 18.5 | 93.4 \pm 18.2 | 10.6 \pm 2.6 |
| DM + Ad.SERCA2a | 4 | 167.4 \pm 16.0 ^b | 201.3 \pm 14.0 ^b | 33.9 \pm 9.3 ^b |
| DM + Ad. β gal | 3 | 112.7 \pm 30.3 | 130.8 \pm 32.7 | 18.1 \pm 5.4 |
| DM + saline | 3 | 93.5 \pm 20.4 | 107.1 \pm 26.6 | 13.6 \pm 8.1 |

All data are shown as means \pm SD. ESP_{mLVV}, end-systolic pressure observed at midrange left ventricular volume (0.05 ml of balloon volume); n, number of hearts. ESP_{mLVV} during Ca²⁺ infusion was obtained in a steady state of hearts after intracoronary infusion of 1% CaCl₂ at a 6 ml/h infusion rate for at least 4 min.

^a P < 0.05 compared to DM group.

^b P < 0.05 compared to DM and DM + saline groups.

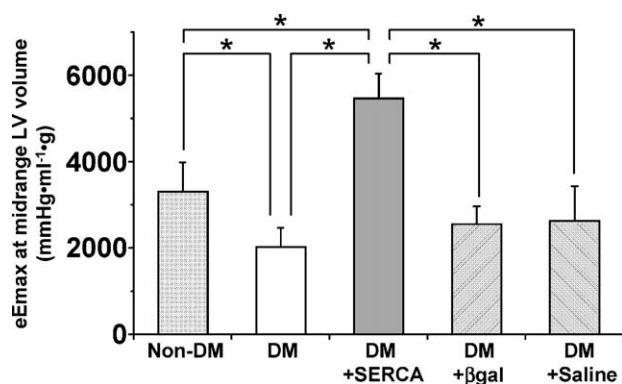


FIG. 2. Comparison of eEmax at midrange LV volume, an index of LV contractility, among all groups. * $P < 0.05$. $n = 6$ in non-DM, $n = 6$ in DM, $n = 4$ in DM + SERCA2a, $n = 3$ in DM + β Gal, $n = 3$ in DM + saline.

of relation between Ca^{2+} -handling VO_2 and eEmax, was increased in the DM hearts, and this increased O_2 cost could be restored to normal levels by SERCA2a gene transfer. The O_2 cost of contractility, which reflects the energy cost of nonmechanical activities from VO_2 to eEmax, is the product of the energy cost from VO_2 to ATP (mitochondrial oxidative phosphorylation) and the energy cost from ATP to eEmax (E-C coupling), which consists of the cost of Ca^{2+} handling and the Ca^{2+} responsiveness of myofilaments [25]. Therefore, the increased O_2 cost of contractility in the present DM hearts appears to be ascribable to the increased energy

cost of Ca^{2+} handling, because the mitochondrial oxidative phosphorylation and Ca^{2+} responsiveness of myofilaments remain unchanged, as mentioned above, even in failing hearts. We have speculated on some mechanisms for the increased energy cost of Ca^{2+} handling, which means the energy wasting in Ca^{2+} handling during E-C coupling. First, in acidotic and ischemic myocardium, the molar coupling ratio of calcium to ATP in SERCA2a was reported to be decreased due to increased calcium permeability of the SR membrane [26]. Therefore, such SERCA2a dysfunction would need more ATP to decrease cytosolic Ca^{2+} during relaxation and to relax the DM heart to the same extent as non-DM heart in each cardiac cycle. Second, an abnormal Ca^{2+} leak through the ryanodine receptor (RyR), caused by RyR conformational change due to a partial loss of RyR-bound FKBP12.6 binding protein (FKBP12.6), was found in a canine model of heart failure [27,28]. In failing human hearts, furthermore, hyperphosphorylation of RyR mediated by protein kinase A caused dissociation of FKBP12.6 from RyR, resulting in defective channel function due to increased sensitivity to Ca^{2+} -induced activation [27,28]. This Ca^{2+} leak via RyR will decrease Ca^{2+} loading of SR and elevate basal cytosolic Ca^{2+} levels during diastole, leading to the futile Ca^{2+} handling in SR, for which extra energy would be required for reuptake of the leaked Ca^{2+} by SERCA2a. However, the increased energy cost of Ca^{2+} handling cannot be fully explained by the first and/or second mechanism, because SERCA2a was down-regulated at

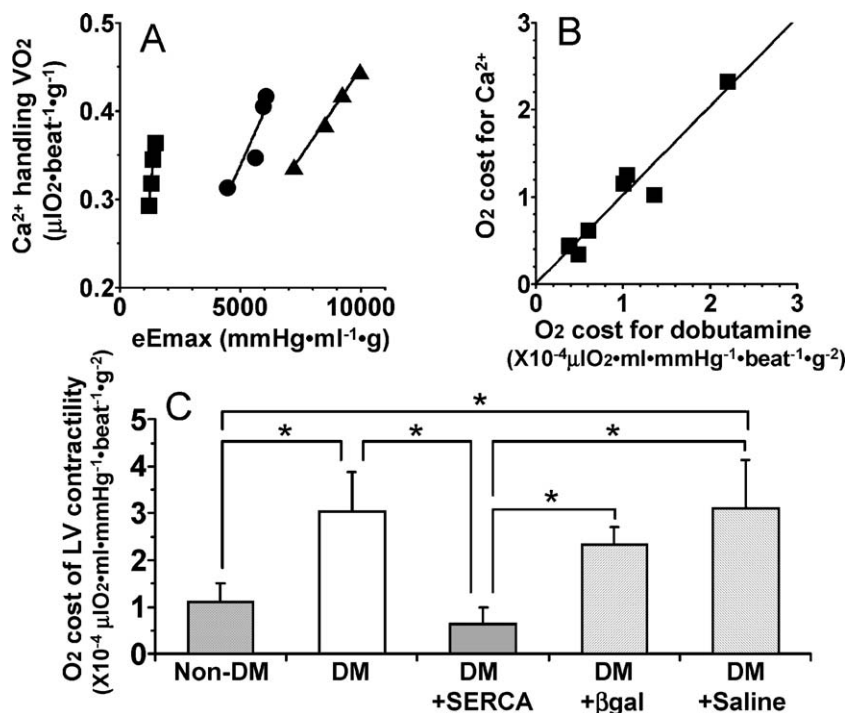


FIG. 3. (A) Linear relations between Ca^{2+} handling VO_2 and eEmax at midrange LV volume during Ca^{2+} inotropic run in non-DM (●), DM (■), and DM + SERCA2a (▲) hearts. The slope of these linear relations describes the oxygen cost of LV contractility. (B) Correlation between O_2 costs for Ca^{2+} and for dobutamine. In eight heart preparations, a dobutamine (78 μM) inotropic run was performed after the Ca^{2+} inotropic run. The equation for the regression line is $f(x) = 1.01x + (4.5 \times 10^{-7})$, $r = 0.963$. (C) Comparison of oxygen costs of LV contractility for Ca^{2+} among all groups. * $P < 0.05$. $n = 6$ in non-DM, $n = 6$ in DM, $n = 4$ in DM + SERCA2a, $n = 3$ in DM + β Gal, $n = 3$ in DM + saline.

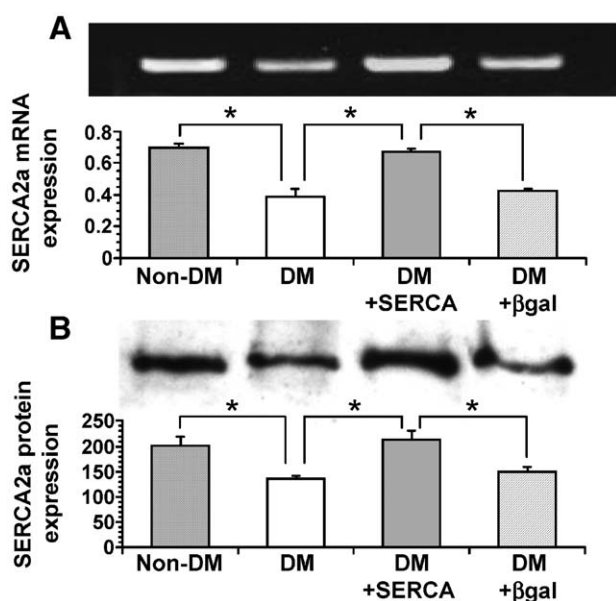


FIG. 4. (A) Comparison of SERCA2a mRNA expression among the four groups. The data were normalized against actin expression ($n = 3$ in each group). $*P < 0.05$. (B) Comparison of SERCA2a protein expression among the four groups ($n = 3$ in each group). $*P < 0.05$.

mRNA and protein levels in our DM hearts. Finally, cardiac NaCaX was reported to be up-regulated at the mRNA, protein, and functional levels in rabbit and human heart failure [20,29–31]. Although NaCaX per se does not consume ATP to remove cytosolic Ca^{2+} in exchange for incoming Na^+ on the basis of a stoichiometry of $3\text{Na}^+ : 1\text{Ca}^{2+}$, the incoming Na^+ will be pumped out by $\text{Na}^+ - \text{K}^+ - \text{ATPase}$ with a stoichiometry of $3\text{Na}^+ : 2\text{K}^+ : 1\text{ATP}$, resulting in the net stoichiometry of $1\text{Ca}^{2+} : 1\text{ATP}$ [32]. On the other hand, SERCA2a removes cytosolic Ca^{2+} on the basis of a stoichiometry of $2\text{Ca}^{2+} : 1\text{ATP}$. Therefore, the Ca^{2+} extrusion via NaCaX leads to a double increase in energy expenditure compared with the Ca^{2+} uptake by SERCA2a into the SR if the same amount of Ca^{2+} is handled by NaCaX versus SERCA2a. Thus it seems most likely that the increased energy cost of Ca^{2+} handling in the DM hearts is caused mainly by the enhanced activity of NaCaX. Nevertheless, overexpression of SERCA2a was capable of restoring the high O_2 cost of LV contractility to non-DM level in the SERCA2a-transferred DM hearts. In normal rat LV, the activity of SERCA2a is high because of a great concentration of SERCA2a molecules, and Ca^{2+} removal via NaCaX is low, resulting in a balance of 92% for SERCA2a, 7% for NaCaX, and 1% for sarcolemmal $\text{Ca}^{2+} - \text{ATPase}$ and mitochondrial Ca^{2+} uniporter [2,32,33]. Because SERCA2a plays a central role in Ca^{2+} transport out of the cytosol, the enhanced expression of SERCA2a in DM + SERCA2a hearts appears to induce both the increase in Ca^{2+} uptake into the SR and,

thereby, the decrease in Ca^{2+} extrusion via NaCaX, leading to the decrease in the energy cost of Ca^{2+} handling, namely, the decrease in the O_2 cost of LV contractility.

Therapeutic Implications

In the treatment of CHF, inotropic agents yield short-term contractile and hemodynamic improvement, but increase VO_2 , as shown by the high O_2 cost of LV contractility for dobutamine in our DM rats. This energy wasting in Ca^{2+} handling is extremely critical in patients with coronary artery disease because of the limited energy supply. Therefore, inotropic interventions increase mortality in CHF [34]. The present study demonstrates that the energy wasting in Ca^{2+} handling in DM failing hearts can be improved by overexpressed SERCA2a. Furthermore, this improved energy utilization by SERCA2a overexpression must have contributed to the improvement of mortality and energy reserve in the aortic banding-induced rat heart failure [23]. Taken together, such a molecular targeting for SERCA2a may be a potential therapeutic modality that has the advantage of efficient energy utilization over pharmacological therapy.

Limitations of the Study

Rescue of LV energetic function by SERCA2a overexpression, demonstrated in the present DM failing hearts using a short-term gene expression system of recombinant adenoviral vectors, may not be applied directly to human failing hearts. The contribution of SERCA2a to decreasing cytosolic Ca^{2+} varies with species. In rats, as mentioned above, 92% of the Ca^{2+} is removed by SERCA2a, while in human about 75% is removed by SERCA2a [32]. However, contractile dysfunction in failing human cardiomyocytes could be improved by SERCA2a overexpression [8]. In addition, high O_2 cost of contractility was found in failing human hearts. Therefore, SERCA2a overexpression would be expected to improve LV energetic function in failing human myocardium. Further confirmation of this concept will be required for clinical gene therapy. It is unclear how long-term overexpression of SERCA2a, using adeno-associated vectors, may influence the energetic function in small and large animal models of pressure- or volume-overload-induced failing hearts. Furthermore, long-term overexpression of SERCA2a may have an antihypertrophic effect, which was not fully clarified in this study. Finally, the energetic function may be modulated by altered expression of other Ca^{2+} -handling proteins through gene transfer.

CONCLUSIONS

Our results demonstrate that in DM failing hearts, the high O_2 cost of LV contractility, as well as contractile dysfunction of LV, can be restored to non-DM levels by SERCA2a gene transfer. The high O_2 cost of LV contractility indicates energy wasting in Ca^{2+} handling

during E-C coupling. However, gene transfer of SERCA2a transforms this inefficient energy utilization into a more efficient state. Overexpression of SERCA2a improves not only mechanical but also energetic function in diabetic hearts. SERCA2a gene transfer may be a potential therapeutic modality that has the benefit of efficient energy utilization.

MATERIALS AND METHODS

Animals

Five-week-old male DM OLETF and normal male LETO rats were obtained from the Tokushima Research Institute, Otsuka Pharmaceutical Co. (Tokushima, Japan). Seventy-to eighty-week-old DM rats were randomized into four groups (group without gene transfer (DM), group with adenoviral SERCA2a (Ad.SERCA2a) transfer (DM + Ad.SERCA2a), group with adenoviral β -galactosidase (Ad. β Gal) transfer (DM + Ad. β Gal), and group with saline injection (DM + saline)).

Adenoviral Vectors

Recombinant adenoviral vectors were used with cytomegalovirus-driven expression cassettes for SERCA2a or β -galactosidase with a second cassette in each adenovirus containing green fluorescent protein substituted for E1 by means of homologous recombination. Ad.SERCA2a and Ad. β Gal had concentrations of 6.2×10^{10} and 4.8×10^{10} pfu/ml, respectively, with a particle/pfu ratio of 40:1. Wild-type adenovirus contamination was excluded by the absence of PCR-detectable E1 sequences.

Adenoviral Delivery Protocol

The three DM groups received Ad.SERCA2a or Ad. β Gal or saline. The adenoviral delivery system has been described previously in detail by our group [14]. Briefly, after the rats were anesthetized and a thoracotomy was performed, a 22-gauge catheter containing 200 μ l of adenovirus and 50 μ l adenosine (3 mg/ml) was advanced from the apex of the left ventricle to the aortic root. The aorta and main pulmonary artery were clamped for 30 s distal to the site of the catheter and the solution was injected, then the chest was closed, and the animal was allowed to recover. The rats underwent LV mechanical and energetic studies 3 days after the injection of the adenovirus or saline.

LV Mechanical and Energetic Studies

Surgical preparations. The LV mechanical and energetic studies were performed on the excised, cross-circulated rat heart preparations. The surgical preparations have been described previously in detail [12,13]. Briefly, in each experiment, two male 500- to 650-g Wistar rats (blood supplier and metabolic supporter) and one DM or non-DM LETO rat (heart donor) were anesthetized with pentobarbital sodium (50 mg/kg, ip) and intubated and heparinized (1000 U, iv). Systolic unstressed volume ($V_0 = 0.08$ ml for non-DM hearts and $V_0 = 0.06$ ml for DM hearts) was determined as the volume at which peak isovolumic pressure was 0. Heart rate was maintained constant at 300 bpm by electrical pacing of the right atrium. Systemic arterial blood pressure of the support rat served as coronary perfusion pressure (80–120 mm Hg). Arterial pH, P_{O_2} , and PCO_2 of the support rat and perfused blood were maintained within their physiological ranges with supplemental oxygen and sodium bicarbonate throughout the experiment.

Calculation for oxygen consumption. Cardiac oxygen consumption was obtained as the product of coronary flow and arteriovenous O_2 content difference. Relation between oxygen consumption per beat (VO_2) and systolic PVA was linear in the rat LV. The VO_2 intercept represents PVA-independent VO_2 . The RV was kept collapsed by continuous hydrostatic drainage of the coronary venous return, so that the RV PVA and PVA-dependent VO_2 were assumed to be negligible. The RV component of

PVA-independent VO_2 was calculated by multiplying biventricular PVA-independent VO_2 with the weight ratio of RV/RV + LV. The RV PVA-independent VO_2 was subtracted from the total VO_2 to yield LV VO_2 . After the experiment, the LV including the septum and RV free walls were separately weighed.

Experimental protocol. LVP, VO_2 , and PVA data were obtained at five different volumes (from 0.06 to 0.16 ml in DM hearts, from 0.08 to 0.18 ml in non-DM hearts), changed with a step of 0.025 ml, without any inotropic interventions (control volume-loading run). After the control volume run, a Ca^{2+} inotropism run was performed at a given mLVV by intracoronary infusion of 1% $CaCl_2$ solution. In some experiments, a dobutamine (78 μ M) inotropism run was also performed after the Ca^{2+} inotropism run. Data sampling was started 4 min after the onset of calcium or dobutamine infusion. The heart steady state was reached 2–3 min after the change in LV volume and 4 min after the change in the infusion rate. Cardiac arrest was induced by infusing 1 M KCl into the coronary perfusion tubing at a constant rate (12 ml/h), which was adjusted to abolish electrical excitation but not to generate any KCl-induced constriction of coronary vessels. In each steady state, data were sampled at 500 Hz for 2 s simultaneously, and the sampling was usually repeated three times at intervals of 0.5–1 min.

Data Analysis

PVA calculation. We calculated PVA as described below, since the ESPVR was an upward convex curve. The end-systolic/diastolic pressure–volume points were fitted by the same exponential functions as used in our previous study to obtain the best fit end-systolic/diastolic pressure–volume relations [12,13]. PVA was defined as the pressure–volume area circumscribed by the curvilinear ESPVR, the EDPVR, and the systolic portion of the ventricular pressure–volume trajectory. The areas under the ESPVR and EDPVR were obtained by integration of the best fit exponential functions. Finally, PVA was normalized to the value per gram by dividing PVA by LV weight.

VO_2 for Ca^{2+} handling in E-C coupling during inotropic run. We first obtained control ESPVR as a best fit exponential function curve. During the Ca^{2+} /dobutamine inotropic run at a mLVV, ESP–volume relations at different infusion rates were obtained as a best fit curve. We calculated PVA at mLVV (PVA_{mLVV}) by integrating each ESPVR from V_0 up to the mLVV. We then obtained the two composite VO_2 – PVA_{mLVV} data points. Next, the lines including each VO_2 – PVA_{mLVV} data point were drawn in parallel to the control VO_2 –PVA relation. The VO_2 -intercept values (PVA-independent VO_2 values), which were increased proportionally to the enhanced LV contractility by Ca^{2+} /dobutamine, were obtained by thus drawing the lines. The VO_2 for Ca^{2+} handling in the E-C coupling was obtained by subtracting the basal metabolic VO_2 per beat from the PVA-independent VO_2 values. The basal metabolic VO_2 was measured in KCl-induced arrested hearts.

LV Contractility. eEmax, an index for LV contractility, was obtained by calculating ESP–volume ratio of the specific virtual triangle, which is energetically equivalent to the real PVA_{mLVV} .

Oxygen cost of LV contractility. During the Ca^{2+} /dobutamine inotropism run, plots of VO_2 for Ca^{2+} handling in the E-C coupling and eEmax data gave us the linear relation. The oxygen cost of LV contractility was obtained as the slope of this linear relation. This slope is considered an index quantifying VO_2 for Ca^{2+} handling in the E-C coupling per unit LV contractility change.

Logistic time constant. To evaluate the LV relaxation rate, we analyzed LV isovolumic relaxation pressure–time curves at mLVV by using the T_L derived from a logistic model [12].

Western blot for SERCA2a protein. Lysates from control and diabetic hearts were matched for protein concentration and then separated by

SDS-PAGE and transferred to nitrocellulose membranes. Blots were incubated with SERCA2a antibodies followed by detection with enhanced chemiluminescence.

Determination of SERCA2a mRNA expression. Myocytes from normal and diabetic hearts were harvested and mRNAs were measured using reverse transcriptase-polymerase chain reaction (RT-PCR). Total RNA was isolated using Trizol reagent (Invitrogen) and cDNA was synthesized from 1 µg of total RNA using iScript reverse transcriptase (Bio-Rad) in a final volume of 20 µl. The mRNA levels of SERCA2a were evaluated by RT-PCR. The level of GAPDH mRNA was evaluated as an internal control. The annealing temperature for the PCR cycles was adjusted according to the optimal annealing temperatures for each specific primer set. Density values of SERCA bands from at least three independent experiments were normalized against GAPDH values.

Statistics. Data are presented as means ± SD. Multiple comparisons were performed by ANOVA with STATVIEW (Abacus Concepts, Berkeley, CA, USA). Statistical significance was accepted at the level of $P < 0.05$.

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