Left ventricular diastolic dysfunction in type 2 diabetes mellitus model rats

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Received 31 May 2001; accepted in final form 11 September 2001

Abe, Takehisa, Yoshimi Ohga, Nobuoki Tabayashi, Shuichi Kobayashi, Susumu Sakata, Hiromi Misawa, Tsuyoshi Tsuji, Hisaharu Kohzuki, Hiroyuki Suga, Shigeki Taniguchi, and Miyako Takaki. Left ventricular diastolic dysfunction in type 2 diabetes mellitus model rats. Am J Physiol Heart Circ Physiol 282: H138–H148, 2002.—To gain insight into the pathogenesis of diabetic cardiomyopathy, we investigated cardiac function in terms of the coupling of left ventricular mechanical work and the energetics in Otsuka Long-Evans Tokushima Fatty rats, which are well known as a model of type 2 diabetes mellitus (DM). Neither left ventricular systolic function and mean coronary flow nor coronary flow reserve differed even in late DM rats. The amount of oxygen required for mechanical work and contraction was unaltered, although myosin isozyme was finally transformed from V1 to V3. The maximum pacing rate was decreased from 300 to 240 beats/min, and the left ventricular relaxation rate was significantly (P < 0.05) slower only in late DM rats, resulting in decreased oxygen consumption per minute for total Ca2+ handling in excitation-contraction coupling mainly consumed by sarco(endo)plasmic reticulum Ca2+-ATPase (SERCA2) without significant changes in basal metabolism or in mitochondrial oxidative phosphorylation. The protein level of SERCA2 in membranes was significantly (P < 0.001) lower in severe DM rats. We conclude that the only lusitropic dysfunction due to the depressed expression of SERCA2 is related to generating diabetic cardiomyopathy even in the present type 2 diabetic rats.

Although the principal pathophysiological feature of diabetes mellitus (DM) is called diabetic microangiopathy, the presence of myocardial dysfunction “diabetic cardiomyopathy” independent of coronary artery disease has been well documented in streptozotocin-induced DM (29, 32). The recently established Otsuka Long-Evans Tokushima Fatty (OLETF) rat model of DM (9) manifests stable clinical and pathological features that resemble human type 2 DM. Mizushige et al. (13) recently elucidated the causal link between microangiopathy and the pathogenesis of diabetic cardiomyopathy in OLETF rats. They found that early diastolic transmitral inflow exhibited prolonged deceleration time and low peak velocity in the prediabetic stage (13). Abnormal relaxation is usually one of the earliest manifestations of cardiac dysfunction, and diastolic impairment is seen before systolic impairment (16), but the pathogenesis of diabetic cardiomyopathy is not well understood. Specifically, the coupling of mechanical work and energetics of the heart in diabetic cardiomyopathy is not yet understood.

We have recently reported a linear relationship between myocardial oxygen consumption per beat (VO2) and the systolic pressure-volume area (PVA: a total mechanical energy per beat) in the rat left ventricle in the blood-perfused whole heart preparation, similar to that observed in canine hearts (24), from a curved end-systolic pressure (ESP)-volume (V) relationship (ESPVR) (3, 4, 23). The VO2 intercept of the linear VO2-PVA relationship is mainly composed of VO2 for Ca2+ handling in the excitation-contraction coupling, which is primarily consumed by sarco(endo)plasmic reticulum Ca2+-ATPase (SERCA2) and basal metabolism (3, 4), in animal hearts (14, 20). We have recently shown (27, 28) that a new “oxygen (O2) cost of left ventricular contractility” index can evaluate changes in VO2 for total Ca2+ handling in the excitation-contraction coupling versus changes in left ventricular contractility in terms of the coupling of mechanical work and energetics. This cost is not dependent on a heart rate between 240 and 300 beats/min (18).

We hypothesized that diastolic dysfunction may manifest itself in early diabetic cardiomyopathy due to dysfunction of SERCA2. If so, it is predicted that VO2 for total Ca2+ handling in the excitation-contraction coupling may decrease, and the O2 cost of left ventricular contractility may increase due to resultant activation of the Na+/Ca2+ exchanger and Na+-K+-ATPase (8, 12). To prove this hypothesis, we investigated left ventricular systolic and diastolic functions linked with intracellular Ca2+ handling in terms of the coupling of mechanical work and energetics in spontaneously DM,
OLETF rats. The use of an appropriate animal model may lead to a better understanding of the processes that cause diabetic cardiomyopathy.

**METHODS**

The investigation conformed with the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH Publication No. 85-23, Revised 1996).

**Animals.** Five-week-old male OLETF rats and control male Long-Evans Tokushima Otsuka (LETO) rats were obtained from Tokushima Research Institute (Tokushima, Japan). A previously reported study (7) shows that OLETF rats drinking 30% sucrose solution for 8 wk decreased the conduction velocity of motor nerves and thus impaired autonomic nerve function. Starting at 25 wk of age, OLETF rats were allowed free access to tap water containing 30% sucrose for 8 wk (up to 32 wk) to cause severe DM with neuropathy.

Variables in left ventricular mechanics (6 L-I OLETF, 3 L-I LETO, 6 L-II OLETF, and 5 L-II LETO rat hearts) as reported previously (3, 4, 27). In each experiment, two retired breeder male crj:Wistar rats weighing 603 ± 42 g (32–40 wk of age, purchased from Charles River Japan; Yokohama, Japan) were anesthetized with pentobarbital sodium (50 mg/kg ip) and used as blood supplier and metabolic supporter rats, respectively. All rats were administered heparin (1,000 units iv). The beating heart was excised from OLETF and LETO rats without interruption of coronary perfusion and supported by cross circulation with the metabolic supporter rat as previously reported in detail (3). The excised heart was maintained at 37°C.

A thin latex balloon (balloon material volume, 0.08 ml) fitted into the left ventricle was connected to a pressure transducer (Life Kit DX-312, Nikohon; Tokyo, Japan) and to a 0.5-ml precision glass syringe with fine scales (minimum scale: 0.005 ml). The maximum unstretched balloon volume was below −0.25–0.30 ml. Thus left ventricular volume (LVV) was changed and measured by adjusting the intraballoon water volume with the syringe in 0.05-ml steps between 0.08 and 0.28 ml. Systolic unstressed volume (V₀) was determined by filling the balloon to the level where peak isovolumic pressure and hence PVA (see Data analysis) were zero. The sum of intraballoon water volume and balloon material volume (0.08 ml) was used as an initial estimate of V₀. This procedure was repeated during different LVV-loading runs. V₀ was then finally determined as the volume-axis intercept of the best-fit ESPVR. We obtained the best-fit ESPVR with the equation stated in Table 1 by means of the least-squares method (Delta-Graph, DeltaPoint; Monterey, CA) on a personal computer (3, 4, 27, 28). Correlation coefficients of the best-fit ESPVRs were higher than 0.99 (Table 1).

The left ventricular epicardial electrocardiogram was recorded, and the heart rate was constantly maintained by electrical pacing of the right atrium. The pacing rate was adjusted to avoid causing incomplete relaxation or arrhythmia (Table 1). The systemic arterial blood pressure of the supporter rat served as the coronary perfusion pressure (100–130 mmHg). Arterial pH, P O₂, and P CO₂ of the supporter rat were maintained within their physiological ranges with supplemental O₂ and sodium bicarbonate.

### Table 1. Variables in left ventricular mechanics

<table>
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<tr>
<th>Rats</th>
<th>n</th>
<th>A₀, mmHg</th>
<th>B₁, 1/ml</th>
<th>R</th>
<th>V₀, ml/g</th>
<th>ESPmax, mmHg</th>
<th>ESPmlLV, mmHg</th>
<th>PVAmlLV, mmHg/ml-beat¹·g⁻¹</th>
<th>mLVV, ml/g</th>
<th>Pacing Rate, beats/min</th>
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<tr>
<td>L-I OLETF (40–46 wk)</td>
<td>6</td>
<td></td>
<td></td>
<td></td>
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<td>136.7</td>
<td>7.3</td>
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<tr>
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<td></td>
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<td>0.078</td>
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<td>± SD</td>
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<td>12.3</td>
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<td>18.8</td>
<td>2.5</td>
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<td>Mean</td>
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<td>167.9</td>
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<td>0.078</td>
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<tr>
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<td>0.005</td>
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<td>7.4</td>
<td>0.16</td>
<td>240(214)†</td>
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<tr>
<td>± SD</td>
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<td>0.002</td>
<td>0.007</td>
<td>36.0</td>
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<tr>
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<td>0.999</td>
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<td></td>
<td></td>
<td>0.15</td>
<td>300(316)†</td>
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<tr>
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<td>0.006</td>
<td>33.9</td>
<td>26.0</td>
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<td>0.01</td>
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OLETF, Otsuka Long-Evans Tokushima Fatty rat; LETO, Long-Evans Tokushima Otsuka rat; L-I, long-term I; L-II, long-term II; A and B, parameters in ESP = A(1 − exp[−BV − V0]); R, correlation coefficients of the best-fit curve for the equation; V₀, volume intercept of end-systolic pressure-volume relationship during different volume loading run; ESPmlLV, end-systolic pressure (ESP) at mean midrange left ventricular volume (mLVV) (see text); ESPmax, observed maximum ESP; PVAmlLV, pressure-volume area at mean mLVV; †our unpublished data; ‡214 and 316 are exceptional ones.
Oxygen consumption. Myocardial oxygen consumption was obtained as the product of coronary flow and coronary arteriovenous O2 content difference (3, 27). Total coronary blood flow was continuously measured with an electromagnetic flowmeter (MFV-3100, Nihon Kohden; Tokyo, Japan) placed in the middle of the coronary venous drainage tubing from the right ventricle. Left ventricular thebesian flow was negligible. The coronary arteriovenous O2 content difference was continuously measured by passing all the arterial and venous cross-circulation blood through the cuvettes of a custom-made arteriovenous O2 content difference analyzer (PWA-200S, Shoe Technica; Chiba, Japan) as previously reported in detail (3, 27). As shown previously (3, 4, 27), the VO2-PVA relationship was linear in the rat left ventricle. Its slope represents the O2 cost of PVA, and its VO2 intercept represents the PVA-independent VO2. The right ventricle was kept collapsed by continuous hydrostatic drainage of the coronary venous return so that the right ventricular PVA and hence PVA-dependent VO2 were assumed to be negligible (3, 27). The right ventricular PVA-independent VO2 (3, 4, 27, 28) was subtracted from the total VO2 to yield left ventricular VO2. The left ventricle (including the septum) and the right ventricle were weighed for normalization of LVV. They were therefore different inotropic run and a second Ca2+ induced different inotropic run (Ca2+–induced different inotropic run (Ca2+–induced inotropic run) was performed at a midrange LVV (mLVV) [0.16 or 0.18 ml/g (n = 20), respectively]. There were no significant differences in left ventricular weight among the four rat groups (L-I OLETF, 1.03 ± 0.08; L-I LETO, 1.04 ± 0.05; L-II OLETF, 1.01 ± 0.07; L-II LETO, 1.16 ± 0.12 g).

Experimental protocol. Left ventricular pressure, VO2, and PVA data during isovolumic contractions were obtained at four to five different LVVs (mean volume range: 0.16 ± 0.03 ml/g; n = 20) in each L-I OLETF and L-I LETO rat heart and L-II OLETF and L-II LETO rat heart. In each L-I OLETF and L-I LETO rat heart, after the control volume run, a Ca2+–induced different inotropic run (Ca2+–induced inotropic run) was performed at a midrange LVV (mLVV) [0.16 or 0.18 ml = water volume infused into the balloon (equals 0.08 or 0.10 ml) plus V0 (equals 0.08 ml)] during intracoronary infusion of 1% CaCl2 solution. In each L-II OLETF and L-II LETO rat heart, after the control volume run, a first dobutamine-induced different inotropic run and a second Ca2+–induced inotropic run were performed at the same mLVV during infusion of 66 μM dobutamine and 1% CaCl2 solution, respectively. Between the two inotropic runs, it took 20 min to attain complete clearance of the blood with dobutamine by the kidney of the supporter rat. Mean values for mLVV and V0 (normalized for 1 g) were 0.15 ± 0.01 and 0.08 ± 0.01 ml/g (n = 20), respectively. These values were not significantly different among the four rat groups (Table 1). The infusion rates of Ca2+ and dobutamine were increased in steps up to 10–20 ml/h (n = 20) and up to 4 ml/h (n = 11), respectively. The blood Ca2+ concentration reached 3.1–8.1 mmol/l. The calculated blood dobutamine concentration reached 1.1–5.5 μmol/l under a coronary flow rate of 3–4 ml/min. Every volume run and inotropic run, a steady state where left ventricular pressure, coronary arteriovenous O2 content difference, and coronary flow were stable was reached 3 min after changing LVV or 5 min after changing the infusion rate of Ca2+ or dobutamine.

Cardiac arrest was induced by infusing KCl (1.0 M) into the coronary perfusion tubing at a constant rate [n = another group of 8 hearts; L-II OLETF (n = 2), L-II LETO (n = 3), Wistar (n = 3)], which was adjusted to abolish electrical excitation under monitoring ventricular electrocardiograms but not to generate any KCl-induced contractions of coronary vessels. VO2 and PVA data were obtained by minimal volume loading to avoid volume-loading effects, if any, on VO2 data. In each steady state, data were sampled at 500 Hz for 2 s, and the sampling was usually repeated three times at intervals of 0.5–1 min.

Data analysis. We attempted to fit experimentally obtained left ventricular pressure-volume data to the exponential equations to obtain ESPVRs (see Table 1) and end-diastolic pressure-volume relationships (EDPVRs) and thus determine PVA by the same method as described previously in detail (3, 4, 27, 28).

Although the EDPVR at 300 beats/min pacing showed an exceptional shift upward due to incomplete relaxation, the area under the EDPVR at 240 beats/min pacing was reasonably assumed to be zero within the same LVV range (3, 4, 27, 28) in L-II OLETF rats. From our previous proposal (3, 4, 10, 23, 27), we obtained control ESPVR and calculated ESP at mLVV (ESPmLVV) and PVAmLVV to assess left ventricular mechanical work and energetics in the four rat groups.

Oxygen cost of left ventricular contractility. We obtained the specific best-fit curves for the observed ESPmLVV and ESP (0 mmHg) at V0 with the ESPVR function in the control volume run by the least squares method, and we calculated PVAmLVV during dobutamine or Ca2+ infusion on a personal computer (18, 27, 28). The parallelism of the VO2-PVA linear relation during dobutamine (1) or Ca2+ infusion (3, 18, 27) has been confirmed in normal Wistar rat hearts. From this parallelism, the lines including all VO2–PVA data obtained during dobutamine or Ca2+ infusion in steps at the mLVV were drawn in parallel to the control VO2–PVA relation line, as described previously (3, 18, 27, 28). The gradually increased VO2–intercept values (PVA-independent VO2 values) of the lines proportional to the enhanced left ventricular contractility induced by dobutamine or Ca2+ were obtained by this procedure. Our recently proposed index for the left ventricular contractility, equivalent ESP-to-volume ratio at mLVV (EESPmax), was calculated from a triangular area equivalent to PVAmLVV (18, 27, 28). Mean ESV (equals mLVV – V0) at mean mLVV (0.15 ml/g) (ESV0.15) was 0.08 ± 0.01 ml/g (n = 20 each). The O2 cost of left ventricular contractility was the slope of the relationship between PVA-independent VO2 and EESP (18, 27, 28).

Logistic time constant. To evaluate the left ventricular relaxation rate or lusitropism, by using our proposed “logistic” time constant (TL) derived from a logistic model (11) and the conventional “exponential” time constant (TK), we analyzed left ventricular isovolumic relaxation pressure-time curves at mLVV in OLETF and LETO rats. Both time constants decrease as the heart rate increases and should therefore be compared under the same pacing rate conditions. We compared the time constants of the hearts paced at ~300 beats/min in L-I OLETF versus L-I LETO rats and also compared those of the hearts paced at 240 beats/min in L-II OLETF versus L-II LETO rats.

Western blots for SERCA2. Membrane proteins from the left ventricular myocardium of each heart were isolated and were used for immunoblotting previously by Yoshida et al. (31). The frozen hearts were homogenized and centrifuged at 1,000 g for 10 min. The supernatants were centrifuged at 100,000 g for 60 min at 4°C. The 100,000-g pellets were cellular membrane fractions and were used for immunoblotting of SERCA2. We confirmed that these membrane fractions did not contain any detectable collagens.

Membrane proteins (20–25 μg/lane) were separated on 10% SDS-polyacrylamide gels in a minigel apparatus (Mini-PROTEAN II, Bio-Rad) and transferred to polyvinylidene difluoride membranes. The membranes were blocked (4% Block Ace, Dainippon Pharmaceutical; Osaka, Japan) and then incubated with anti-SERCA2 antibody (1:1,000 dilution, 24/26 January 2002 • V OL 282
Affinity Bio Reagents). The antigens were detected by the luminescence method (ECL Western blotting detection kit, Amersham) with peroxidase-linked anti-mouse IgG (1:2,000 dilution). After immunoblotting, the film was scanned with a scanner, and the intensity of the bands was calculated by NIH Image analysis. Each value of the calculated area was finally expressed by the relative value to that in the Wistar rat heart (equalling 1.0).

Polyacrylamide gel electrophoresis. The left ventricular myocardium from each heart was frozen and stored at −20°C. Myosin was extracted from 50 mg of the left ventricular myocardium, and three myosin isozymes (V1, V2, and V3) were separated by 3.7% polyacrylamide gel electrophoresis in the presence of pyrophosphate according to a slightly modified procedure (10) of Hoh et al. (6).

Mitochondrial respiratory function. A differential centrifugation was used according to the method of Hatof et al. (5). Immediately after the mitochondrial preparation, oxygen consumption of mitochondria was measured polarographically with an oxygen electrode installed in a closed cell (UC-12, Central; Tokyo, Japan) at 25°C. The incubation medium contained 0.3 M mannitol, 10 mM KH2PO4, 2.5 mM MgCl2, and 0.1 mM EDTA at pH 7.4 in a total of 0.7 ml. A mitochondrial suspension of 0.03 ml (6.27 ± 2.08 mg protein/ml) was first added, and then 0.2 M sucinic acid (0.03 ml) was added to the incubation medium as a substrate. One minute later, 10 mM ADP (0.01 ml) was added. The rate of oxygen consumption in state III (state III O2) and the respiratory control index (RCI) were determined. State III O2 was also calculated. RCI (dimensionless) was taken as the ratio of the rates of oxygen consumption before and after the addition of ADP.

We have previously confirmed >95% intactness of the mitochondria prepared by the present differential centrifugation method from the heart by means of electron microscopic examination, although in normal Wistar rat hearts (26).

Statistics. Comparison of paired and unpaired individual values was performed by paired and unpaired t-test, respectively. Multiple comparisons were performed by analysis of variance (ANOVA) with post hoc Bonferroni’s test or Fisher’s protected least-significant difference method. A value of P < 0.05 was considered statistically significant. All data are expressed as means ± SD.

RESULTS

ESPVRs, EDPVRs, and VO2-PVA relationships in control and during dobutamine or Ca2+ infusion in OLETF and LETO rats. We simultaneously recorded the electrocardiogram, left ventricular pressure, coronary flow, arteriovenous oxygen content difference, and LVV of each heart from the four rat groups at different pacing rates. Left ventricular pressure increased with the increase in LVV. The best-fit ESPVR in the control volume run was an upward convex curve in each heart of L-I OLETF and L-I LETO and L-II OLETF and L-II LETO rats. Although the EDPVR by pacing at 300 beats/min showed a shift upward due to incomplete relaxation in L-II OLETF rats, the EDPVR at 240 beats/min pacing did not shift upward, and the area under the EDPVR is reasonably considered to be zero. An example of control ESPVR and EDPVR in L-II OLETF rats is shown in Fig. 1A. The ESP at a LVV of 0.17 ml/g (ESP0.17) gradually increased, and the specific best-fit curves for the observed ESP0.17 and ESP (0 mmHg) at V0 were obtained with the ESPVR function in the control volume run (Fig. 1B). PVA at a LVV of 0.17 ml/g (PVA0.17) was then calculated during the first dobutamine (1–4 ml/h) and second Ca2+ infusion (1–20 ml/h) in the same heart (Fig. 1B). Mean best-fit parameters, ESPmlLV, and PVAmlLV were not significantly different among the four rat groups (Table 1). The mean ESP at mean mLV (0.15 ml/g) was 76.7 ± 12.4% of the maximum ESP (ESPmax) (n = 20).

VO2 increased with increases in left ventricular pressure and LVV, and hence with an increase in PVA. An example of the control VO2-PVA relation line is shown in Fig. 2A in the same heart as shown in Fig. 1. VO2-PVA0.17 data points deviated right upward on the composite VO2-PVA lines during the first dobutamine and the second Ca2+ infusion (1–20 ml/h) (Fig. 2A). Mean VO2 and PVA at mLV were maximally increased by −64 and 75% (P < 0.001, paired t-test) with dobutamine (n = 11) and by −63 and 61% (P < 0.001, paired t-test) with Ca2+ (n = 20). The dotted and thin lines in Fig. 2A, including all VO2-PVA data obtained during the first dobutamine and second Ca2+ in steps at the mLV (0.17 ml/g), were drawn in parallel to the control
VO2-PVA relation line. The gradually increased VO2-intercept values (PVA-independent VO2 values) for the thin or dotted lines proportional to the enhanced left ventricular contractility induced by first dobutamine and second Ca2+ were obtained by this procedure. eE\text{max} was calculated from a triangular area equivalent to PVA0.17. The linear relationship between PVA-independent VO2 and eE\text{max} during the first dobutamine infusion could be superimposed on the relation line during the second Ca2+ infusion in the same heart, indicating a similar O2 cost of left ventricular contractility and Ca2+ (Fig. 2B). This result is consistent with that for Wistar rats (1).

Mean slopes and VO2 intercepts (PVA-independent VO2 values) for the composite linear relationships between PVA-independent VO2 and eE\text{max} were obtained by this procedure. LV control linear relationships between VO2 and PVA (thin line) during Dob and Ca2+ were obtained by this procedure. Therefore, the decrease in PVA-independent VO2 per minute was not due to the decrease in basal metabolic VO2 measured during KCl-induced arrest was unaltered in L-II OLETF rats (25.2 ± 5.1 \( \mu \text{L} \text{O}_2 \cdot \text{min}^{-1} \cdot \text{g}^{-1} \); \( n = 2 \)) compared with 22.8 ± 6.6 \( \mu \text{L} \text{O}_2 \cdot \text{min}^{-1} \cdot \text{g}^{-1} \) in L-II LETO rats (\( n = 3 \)) (Fig. 3B). Furthermore, these mean values were not significantly different from that in three normal Wistar rats (28.2 ± 3.2 \( \mu \text{L} \text{O}_2 \cdot \text{min}^{-1} \cdot \text{g}^{-1} \)). Therefore, the decrease in PVA-independent VO2 per minute was not due to the decrease in basal metabolic VO2 but due to the decrease in VO2 for Ca2+ handling in excitation-contraction coupling per minute in L-II OLETF rat hearts compared with that in L-II LETO rat hearts.

The mean coronary flow values at mLVV were not significantly different among the four rat groups in control conditions. The maximum value for mean coronary flow at mLVV increased by Ca2+ infusion in each group was significantly larger (\( P < 0.05 \) by paired \( t \)-test) than that in the control condition (L-I OLETF, where there were no significant differences in PVA-independent VO2 per minute between L-I OLETF and L-I LETO rat hearts (Fig. 3A).

Table 2. Variables in left ventricular energetics

<table>
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<th>Rats</th>
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<th>Slope, ( \times 10^{-2} \mu \text{L} \text{O}_2 \cdot \text{mmHg}^{-1} \cdot \text{ml}^{-1} )</th>
<th>VO2 intercept, ( \mu \text{L} \text{O}_2 \cdot \text{beat}^{-1} \cdot \text{g}^{-1} )</th>
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<tr>
<td>L-I OLETF (40–46 wk)</td>
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<td>1.06</td>
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<td>1.34</td>
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<td>L-II OLETF (62–66 wk)</td>
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<td>L-II LETO (60–72 wk)</td>
<td>5</td>
<td>1.54</td>
<td>0.31</td>
<td>0.976</td>
</tr>
<tr>
<td>Wistar*</td>
<td>14</td>
<td>1.34</td>
<td>0.30</td>
<td>0.986</td>
</tr>
</tbody>
</table>

VO2, myocardial oxygen consumption per beat. *Our unpublished data.

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4.0 ± 0.8 vs. 3.4 ± 0.5 ml/min; L-I LETO, 4.1 ± 1.4 vs. 3.1 ± 1.4 ml/min; L-II OLETF, 3.9 ± 0.9 vs. 3.1 ± 0.6 ml/min; L-II LETO, 5.4 ± 0.5 vs. 3.9 ± 0.4 ml/min).

**Oxygen cost of eE max for dobutamine and Ca\(^{2+}\).** Figure 4A shows that the mean O\(_2\) cost of eE\(_{\text{max}}\) for Ca\(^{2+}\) showed no significant difference between L-I OLETF and L-I LETO rats or between L-II OLETF and L-II LETO rats. Figure 4B shows that the mean O\(_2\) cost for dobutamine did not show any difference between L-II OLETF and L-II LETO rats. Furthermore, O\(_2\) cost in L-II OLETF and L-II LETO rats did not differ between Ca\(^{2+}\) (Fig. 4A,a) and dobutamine (Fig. 4B,b) in Wistar rat hearts (n = 14) were similar and neither of them differed from the costs in any other group of hearts (Fig. 4A,a and B,b, our unpublished data).

**Logistic time constants for the hearts of OLETF and LETO rats.** Left ventricular representative isovolumic relaxation pressure-time curves at mLVV are shown in Fig. 5, A and B. \(T_L\) and \(T_E\) for these curves were 18.2 and 28.9 ms in the L-II OLETF rat (Fig. 5A), respectively, and 13.5 and 19.1 ms in the L-II LETO rat (Fig. 5B), respectively. Mean \(T_L\) and \(T_E\) at 240 beats/min pacing in L-II OLETF rats were significantly (\(P < 0.05\) unpaired \(t\)-test) longer than the constants in L-II LETO rats, but in L-I OLETF rats, neither mean \(T_L\) nor \(T_E\) at 300 beats/min pacing was significantly different from the corresponding value in L-I LETO rats (Fig. 5C).

**Western blotting of SERCA2.** The protein levels of SERCA2 from two hearts in each of the two groups (L-II OLETF and L-II LETO rats) are shown in Fig. 6A. Although the mean protein level of SERCA2 in L-I OLETF rat hearts was 0.82 ± 0.05 (n = 6) and did not differ significantly from other rat hearts, the mean protein level of SERCA2 in L-II OLETF rat hearts was significantly (ANOVA and Bonferroni’s test) lower than that in L-II LETO rat hearts (Fig. 6).

**Myosin isozyme.** Myosin isozyme in the left ventricular myocardium was V\(_3\) in L-II OLETF rats (Fig. 7A) and V\(_1\) in L-II LETO rats (Fig. 7B), whereas it was V\(_1\) in L-I OLETF and L-I LETO rats. In adult Wistar rats

**Fig. 4.** Mean O\(_2\) costs of LV eE\(_{\text{max}}\) for Ca\(^{2+}\) and Dob. Mean O\(_2\) costs of LV eE\(_{\text{max}}\) for Ca\(^{2+}\) (A) in L-I OLETF (n = 6) and age-matched L-I LETO (n = 3), in L-II OLETF (n = 6) and age-matched L-II LETO rat hearts (n = 5), and those for Dob (B) in L-II OLETF (n = 6) and age-matched L-II LETO rat hearts (n = 5). a and b, mean O\(_2\) cost of eE\(_{\text{max}}\) for Ca\(^{2+}\) (a) and Dob (b) in Wistar rat hearts (n = 14) (unpublished data). *Significantly different (\(P < 0.01\) ANOVA with post hoc Bonferroni’s test).
(32–40 wk), we have previously confirmed a predominance of V1 (our unpublished data).

Mitochondrial respiratory function. Figure 8 summarizes the data on the ADP-to-O ratio (ADP/O ratio, dimensionless), RCI (dimensionless), and state III O2 (ng atom·min⁻¹·mg protein⁻¹) of myocardial mitochondria from L-II OLETF (n = 6) and L-II LETO rats (n = 4). Both the ADP/O ratio and state III O2 in L-II OLETF rat hearts showed a tendency to decrease but to not differ significantly from those in L-II LETO rats, although RCI in L-II OLETF rat hearts was significantly (P < 0.05) smaller than that in L-II LETO rat hearts. State II O2 and state IV O2 in L-II OLETF rat hearts also did not differ from those in L-II LETO rats (state II O2, 141.0 ± 23.6 vs. 121.7 ± 14.8; state IV O2, 208.1 ± 4.0 vs. 221.1 ± 26.3 ng atom·min⁻¹·mg protein⁻¹).

In L-I OLETF rats (n = 6), the ADP/O ratio, RCI, and state III O2 did not differ from those in L-I LETO rat hearts (state II O2, 141.0 ± 23.6 vs. 121.7 ± 14.8; state IV O2, 208.1 ± 4.0 vs. 221.1 ± 26.3 ng atom·min⁻¹·mg protein⁻¹). In L-I OLETF rats (n = 6), the ADP/O ratio, RCI, and state III O2 did not differ from those in L-I LETO rat hearts (state II O2, 141.0 ± 23.6 vs. 121.7 ± 14.8; state IV O2, 208.1 ± 4.0 vs. 221.1 ± 26.3 ng atom·min⁻¹·mg protein⁻¹). In L-I OLETF rats (n = 6), the ADP/O ratio, RCI, and state III O2 did not differ from those in L-II OLETF rat hearts (n = 4) (ADP/O ratio, 1.45 ± 0.51 vs. 1.28 ± 0.19; RCI, 2.82 ± 0.32 vs. 2.82 ± 0.13; state III O2, 440.8 ± 146.0 vs. 471.4 ± 156.0). The myocardial mitochondrial ADP/O ratio, RCI, and state III O2 in L-II OLETF rats (see Fig. 8) did not differ from those in normal Wistar rats (n = 7; ADP/O ratio, 1.28 ± 0.22; RCI, 3.59 ± 0.36; state III O2, 425.7 ± 26.5).

DISCUSSION

L-I OLETF rats (40–46 wk) showed no slowing of the left ventricular relaxation rate and had unchanged expression of SERCA2 and V1 myosin isozyme. It seems likely that left ventricular diastolic dysfunction...
does not occur, although the left ventricular diastolic dysfunction associated with increased left ventricular collagen content has been observed in OLETF rats at a similar age (13). The discrepancy between our results and those of a previous study (13) may derive from the difference between the methods for evaluating left ventricular diastolic function (i.e., our direct measurements vs. indirect, noninvasive measurements) and the difference between preparations (excised hearts vs. in situ hearts). In in situ hearts, the interferences of preload and afterload with left ventricular functions may result in left ventricular diastolic dysfunction at the prediabetic stage (15–27 wk) as shown by Mizushige et al. (13).

In L-II (62–66 wk) OLETF rats, we found diabetic cardiomyopathy with lusitropic dysfunction and with unchanged coronary flow and coronary flow reserve, but up to 66 wk, no systolic dysfunction was observed at 240 beats/min pacing, as shown by comparison with L-II LETO rats. The left ventricular lusitropic dysfunction in L-II OLETF rats may be related to lower protein levels of SERCA2. The functional defect in SERCA2 also has been observed in streptozotocin-induced DM rats, but this defect has been reported not to be due to changes at the transcriptional or translational level (33). The discrepancy between our results and those in the previous report may be explained by the different DM type and time course (62–66 wk of age in L-II OLETF rats vs. diabetic with streptozotocin for 3–5 wk).

On the other hand, Mizushige et al. (13) suggested that the left ventricular diastolic dysfunction was associated with an increase in cardiac collagen content at the prediabetic stage (15–27 wk). Although the ratio of extracellular matrix (including collagen) to cardiac myocyte content had further increased at 31–47 wk in both OLETF and LETO rats, there was no significant difference between the two groups in this ratio (13), but in the present study, L-II OLETF rats at 62–66 wk showed a significant slowing of the left ventricular relaxation rate compared with that in L-II LETO rats. Therefore, it seems likely that left ventricular diastolic dysfunction in the L-II OLETF rats is not simply related to the increased collagen content but is related to the decreased protein level of SERCA2. The decreased protein level of SERCA2 did not result from the increased collagen content, because the cellular membrane fractions did not contain any collagen.

Fig. 7. Myosin isozyme. A: myosin isozyme in LV myocardium is V3 in a L-II OLETF rat. B: myosin isozyme is V1 in an age-matched L-II LETO rat.

Fig. 8. Myocardial mitochondrial respiratory function. ADP-to-O ratio (ADP/O) ratio (A), respiratory control index (RCI, B), and oxygen consumption at state III (C) in L-II OLETF (n = 6) and age-matched L-II LETO rat hearts (n = 4). *P < 0.05 (unpaired t-test).
Curved ESPVR, linear VO₂-PVA relationship, and oxygen cost of PVA. No significant differences were found between L-II OLETF and L-II LETO rats in ESP_mLVV or PVA_mLVV. Each mean slope of the VO₂-PVA relationship (O₂ cost of PVA) in the four rat groups is almost the same as that previously reported (3, 4, 18, 27, 28). In hyperthyroid rabbits, where myosin isozyme is V₃, the increased O₂ cost of PVA is observed (2). In hyperthyroid dogs, where myosin isozyme remains V₃, unaltered O₂ cost of PVA is observed (21). Therefore, it seems likely that there exists a correlation between O₂ cost of PVA and myosin isozyme, but the present results showing uncharged O₂ cost of PVA indicate that the efficiency of chemomechanical energy transduction (the inverse of O₂ cost of PVA) was unchanged among the four rat groups, despite the different myosin isozymes. Mitochondrial oxidative phosphorylation appeared to be unchanged between L-I OLETF and L-I LETO rat hearts and between L-II OLETF and L-II LETO rat hearts. This finding further supports the unchanged efficiency of chemomechanical energy transduction.

**PVA-independent VO₂.** All mean VO₂ intercepts of the VO₂-PVA relationship (PVA-independent VO₂) in the four rat groups were similar, and this is in agreement with our previous results (3, 4, 18, 27, 28). The PVA-independent VO₂ corresponds primarily to the VO₂ for total Ca²⁺ handling in the excitation-contraction coupling and that for basal metabolism (3, 4, 27, 30). The significantly decreased PVA-independent VO₂ per minute with unchanged basal metabolism in L-II OLETF rat hearts suggests a decrease in VO₂ for total Ca²⁺ handling in the excitation-contraction coupling per minute. VO₂ for Ca²⁺ handling in the excitation-contraction coupling is primarily consumed by SERCA2 (4, 26). Therefore, the decrease in VO₂ for Ca²⁺ handling indicates a decrease in O₂ consumption by SERCA2 per minute. This is causally related to the decreased pacing rate so as not to cause incomplete left ventricular relaxation. This decrease in the pacing rate seems to be due to dysfunction of SERCA2. In fact, in L-II OLETF rat hearts, depressed expression of SERCA2 was found.

**Oxygen cost of left ventricular eB_max.** Dobutamine is known to stimulate β-adrenergic receptors and thereby to activate protein kinase A (17), resulting in enhanced Ca²⁺ handling in the excitation-contraction coupling, whereas Ca²⁺ directly enhances the Ca²⁺ handling by increasing Ca²⁺ influx. Although the mechanism of enhancing the Ca²⁺ handling is different, the paired O₂ costs of left ventricular contractility (changes in total Ca²⁺ handling VO₂ in the excitation-contraction coupling per beat against unit change in left ventricular contractility) for Ca²⁺ and for dobutamine were similar in each L-II OLETF and L-II LETO rat heart. This result is consistent with that for Wistar rats (1). In non-insulin-dependent diabetic hearts, Schaffer et al. (19) could not find any changes in the myocardial β-receptor population but reported reduced responsiveness to a β-adrenergic agonist. Nevertheless, the O₂ cost of left ventricular contractility for dobutamine was similar for L-II OLETF and L-II LETO rats. Furthermore, the O₂ cost of left ventricular contractility for Ca²⁺ was similar for L-I OLETF and L-I LETO rats and for L-II OLETF and L-II LETO rats. All these results indicate that changes in the total Ca²⁺ handling VO₂ in the excitation-contraction coupling against unit change in left ventricular contractility induced by Ca²⁺ or dobutamine did not alter on a beat-to-beat basis in the present long-lasting, severe type 2 diabetic rat hearts. Decreasing the pacing rate from 300 to 240 beats/min made it possible to maintain unchanged systolic function and unchanged O₂ cost of left ventricular contractility in L-II OLETF rats.

**Left ventricular diastolic dysfunctions in L-II OLETF rat hearts.** In L-II OLETF rat excised hearts, at a lower pacing rate (240 beats/min) than normal (300 beats/min), a significant slowing of the left ventricular relaxation rate was observed, but systolic function was unchanged. These results indicate that the present long-lasting, severe type 2 diabetic rat hearts do not show terminal diabetic cardiomyopathy. In situ hearts, the preload and afterload and hormonal and neuronal influences could interfere with left ventricular function, resulting in left ventricular diastolic dysfunction at the prediabetic stage (15–27 wk) as shown by Mizushige et al. (19). In contrast, in the present cross-circulated (blood-perfused) excised hearts, left ventricular function can be characterized by myocardium per se. Therefore, it seems unlikely that the present long-lasting, severe diabetic rat hearts causes terminal diabetic cardiomyopathy.

In the present long-lasting, severe type 2 diabetic rats, the left ventricular myosin isozyme was transformed to V₃ from V₁. This transformation might be causally related to a significant slowing of the left ventricular relaxation rate, but in the same type of heart preparations as the present one of hypothyroid rats, where myosin isozyme was V₃, both left ventricular systolic and diastolic dysfunctions are found under the decreased pacing rate and the decreased VO₂ intercept without any change in O₂ cost of PVA (15). The decreased VO₂-intercept is due to the decreased VO₂ for Ca²⁺ handling in excitation-contraction coupling and the decreased basal metabolism (15). These findings indicate that the left ventricular mechanical work and energetic characterization quite differ between the hypothyroid and the present type 2 diabetic rats, in both of which myosin isozymes were the same V₃. Therefore, it seems unlikely that the transformation of myosin isozyme into V₃ is causally related to the left ventricular diastolic dysfunction in the present DM rats, although the possibility for its partial contribution could not be excluded.

In contrast to our hypothesis and previous report (13), we found that diastolic dysfunction did not manifest itself in prediabetic or early diabetic stage of a type 2 diabetic rat model. Although the left ventricular systolic function was not impaired and the O₂ cost of left ventricular contractility did not increase, we proved that diastolic dysfunction manifested itself due to depressed expression of SERCA2 and that the de-
increase in VO₂ for Ca²⁺ handling in excitation-contraction coupling per minute manifested itself due to decreased pacing rate in late diabetic stage of a type 2 diabetic rat model. We conclude that left ventricular lusitropic function in the present diabetic rat hearts is impaired by depressed expression of SERCA2, and that the impaired Ca²⁺ uptake function by SERCA2 has an important role on the pathogenesis of diabetic cardiomyopathy in a type 2 diabetic rat model.

This study was partly supported by Grant-in-Aid for Scientific Research 11470277 from the Ministry of Education, Science, Sports and Culture, Japan, and 2000 Health Science Research Grant for Human Genomics and Regenerative Medicine from the Ministry of Health, Labor, and Welfare, Japan.

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