Alteration in Left Ventricular Diastolic Filling and Accumulation of Myocardial Collagen at Insulin-Resistant Prediabetic Stage of a Type II Diabetic Rat Model

Katsufumi Mizushige, MD; Li Yao, MD; Takahisa Noma, MD; Hideyasu Kiyomoto, MD; Yang Yu, MD; Naohisa Hosomi, MD; Koji Ohmori, MD; Hirohide Matsuo, MD

Background—Considerable controversy exists regarding impairment of cardiac function in diabetes mellitus (DM). We investigated the serial changes in left ventricular (LV) histopathology and LV filling dynamics in Otsuka Long-Evans Tokushima Fatty (OLETF) rats, which have been established as an animal model of type II DM.

Methods and Results—In 54 OLETF and 54 non-DM rats, body weight, blood pressure, heart rate, and transmitral pulsed Doppler examinations were performed from 5 to 47 weeks of age. An oral glucose tolerance test was performed at 10, 20, and 30 weeks of age. The hearts were excised for histopathology, including immunohistochemistry and histomorphometry of collagen, and measurement of hydroxyproline at baseline and each stage of developing DM. In the prediabetic stage (15 weeks of age), in which fast blood glucose remained normal, OLETF rats manifested mild obesity, postprandial hyperglycemia, and hyperinsulinemia, and early diastolic transmitral inflow exhibited prolonged deceleration time (OLETF, 59 ± 10 ms versus non-DM, 49 ± 8 ms, P<0.01) and low peak velocity (OLETF, 73 ± 11 cm/s versus non-DM, 88 ± 11 cm/s, P<0.01). Histopathology revealed extracellular fibrosis and abundant transforming growth factor-β1 receptor II in LV myocytes of OLETF rats. At 15 weeks of age, the ratio of collagen area/visual field of LV wall in OLETF rats (8.3 ± 1.3%) was larger than that in non-DM rats (4.9 ± 1.8%, P<0.001), and the collagen content/dry tissue weight ratio of heart was significantly higher in OLETF (2.0 ± 0.5 mg/g) than non-DM (1.3 ± 0.2 mg/g, P<0.01) rats.

Conclusions—A metabolic abnormality present in the prestage of type II DM may produce LV fibrosis and alteration in cardiac function. (Circulation. 2000;101:899-907.)

Key Words: diabetes mellitus ■ diastole ■ collagen ■ echocardiography

The principal pathophysiological feature of diabetes mellitus (DM) is the morphological and functional alteration of microvessels, called diabetic microangiopathy. Conversely, the presence of myocardial dysfunction independent of coronary artery disease in DM, “diabetic cardiomyopathy,” has been well documented.7 Such focal changes in microvessels are not sufficient to account for the diffuse myocardial degeneration with interstitial fibrosis in diabetic cardiomyopathy. The causal link between microangiopathy and the pathogenesis of diabetic cardiomyopathy remains to be elucidated.1

Usually, an abnormality of relaxation is the earliest manifestation of cardiac dysfunction, and diastolic impairment presents before systolic impairment.2–4 Recently, Doppler echocardiography has become well accepted as a reliable, reproducible, and noninvasive method for diagnosis and longitudinal follow-up of subjects with diastolic dysfunction.5 Doppler flow velocity pattern represents the overall diastolic filling characteristics of the heart and can be useful in the diagnosis of diastolic dysfunction.6 However, definition of the natural history of DM heart function with Doppler echocardiography is limited, because most patients do not have a prediabetic study, and most patients receive treatments in the full-blown stage of DM or after having DM complications. Therefore, the use of an appropriate animal model may allow a better understanding of the processes that lead to diabetic cardiac dysfunction. Up to now, longitudinal follow-up animal studies looking at the progression of DM are rare. The Otsuka Long-Evans Tokushima Fatty (OLETF) rat was recently established as an animal model of congenital DM by selective mating.7 The strain manifests stable clinical and pathological features that resemble human type II DM. Briefly, the characteristics of this strain are (1) late onset of hyperglycemia, (2) a mild and chronic course of DM, (3) mild obesity, (4) inheritance by males, and (5) renal and cardiac complications.

Using this model, we aimed to investigate the serial changes in left ventricular filling dynamics and histopathol-
Immunohistochemistry (O
L)
Histopathology, heart weight, and lipid measurements (O
L)
OGTT (O=L)
Early diastolic and late atrial waveforms separated in Doppler (O/L)
Echocardiography. The transmitral flow velocity profile was deter-
mined by positioning a sample volume at the tip of the mitral valve on the para-apical long-axis view. The Doppler beam was set with <30° of the incident angle to flow direction identified on color Doppler image. The angle correction was performed by a semiauto-
mated system installed in the machine.

The peak velocity and deceleration time of the early diastolic filling wave were measured. The deceleration time was obtained by extrapolating the initial slope of early diastolic filling wave decel-
eration to the baseline. Transmitral inflow pattern was recorded on a strip chart at 100-mm/s sweep speed with simultaneous 3-lead ECG for offline analysis. All measurements represent the mean of 5 consecutive cardiac cycles, and heart rate was calculated on the basis of the strip chart of Doppler echocardiography.

Euthanasia and Histopathological Methods
Rats were anesthetized with 50 mg/kg sodium pentobarbital. The hearts were excised, weighed, and separated into 2 halves along the anterior longitudinal middle line. Half of the heart was fixed with formalin solution, and the specimens were embedded in paraffin and cut into sections 4 μm thick for Azan-Mallory staining and immu-
nostaining of transforming growth factor (TGF)-β1 receptor II (streptavidin/biotin immunoperoxidase method, VECTASTAIN Elite ABC kit, Vector Laboratories, Inc). Tissues expressing TGF-β1 receptor II appeared brown.

The other half of the heart was frozen in liquid nitrogen and stored at −80°C for measurement of hydroxyproline content. Trunk blood was sampled for measurement of plasma total cholesterol (choles-
terol oxidase-peroxidase method) and triglyceride (glycerokinase-glycerol-3-phosphatase method).

Histomorphometry in Ventricular Interstitial Collagen Percentage
The specimens from OLETF and LETO rats at each age were simultaneously stained by Azan-Mallory staining in the same bath for an accurate histopathological comparison except for technical differences between the 2 groups. Collagen deposition in myocardium of the left and right ventricular walls of each visual field was quantitatively measured on the sections stained. Each field was entered into a computer as an image with 256 gray levels on which the collagen that stained blue appeared black. We measured the collagen area at 3 sites on each slide using a threshold function of NIH software. A correct surface detection of collagen area was visually confirmed on the gray image by comparing the original color image before measurement. The area darker than threshold.
value was measured, and the percent collagen area per visual field area was automatically computed. The mean value of the 3 measurements was used for analysis.

**Myocardial Hydroxyproline and Collagen Content**
Specimens were dried and hydrolyzed in 6N HCl at 110°C for 24 hours. The hydrolyzed material was dried and reconstituted in 3 mL H2O. Hydrolysate was mixed with ethanol and chloramine T solution and oxidized for 20 minutes at room temperature. The oxidized product was reacted with D-methylaminobenzaldehyde in ethanol and H2SO4 solution at 60°C for 15 minutes, and the resulting chromophore was quantified spectrophotometrically at 572 nm against a hydroxyproline standard curve. Because hydroxyproline is incorporated only into collagen and assuming that collagen contains 14% hydroxyproline, the total collagen content per dry weight can be calculated.\textsuperscript{12–14}

**Statistical Analysis**
All values are represented as mean±SD. Statistical differences were determined by ANOVA with Statview software (Power PC version, Abacus Concepts Inc). Comparison of values was done between the age-matched OLETF and LETO rats by the Mann-Whitney \textit{U} test. Statistical comparison of serial changes was performed by 1-way ANOVA. Paired Student’s \textit{t} test was used to compare serial changes in serum glucose and insulin concentration during OGTT. A probability value of <0.05 was considered statistically significant.

**Results**

**Blood Pressure, Heart Rate, and Body and Heart Weight**
There was no constant tendency in the systolic blood pressure or heart rate despite significant but small differences at all ages. After 9 weeks of age, body weight showed a significant difference. At 17, 26, and 47 weeks, the heart weight of OLETF rats was significantly larger than that of LETO rats. No significant difference in ratio of heart weight to body weight between OLETF and LETO rats was observed (Figure 1).

**Glucose and Lipid Metabolism**
From 9 to 47 weeks of age, the fast blood glucose in OLETF rats was always higher than that in LETO rats. At only 37 weeks, the fast blood glucose of OLETF rats was >120 mg/dL (Figure 2).

On OGTT, baseline blood glucose of OLETF and LETO rats was within the normal range, although baseline blood glucose of OLETF was significantly higher than that of LETO rats. At 10 and 20 weeks of age, the 2-hour blood glucose of OLETF rats was significantly higher than that of
LETO rats and did not return to baseline level. At 30 weeks, OLETF rats could be diagnosed as impaired glucose tolerance. Plasma insulin concentrations of OLETF rats were markedly higher than those of LETO rats (Table 2). At 31 and 47 weeks, the plasma total cholesterol and triglyceride levels of OLETF rats were higher than those of LETO rats (Figure 2).

**Doppler Echocardiography**

Because it was impossible to separate the early diastolic filling wave and late filling wave of transmitral flow, we have to exclude the data of heart rate >390 bpm. Heart rate and blood pressure values were not significantly different between OLETF and LETO rats used for analysis of transmitral inflow.

The deceleration time was prolonged and peak velocity was decreased in OLETF rats compared with those in LETO rats from 11 to 27 weeks of age. At 15 weeks, the deceleration times of OLETF versus LETO rats were 59 ± 10 versus 49 ± 8 ms (P < 0.01), and the peak velocities of OLETF versus LETO rats were 73 ± 11 versus 88 ± 11 cm/s (P < 0.01), respectively (Figures 3 and 4).

**Myocardial Collagen Content**

The collagen content/dry weight ratio in OLETF rats (2.0 ± 0.5 mg/g) was significantly larger than that in LETO rats (1.3 ± 0.2 mg/g, P < 0.01) at 15 weeks of age, whereas no significant difference was observed at 5, 31, and 47 weeks (Figure 5).

**Histopathological Findings**

At 5 and 7 weeks of age, no significant histopathological differences between OLETF and LETO rats were observed. Figure 6 demonstrates the histopathological sections by the Azan-Mallory staining and threshold images at 5 and 15 weeks of age. Interstitial fibrosis was marked after 15 weeks of age, and fibroblasts were more abundant at the ages of 31 and 47 weeks in the right and left ventricular walls of OLETF rats. Collagen accumulation and no atherosclerotic changes were observed.

The left ventricular collagen area per visual field (%) gradually increased in OLETF and did not change significantly in LETO rats throughout the protocol. At 5 weeks, the values were not significantly different between OLETF (2.9 ± 0.8) and LETO (4.3 ± 1.4) rats. At 15 weeks, the value of OLETF (8.3 ± 1.3) was larger than that of LETO (4.9 ± 1.8, P < 0.0001) rats (Figure 7). Right ventricular collagen area per visual field (%) showed a tendency similar to that in the left ventricle (5 weeks: OLETF, 4.8 ± 1.2 versus LETO, 7.1 ± 1.8, P < 0.001; 15 weeks: OLETF, 14.8 ± 1.8 versus LETO, 7.7 ± 2.3, P = 0.0001).

The immunohistochemical stain of the left ventricle showed much more TGF-β1 receptor II expression in the myocytes and endothelium of small arteries of OLETF rats than did that in LETO rats at 15 weeks of age (Figure 8).

**Table 2. Blood Glucose and Blood Insulin Concentration on OGTT**

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<thead>
<tr>
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<th>10 Weeks</th>
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<th>20 Weeks</th>
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<td></td>
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<td>2 Hours</td>
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<td>2 Hours</td>
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<td>Glucose, mg/dL</td>
<td>115±7*†</td>
<td>168±30†</td>
<td>103±6</td>
<td>197±45*</td>
<td>112±12*</td>
<td>294±41*</td>
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<tr>
<td>Insulin, pg/mL</td>
<td>784±392†</td>
<td>824±337†</td>
<td>3233±493†</td>
<td>2400±1929</td>
<td>4667±971</td>
<td>7967±1436*</td>
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<tr>
<td>Glucose, mg/dL</td>
<td>87±5</td>
<td>118±13</td>
<td>103±11</td>
<td>111±12</td>
<td>90±5</td>
<td>165±33</td>
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<tr>
<td>Insulin, pg/mL</td>
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<td>156±145</td>
<td>1080±383</td>
<td>883±176</td>
<td>2933±1858</td>
<td>2700±1609</td>
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</table>

*P < 0.05 vs LETO; †P < 0.01 vs LETO.
Discussion

In patients with DM, Doppler echocardiographic studies have demonstrated that not only diabetes but also a minor degree of hyperglycemia will influence cardiac function. In these studies, the authors mentioned the possibility that other metabolic abnormalities associated with hyperglycemia might play a role in cardiac dysfunction. To the best of our knowledge, this is the first study that provides serial data on left ventricular diastolic function from the prediabetic stage in an animal model of developing DM along with data on glucose and lipid metabolism, ventricular collagen content, and cardiac histopathology.

Stages of DM in OLETF Rats

The fast blood glucose of OLETF rats was in the normal range and was slightly but significantly higher than that in LETO rats after 9 weeks of age. On the basis of OGTT data, from 10 to 20 weeks of age, the OLETF rats showed minor abnormalities in 2-hour serum glucose (postprandial hyperglycemia) and relatively higher plasma insulin levels both at

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**Figure 3.** Alteration in transmitral inflow pattern of OLETF and LETO rats at 15 and 31 weeks of age. Flow pattern was recorded under color Doppler guidance. Prolongation of deceleration time and decrease in peak velocity of early transmitral inflow were observed at 15 weeks in OLETF rats. LV indicates left ventricle; LA, left atrium.

**Figure 4.** Deceleration time and peak velocity of early transmitral inflow during protocol in OLETF and LETO rats. Prolongation of deceleration time and decrease in peak velocity of early transmitral inflow were observed in prediabetic stage in OLETF rats. All data are expressed as mean ± SD. *P < 0.05 vs LETO; **P < 0.01 vs LETO.

**Figure 5.** Left ventricular collagen content/dry weight at 5, 15, 31, and 47 weeks of age in OLETF and LETO rats. An increase in left ventricular collagen content/dry weight was observed at 15 weeks in OLETF rats. All data are expressed as mean ± SD. *P < 0.01 vs LETO.
Figure 6. Azan-Mallory staining and its threshold image of OLETF and LETO rats at 5 and 15 weeks of age. Interstitial collagen area of left ventricular wall showing as black on threshold image of OLETF rats (top) was significantly larger than that of LETO rats (bottom) at 15 weeks of age.
baseline and 2 hours after loading compared with age-matched LETO rats. Two-hour serum glucose of OLETF rats at 30 weeks of age was 200 mg/dL. This demonstrates that the OLETF strain of rats was in the stage of impaired glucose tolerance with hyperglycemia (by World Health Organization diagnostic criteria) at 30 weeks of age. After 9 weeks of age, OLETF rats showed mild obesity, which was sustained up to 47 weeks of age. Both the plasma cholesterol and triglyceride levels of OLETF rats were significantly higher than those of LETO rats at 31 and 47 weeks of age, which suggested that from 30 to 47 weeks of age, metabolic disorders were evident in OLETF rats. According to previous reports and the present data, our subjects were in the stage of prediabetes and insulin resistance at 10 to 20 weeks, impaired glucose tolerance at 30 weeks, and type II DM at 37 weeks.

Remodeling of Left Ventricle

Our results of a decrease in the ratio of collagen content/dry weight of left ventricle from 5 to 15 weeks seemed to contradict previous studies in myocardial fibrosis, which showed the ratio to increase along with disease progression. We speculate that from 5 to 15 weeks of age, the rats are undergoing developmental transition. During this stage, both extracellular matrix (including collagen) and cardiac myocyte content are increased, so it is reasonable that the ratio decreased from 5 to 15 weeks of age. At 31 and 47 weeks, the ratio increased in both groups compared with that at 15 weeks, and there is no significant difference between the 2 groups. Slightly augmented collagen deposition in the myocardial interstitium and perivascular area during the maturational period of life was reported previously in rat heart. Cardiac fibrosis and collagen deposition may be related to premature aging and the processes involved in cardiac extracellular remodeling in OLETF rats. Cardiac fibrosis might play a role in progressive deterioration of cardiac hemodynamics in aging and an explanation for the diastolic dysfunction.

Transmitral Inflow Change

Usually, an abnormality of relaxation is the earliest manifestation of a disease process. A primary abnormality of relaxation produces specific changes in the mitral flow velocity curve, consisting of a low velocity of early filling wave, a high velocity of late filling wave, and a prolonged deceleration time of early filling wave. In the present study, the deceleration time of OLETF rats was prolonged, and peak velocity of early filling wave was decreased from 11 to 27 weeks of age, which represents an early manifestation of abnormality of left ventricular diastolic function. During this stage, OLETF rats showed postprandial hyperglycemia and hyperinsulinemia, although the average fasting serum glucose was normal. This impairment was evident at a preclinical stage of DM (prediabetes or impaired glucose tolerance) and was accompanied by serological or pathological findings.
was likely that alteration in the diastolic function in the pre-diabetic stage was related to the remodeling of myocardial tissue structure.21–23

The normal mitral flow velocity curve varies with age, loading conditions, and heart rate.5 In our study, therefore, we age-matched our control rats, measured the blood pressure immediately after finishing Doppler echocardiography, and subclassified the OLETF rats and LETO rats according to their heart rate.

The pattern of left ventricular filling that we have documented in OLETF rats with DM may be a result of abnormal left ventricular relaxation or decreased left ventricular compliance. Our research subjects are in the prestage of type II DM, and left ventricular compliance impairment is usually evident in the late stages of diastolic dysfunction. It is clear that at this stage, OLETF rats are free of premature atherosclerosis (histological and serological findings) and hypertension as alternative explanations, although these factors are plausible explanations for some adult rats with DM and diastolic dysfunction.

**Mechanisms of Alteration in Left Ventricular Diastolic Function in Prediabetes**

The TGF-β family has been identified in mammals, where it modulates cell growth and differentiation as well as extracellular matrix deposition and degradation. In this family, TGF-β1 is shown to play a critical role in organ morphogenesis, development, growth regulation, cellular differentiation, gene expression, and tissue remodeling. In rat heart, this cytokine is involved in fibrous tissue formation and may upregulate collagen expression during tissue repair. TGF-βs mediate their activity by high-affinity binding to the type II receptor with a cytoplasmic serine-threonine kinase domain.8–10 On the basis of this concept, we stained the TGF-β1 receptor II of myocardium.

In the present study, TGF-β, receptor II increased significantly in the left ventricle of OLETF rats, and the ratio of collagen content/dry weight of left ventricle was significantly higher in OLETF rats than in LETO rats at 15 weeks of age. This suggested that the gene expressions of this cytokine demonstrated by the previous report18 might be induced by metabolic abnormalities (chronic postprandial hyperglycemia, hyperinsulinemia, insulin resistance) and participate in the onset of cardiac fibrosis by stimulating extracellular matrix synthesis.

On the basis of our results, the stage from 10 to 30 weeks seems to be the critical stage of DM formation, because postprandial hyperglycemia and hyperinsulinemia coexist, which reflects the existence of insulin resistance. The pathogenesis is the downregulation of the expression of insulin receptors on adipocytes and skeletal muscle cell surfaces and resultant diminished insulin binding. Although additional work is required for clarification of the influence of insulin resistance on left ventricular diastolic function, our study demonstrated that the alteration in left ventricular diastolic function was associated with the increase in cardiac collagen content at the pre-diabetic stage.

**Limitations and Clinical Implications**

The effects of anesthesia on transmitral inflow pattern could not be completely neglected if it was possible that the 2 groups of rats responded differentially to anesthesia. Therefore, we used only as much anesthesia as necessary to keep the body position of the rats stable and tried to eliminate an excessive increase in heart rate due to excitation. In addition to the possibility of this excessive heart rate being a result of inadequate sedation, the elimination of Doppler data for animals with heart rates >390 bpm could have selected out a portion of the animals who were capable of that heart rate response, ie, a greater number of control group LETO animals were rejected because of the heart rate response at 5 weeks and at 15 weeks. In the mildly anesthetized condition, the number of rats with an adequate heart rate separating early and late filling waves of transmitral inflow velocity curve was great enough for statistical analysis.

In this study, young rats (5 weeks of age) were used. The serial data of body weight, blood pressure, serum glucose, triglyceride, cholesterol, left ventricular inflow pattern, and histopathology were obtained from 5 weeks until 47 weeks of age. This study implies an optimal timing of interventional measures. Individuals who have a genetic background of DM, even though they are young and asymptomatic, should be included in interventional procedures to prevent the development of type II DM and its complications.

**Conclusions**

In the prestage of type II DM, the OLETF rats showed alteration in left ventricular diastolic function. The alteration includes a prolongation in deceleration time and a decrease in amplitude of peak velocity of the early diastolic filling wave without obvious differences in blood pressure and heart rate in OLETF rats compared with LETO rats. This change can be tracked longitudinally with transthoracic Doppler echocardiography and is accompanied by serological and pathological changes of the heart. These changes may be a result of metabolic abnormalities or a simultaneous alteration parallel to the metabolic abnormalities in prediabetes. The OLETF rat seems to be a useful model to study the mechanism of alteration in cardiac function in type II DM.

**References**

6. Harrell DG, Nishimura RA, Ilstrup DM, Appleton CP. Utility of preload alteration in assessment of left ventricular filling pressure by Doppler


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