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Effects of Pravastatin on Progression of Glucose Intolerance and Cardiovascular Remodeling in a Type II Diabetes Model

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OBJECTIVES We examined the effects of early treatment with a 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitor pravastatin on the progression of glucose intolerance and cardiovascular remodeling in a model of spontaneously developing type II diabetes mellitus (DM), the Otsuka Long-Evans Tokushima Fatty (OLETF) rats.

BACKGROUND Clinical trials showed that pravastatin prevented new-onset DM in hypercholesterolemic patients, and that it was effective in prevention of cardiovascular events in diabetics.

METHODS The OLETF rats were treated with pravastatin (100 mg/kg/day) from 5 weeks of age and compared with age-matched untreated OLETF rats and normal Long-Evans Tokushima Otsuka (LETO) rats on serial oral glucose tolerance tests (OGTT) and Doppler echocardiography and on histopathological/biochemical analyses of the heart at 30 weeks.

RESULTS The OGTT revealed that 40% and 89% of untreated OLETF rats were diabetic at 20 and 30 weeks, respectively, but 0% and only 30%, respectively, were diabetic in the treated OLETF. Left ventricular diastolic function was found impaired from 20 weeks in untreated OLETF but remained normal in the treated-OLETF. The wall-to-lumen ratio and perivascular fibrosis of coronary arteries were increased in untreated-OLETF but were limited in the treated-OLETF at 30 weeks. Moreover, cardiac expressions of a fibrogenic growth factor, transforming growth factor-β1 (TGF-β1), and a proinflammatory chemokine, monocyte chemoattractant protein-1 (MCP-1), were increased in untreated-OLETF. However, in the treated-OLETF, overexpressions of TGF-β1 and MCP-1 were attenuated, which was associated with overexpression of endothelial nitric oxide synthase (eNOS) (2.5-fold of control LETO).

CONCLUSIONS Early pravastatin treatment prevented cardiovascular remodeling in the spontaneous DM model by retarding the progression of glucose intolerance, overexpressing cardiac eNOS, and inhibiting overexpressions of fibrogenic/proinflammatory cytokines. (J Am Coll Cardiol 2004;44:904–13) © 2004 by the American College of Cardiology Foundation

Abnormal glucose tolerance is a major risk factor for cardiovascular diseases (1). Metabolic abnormalities including hyperglycemia (2), impaired nitric oxide (NO)-mediated pathways (3), oxidative stress (4), and advanced glycation end products (5,6) have been proposed as the pathogenesis of arterial remodeling in diabetes mellitus (DM). Diabetic cardiomyopathy, which manifests left ventricular (LV) diastolic dysfunction (7–9) was also attributed to impaired NO pathway associated with hyperglycemia (7). Then, several antidiabetic or antioxidative agents have been shown to prevent cardiovascular remodeling including myocardial fibrosis (10,11), LV diastolic dysfunction (9), or arteriosclerosis (4,9) during the progression of glucose intolerance.

The 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors have been shown to exert antioxidative and anti-inflammatory actions and to enhance endothelial NO synthesis (12), which may underlie the usefulness of statins in the secondary prevention of cardiovascular diseases in diabetic patients with (13) and without hypercholesterolemia (14) in large clinical trials. Moreover, pravastatin given before the onset of DM significantly reduced new-onset DM in patients with hypercholesterolemia (15). Thus, it is hypothesized that early treatment with pravastatin may retard the development of DM and prevent subsequent cardiovascular remodeling in patients who are prone to develop DM.

The Otsuka Long-Evans Tokushima Fatty (OLETF) rat is an established genetic model of human type II DM (16). We recently characterized interstitial fibrosis in the myocardium (8) and structural alterations of coronary microvasculature (17) in the early stage of DM in this model, which correlated with diastolic dysfunction (8) and reduced coronary flow reserve (17).

In the present study, we sought to clarify whether early treatment with pravastatin can retard the development of DM in this spontaneous DM model and whether such effects are indeed associated with limitation of coronary arteriosclerosis and LV diastolic dysfunction. We examined the effects of pravastatin on the metabolic abnormalities causing insulin resistance and serially assessed the LV diastolic function by Doppler echocardiography. In addition

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Abbreviations and Acronyms

DM = diabetes mellitus
DT = deceleration time of early diastolic left ventricular inflow
E/A = early-to-late diastolic left ventricular inflow velocity ratio
eNOS = endothelial nitric oxide synthase
GAPDH = glyceraldehyde 3-phosphate dehydrogenase
LETO = Long-Evans Tokushima Otsuka
LV = left ventricular/ventricle
MCP = monocyte chemoattractant protein
mRNA = messenger ribonucleic acid
NO = nitric oxide
OGTT = oral glucose tolerance test
OLETF = Otsuka Long-Evans Tokushima Fatty
TGF = transforming growth factor
TNF = tumor necrosis factor
RT-PCR = reverse transcription-polymerase chain reaction

to the histomorphometry to evaluate coronary arterial remodeling, we especially investigated cardiac tissue endothelial-NO-synthesis and expressions of factors causing cardiovascular remodeling, monocyte chemoattractant protein (MCP)-1, which is a proinflammatory cytokine (5,12), and transforming growth factor (TGF)-β1, which promotes tissue fibrosis (11,18).

METHODS

Animals and experimental protocol. The protocol of this study was in accordance with the institutional guidelines for animal research. The OLETF rats were used as a model of type II DM, whereas age-matched Long-Evans Tokushima Otsuka (LETO) rats, which were developed from the same colony but do not develop DM, were used as a normal control (4,8–11,16,17). The control LETO group and the untreated-OLETF group received normal drinking water; and the treated-OLETF group received pravastatin sodium (Sankyo Co. Ltd., Tokyo, Japan) in drinking water at a dose of 100 mg/kg/day, which is known to yield a plasma level of pravastatin in the clinical range (12). Moreover, to determine whether the oral administration of this dose of pravastatin directly affects food intake and body weight through possible gastrointestinal disturbance, a group of LETO rats were treated with the same dose of pravastatin, which formed the treated-LETO group. All animals were maintained at the Kagawa University animal experiment center from 5 weeks to 30 weeks of age, and housed by twos in a specific pathogen-free facility under controlled temperature (23°C) and humidity (55%) with a 12-h light and dark cycle. Animals were given free access to standard laboratory rat chow (MF, Oriental Yeast Corp., Tokyo, Japan) while body weight and amount of food intake were recorded.

At 10, 20, and 30 weeks of age, in untreated-LETO, untreated-OLETF, and treated-OLETF groups, blood pressure and heart rate were measured awake. After anesthesia with intraperitoneal sodium pentobarbital (50 mg/kg), transthoracic Doppler echocardiography was performed with a SONOS5500 equipped with an s12 (5–12 MHz) phased-array transducer (Philips Medical Systems, Andover, Massachusetts). The LV systolic function was assessed with a percent fractional shortening of LV diameter, and LV diastolic function was assessed with an early-to-late diastolic peak left ventricular inflow velocity ratio (E/A) (7,8) and a deceleration time of early diastolic left ventricular inflow (DT) (8).

At the same weeks of ages, following anesthesia with ether, blood was obtained from the right carotid vein for blood chemistry, and oral glucose tolerance tests (OGTT) were performed by oral administration of glucose solution (2 g/kg body weight), in which serum glucose concentrations before and 120 min after the glucose loading were measured (8).

At 30 weeks, all rats were anesthetized by intraperitoneal sodium pentobarbital (50 mg/kg) and killed, and the hearts were excised for morphometric, immunohistochemical, or biochemical analyses.

Biochemical analyses. Glucose levels were measured immediately after sampling with a glucose test meter (Glutest EII, Kyoto First Scientific, Kyoto, Japan). Total cholesterol and triglyceride levels were determined with commercially available kits (Wako Pure Chemical Industries, Osaka, Japan). Serum leptin, insulin, and tumor necrosis factor (TNF)-α levels were measured with commercial ELISA kits (Morinaga, Kanagawa, Japan; Shibayagi, Gunma, Japan; Biosource International, Camarillo, California, respectively).

Histopathology and immunohistochemistry. The LV was isolated and cut into three pieces perpendicular to the long axis. One part of the heart was fixed with formalin, embedded in paraffin, and cut into sections 4-μm thick for Azan-Mallory staining. Another part of the heart was embedded immediately in OCT compound, frozen, cut into 8-μm-thick slices for immunostaining of TGF-β1, MCP-1, and endothelial nitric oxide synthase (eNOS). The other part of the heart was frozen in liquid nitrogen and stored at −80°C for the extraction of total ribonucleic acid (RNA) of TGF-β1, MCP-1, and for Western blot analysis of eNOS protein.

Immunohistochemical studies were done with commercially available monoclonal antibodies: anti-rat TGF-β1 and anti-rat MCP-1 antibodies (both Santa Cruz Biotechnology, Santa Cruz, California), and anti-rat eNOS antibody (Transduction Laboratories, Lexington, Kentucky).

Evaluation of the medial thickness and perivascular fibrosis of coronary arteries at 30 weeks of age was performed employing Azan-Mallory staining as previously described (17).

Quantification of messenger RNA. Quantification of messenger ribonucleic acid (mRNA) expressions of TGF-β1 and MCP-1 was performed with reverse transcription polymerase chain reaction (RT-PCR) method.

Total RNA was extracted from tissue by the guanidinium thiocyanate method using an Ultraspec RNA isolation system (Biotecx Laboratories, Houston, Texas). The RNA concentration was measured by a spectrophotometer at 260 nm. An 8-μl aliquot (8 μg) of total RNA was used to synthesize cDNA using the First-Strand cDNA Synthesis Kit (Amersham Biosciences, Piscataway, New Jersey). The RT mixture (1 μl) was amplified by the hot-starting PCR in a 50-μl reaction using Taq DNA polymerase (Amersham Biosciences) for TGF-β1, MCP-1, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as control, respectively. The sequences of the primers were as follows: TGF-β1 forward, 5′-CTGCTGGCAATAGCTTC-CTA-3′; TGF-β1 reverse, 5′-CGAGCCTTAGTTTG-GACAGGAT-3′; MCP-1 forward, 5′-ATGCAG-GTCTCTGTCACG-3′; MCP-1 reverse, 5′-CTAGTTCTCTGTCATACT-3′; GAPDH forward, 5′-TGAACGGGAAGCTCACTGG-3′; GAPDH reverse, 5′-TCCACCACCCTGTTGCTGTA-3′. The PCR conditions were as follows: 37 cycles at 94°C for 30 s, at 50°C for 30 s, and 72°C for 1 min for MCP-1; 35 cycles at 94°C for 1 min, at 62°C for 1 min, and 72°C for 1 min for TGF-β1 and GAPDH. The PCR products were electrophoresed on a 1.5% agarose gel, visualized by ethidium bromide under ultraviolet light, and photographed. The intensity of each band was analyzed by the National Institute of Health (NIH) Image-analysis system and normalized by the GAPDH band density.

Western blot analysis. The frozen tissue was homogenized at 4°C in a lysis buffer containing a protease inhibitor and centrifuged at 12,000 rpm for 10 min at 4°C. The protein concentration of supernatants was determined using a BCA Protein Assay Kit (Pierce Chemical, Rockford, Illinois). The cytosolic protein (20 μg) was separated by electrophoresis on an 8% sodium dodecyl sulfate–polyacrylamide gel. Proteins were transferred to an Immobilon PVDF membrane (Millipore, Bedford, Massachusetts). The membrane was incubated overnight at 4°C in blocking buffer (Tris-buffered saline with 5% nonfat dried milk, 2% BSA, and 0.1% Tween 20). The blot was incubated with primary monoclonal antibody (1:1,000 dilution) to eNOS for 90 min, and then incubated with goat anti-mouse horseradish peroxidase-conjugated secondary antibody (1:2,000 dilution) for 60 min at room temperature. The immunoreactive bands were detected by enhanced chemiluminescence (NEN Life Science Products, Boston, Massachusetts) and quantified by the NIH Image. The intensity of the band was presented as a percentage of the mean values of the LETO rats.

Statistical analysis. Data are expressed as mean ± SD. We used UNISTAT 5.5 in statistical analyses. Differences in measured values among the multiple groups were analyzed by analysis of variance with Bonferroni's multiple comparison test and those in incidences of OGTT abnormalities were assessed by contingency table analysis with the chi-square test. A level of $p < 0.05$ was considered statistically significant.

Table 1. Comparison of Body Weight and Food Intake

	Body Weight (g)	Food Intake (g/day)
10 weeks		
LETO rat	276 ± 18	16.6 ± 1.0
Treated-LETO rat	289 ± 12	16.6 ± 0.8
OLETF rat	317 ± 14*	20.3 ± 1.4*
Treated-OLETF rat	300 ± 11*†	18.9 ± 0.1*
20 weeks		
LETO rat	431 ± 24	19.3 ± 2.0
Treated-LETO rat	436 ± 22	18.6 ± 0.6
OLETF rat	509 ± 18*	26.1 ± 1.6*
Treated-OLETF rat	487 ± 19*†	24.0 ± 1.7*
30 weeks		
LETO rat	514 ± 20	21.8 ± 0.8
Treated-LETO rat	520 ± 20	22.1 ± 0.5
OLETF rat	607 ± 17*	25.5 ± 0.3*
Treated-OLETF rat	578 ± 19*†	24.4 ± 0.5*†

Data are expressed as mean ± SD; n = 12–16 for each group. *$p < 0.05$ vs. LETO rats. †$p < 0.05$ vs. OLETF rats.
LETO = Long-Evans Tokushima Otsuka; OLETF = Otsuka Long-Evans Tokushima Fatty.

RESULTS

Body weight, hemodynamic variables, and blood chemistry. As shown in Table 1, the body weight and food intake were greater in both OLETF groups than in both age-matched LETO groups from 10 weeks of age, whereas they were less in the pravastatin-treated-OLETF group than in the untreated-OLETF group through the observation. However, no difference was seen in the food consumption or in body weight between the untreated-LETO rats and treated-LETO rats.

Table 2 summarizes the results of blood chemistry. The serum triglyceride in both OLETF groups was significantly higher than the LETO rats from 20 to 30 weeks of age regardless of pravastatin treatment. Although no significant difference was observed in the serum total cholesterol level between the untreated-OLETF and the LETO rats at any age, pravastatin tended to lower the total cholesterol levels in OLETF rats. Whereas insulin levels were elevated in both OLETF groups as compared with the LETO group at 10 weeks and thereafter, the elevation was less in the treated-OLETF group through the observation. Although the TNF-α level tended to be higher in the OLETF groups than in the LETO rats, the elevation reached statistical significance only in the untreated-OLETF rats at 30 weeks. The serum leptin concentrations were significantly increased from 10 weeks of age in both OLETF groups compared with the LETO rats. However, the leptin increase was significantly attenuated by treatment with pravastatin at all ages.

Effects of pravastatin on glucose metabolism. As summarized in Table 3, as compared with the LETO group, both OLETF groups showed higher baseline and 2-h glucose levels on the OGTT at 10 weeks of age and

Table 2. Comparison of Blood Chemistry

	T-Chol (mg/dl)	TG (mg/dl)	Insulin (ng/ml)	TNF-α (pg/ml)	Leptin (ng/dl)
10 weeks					
LETO rat	91 ± 12	33 ± 8	0.12 ± 0.06	10.6 ± 7.6	1.82 ± 0.70
OLETF rat	95 ± 7	53 ± 15*	0.37 ± 0.14*	15.8 ± 8.9	6.33 ± 1.59*
Treated-OLETF rat	83 ± 9†	43 ± 10	0.27 ± 0.09*†	14.9 ± 8.1	3.26 ± 0.91*†
20 weeks					
LETO rat	92 ± 8	31 ± 15	0.14 ± 0.06	13.1 ± 9.4	2.42 ± 0.59
OLETF rat	89 ± 9	100 ± 16*	0.61 ± 0.18*	18.3 ± 9.7	7.10 ± 1.85*
Treated-OLETF rat	80 ± 8*†	98 ± 28*	0.31 ± 0.08*†	18.0 ± 7.1	5.09 ± 1.50*†
30 weeks					
LETO rat	77 ± 5	16 ± 5	2.55 ± 0.84	14.6 ± 11.6	4.97 ± 1.53
OLETF rat	83 ± 8	89 ± 39*	4.29 ± 0.68*	26.9 ± 11.3*	14.24 ± 2.65*
Treated-OLETF rat	78 ± 8	86 ± 28*	3.61 ± 0.80*†	23.4 ± 10.9	11.7 ± 2.87*†

Data are expressed as mean ± SD; n = 12–16 for each group. *$p < 0.05$ vs. LETO rats. †$p < 0.05$ vs. OLETF rats.
T-Chol = total cholesterol; TG = triglyceride; TNF = tumor necrosis factor; other abbreviations as in Table 1.

thereafter. However, the extent of elevation, especially in 2-h glucose, was lower in the treated-OLETF than in the untreated group. Defining DM based on the American Diabetic Association threshold (19) as fasting glucose ≥126 mg/dl and/or 2-h glucose ≥200 mg/dl, none of the LETO rats were diabetic until 30 weeks. Forty percent of rats in the untreated-OLETF group but none in the treated group were diabetic at 20 weeks ($p < 0.01$), and 89% and 30% of rats were diabetic at 30 weeks in the respective groups ($p < 0.001$). Defining normal glucose tolerance as fasting glucose <110 mg/dl and 2-h glucose <140 mg/dl (19), only 11% of untreated-OLETF rats remained normal, while 64% of treated-OLETF animals remained normal at 10 weeks ($p < 0.001$). At 20 weeks, none of the untreated-OLETF rats remained normal, whereas 55% of the treated-OLETF rats still showed a normal response ($p < 0.01$), although none of the OLETF rats were normal at 30 weeks. Thus, pravastatin improved glucose metabolism and prevented the development of DM in OLETF rats that exhibit insulin resistance at 10 weeks and definite DM at 30 weeks of age if untreated.

Effects of pravastatin on hemodynamics and LV function. As shown in Table 4, no significant differences existed in either mean blood pressure or heart rate among the three groups at any age. Figure 1 displays representative recordings of LV inflow patterns at 30 weeks. The untreated-OLETF rats (middle) exhibited a reduced E/A ratio and increased DT compared with LETO rats (Fig. 1, left), which were restored in the treated-OLETF group (Fig. 1, right). The diastolic dysfunction was noted in the OLETF rats from 20 weeks as a prolonged DT and at 30 weeks as a reduced E/A, both of which were restored by pravastatin treatment. There were no significant differences in percent fractional shortening of LV diameter among the three groups (Table 4).

Effects of pravastatin on remodeling of small coronary artery and perivascular fibrosis. Figure 2A compares representative sections of small coronary arteries from the three groups at 30 weeks of age. The coronary arterial wall was thickened in an untreated-OLETF rat (Fig. 2A, middle), which was accompanied by marked perivascular fibrosis as compared with an age-matched LETO rat (Fig. 2A, left). However, such structural remodeling was limited in a

Table 3. Oral Glucose Tolerance Test

		Glucose (mg/dl)		Incidences			
	n	Baseline	2 h	DM	IGT	Normal	p Value
10 weeks							
LETO rat	9	88 ± 8	88 ± 11	0 (0)	0 (0)	9 (100)	
OLETF rat	9	103 ± 5*	147 ± 6*	0 (0)	8 (89)	1 (11)	<0.005
Treated-OLETF rat	11	104 ± 14*	114 ± 13*†	1 (9)	3 (27)	7 (64)	
20 weeks							
LETO rat	9	106 ± 11	104 ± 8	0 (0)	4 (44)	5 (56)	
OLETF rat	10	121 ± 12*	158 ± 10*	4 (40)	6 (60)	0 (0)	<0.01
Treated-OLETF rat	11	103 ± 12†	122 ± 15*†	0 (0)	5 (45)	6 (55)	
30 weeks							
LETO rat	9	98 ± 9	115 ± 11	0 (0)	1 (11)	8 (89)	
OLETF rat	9	129 ± 7*	211 ± 26*	8 (89)	1 (11)	0 (0)	<0.005
Treated-OLETF rat	10	117 ± 14*	176 ± 29*†	3 (30)	7 (70)	0 (0)	

Glucose concentrations are expressed as mean ± SD, whereas incidences of diabetes mellitus (DM), impaired (IGT), and normal glucose tolerance are presented as n (% of rats in each group) with p values by chi-square test. *$p < 0.05$ vs. LETO rats. †$p < 0.05$ vs. OLETF rats.
Abbreviations as in Table 1.

Table 4. Comparison of Hemodynamic and Echocardiographic Parameters

	mBP (mm Hg)	Heart Rate (beats/min)	E (cm/s)	A (cm/s)	E/A	DT (ms)	%FS (%)
10 weeks							
LETO rat	124 ± 9	406 ± 38	103 ± 11	44 ± 5	2.4 ± 0.4	36 ± 3	38 ± 1
OLETF rat	127 ± 8	389 ± 33	105 ± 12	46 ± 4	2.3 ± 0.4	39 ± 3	38 ± 3
Treated-OLETF rat	126 ± 6	404 ± 21	102 ± 9	44 ± 4	2.3 ± 0.3	37 ± 2	39 ± 2
20 weeks							
LETO rat	120 ± 6	453 ± 54	105 ± 8	41 ± 5	2.6 ± 0.3	37 ± 4	42 ± 2
OLETF rat	128 ± 9	433 ± 56	100 ± 9	44 ± 4	2.3 ± 0.2	46 ± 5*	40 ± 3
Treated-OLETF rat	126 ± 8	421 ± 33	104 ± 7	42 ± 5	2.5 ± 0.3	38 ± 5†	41 ± 5
30 weeks							
LETO rat	122 ± 13	436 ± 32	106 ± 7	42 ± 4	2.5 ± 0.3	39 ± 3	40 ± 6
OLETF rat	130 ± 11	420 ± 31	96 ± 13	45 ± 4	2.1 ± 0.3*	48 ± 7*	41 ± 4
Treated-OLETF rat	126 ± 10	423 ± 26	100 ± 6	41 ± 5	2.5 ± 0.4	39 ± 4†	41 ± 6

Data are expressed as mean ± SD; n = 9–11 for each group. *$p < 0.05$ vs. LETO rats. †$p < 0.05$ vs. untreated-OLETF rats.
mBP = mean blood pressure; E = early diastolic peak velocity of LV inflow; A = peak velocity of LV inflow due to atrial contraction; DT = deceleration time of early diastolic LV inflow; %FS = % fractional shortening of LV diameter; other abbreviations as in Table 1.

treated-OLETF rat (Fig. 2A, right). Thus, both the wall-to-lumen ratio and the degree of perivascular fibrosis in small coronary arteries were significantly greater in the untreated-OLETF group than in the LETO rats, but the increases in these histomorphometric parameters were significantly inhibited by pravastatin (Fig. 2B).

Effects of pravastatin on TGF-β1 and MCP-1 gene expression. As shown in Figure 3, both TGF-β1 and MCP-1 mRNA expressions were significantly increased in the untreated-OLETF rats at 30 weeks of age, but they were suppressed to the levels similar to those in the age-matched LETO rats with pravastatin.

Immunohistochemical analyses at 30 weeks (Fig. 4) revealed much greater immunoreactivities of TGF-β1 (Fig. 4, top) and MCP-1 (Fig. 4, bottom) in small coronary arteries in untreated-OLETF rats (Fig. 4, middle) than those in LETO rats (Fig. 4, left), which were limited by pravastatin (Fig. 4, right).

Effects of pravastatin on endothelial NO synthase expression. Figure 5 compares the eNOS protein expression in the LV tissue examined by Western blotting at 30 weeks of age. Although the eNOS protein expression was similar between the untreated-OLETF and the control LETO rats, it was 2.5 times higher in the pravastatin-treated OLETF rats (Fig. 5B). As demonstrated in Figure 6, eNOS immunoreactivity at 30 weeks was localized in the endothelial layer of coronary arteries, and the magnitude was similar between the LETO (Fig. 6, left) and untreated-OLETF rats (Fig. 6, middle). Notably, pravastatin markedly increased the eNOS immunoreactivity above that in the LETO rats (Fig. 6, right).

DISCUSSION

The major findings of the present study were 1) early treatment with pravastatin limited the progressive elevation of plasma leptin and TNF-α levels and retarded the development of DM in a rat model of spontaneously developing type II DM, the OLETF rats; 2) the increased cardiac expressions of TGF-β1 and MCP-1 were associated with the coronary arterial remodeling and diastolic dysfunction; and 3) pravastatin markedly enhanced eNOS expression in the coronary endothelium and inhibited the cardiac overexpressions of TGF-β1 and MCP-1, which were asso-

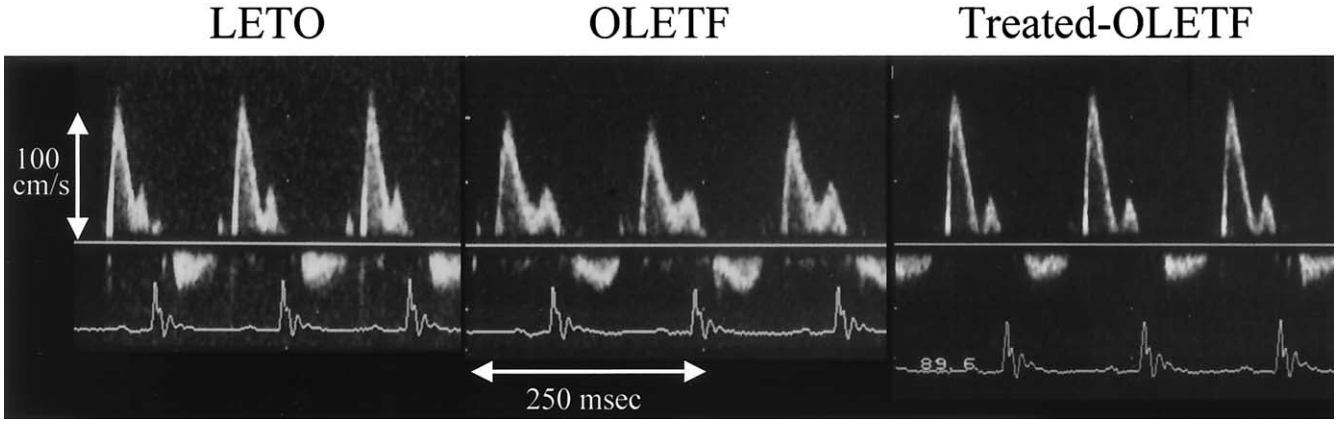

Figure 1. Comparison of left ventricular inflow velocity patterns at 30 weeks. Early to late diastolic peak velocity ratio was reduced and deceleration of early diastolic inflow was slow in the untreated Otsuka Long-Evans Tokushima Fatty (OLETF) rats **(middle).** These were normalized in the treated-OLETF rats **(right).** LETO = Long-Evans Tokushima Otsuka.

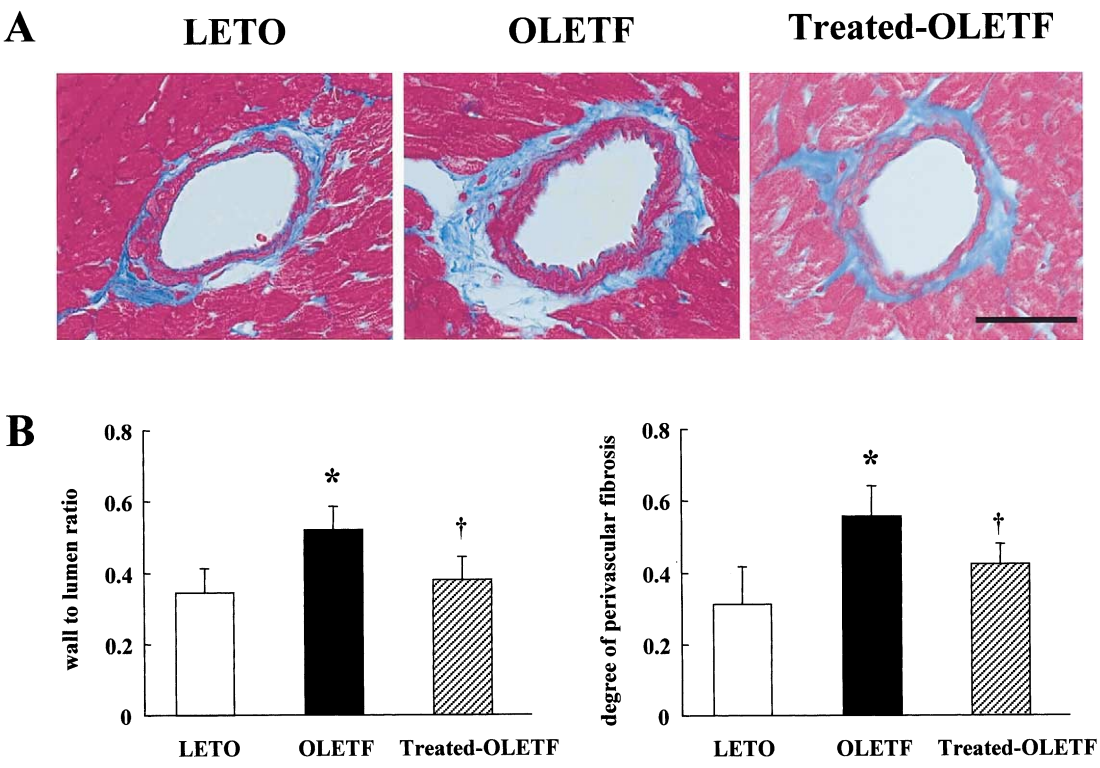

Figure 2. A. Representative micrographs of coronary arteries with Azan-Mallory stain at 30 weeks. Arterial wall thickness and perivascular fibrosis were greater in the OLETF rat **(middle)** than in the LETO rat **(left).** However, the coronary arterial remodeling was less in the treated-OLETF rat. **(right). Scale bar** is 50 μm. **B.** Wall-to-lumen ratio **(left)** and degree of perivascular fibrosis **(right)** in LETO, untreated-OLETF, and treated-OLETF rats. Values are expressed as mean ± SD (n = 5 for each group). *p < 0.05 versus LETO rats; †p < 0.05 versus untreated-OLETF rats. Abbreviations as in Figure 1.

ciated with a significant inhibition of coronary arteriosclerosis and diastolic dysfunction.

Retardation of DM development. Although the total cholesterol level was significantly lower in the treated-OLETF rats than in the untreated group at 10 and 20 weeks, the difference was no longer statistically significant at 30 weeks. Because the low-density lipoprotein cholesterol/high-density lipoprotein cholesterol ratio is generally low in rats including OLETF rats (20), low-density lipoprotein cholesterol-lowering effect and high-density lipoprotein cholesterol-increasing effect of pravastatin might have resulted in the inconsistent effects in total cholesterol levels in the treated rats. Although triglyceride in the treated-OLETF group remained at a similar level to that in the LETO rats at 10 weeks, levels in both OLETF groups were elevated at 20 and 30 weeks regardless of pravastatin treatment. Thus, there was no consistent lipid-lowering effect of pravastatin in the present study. Therefore, it is unlikely that the lipid-lowering effect including triglyceride lowering may be a central mechanism for the prevention of DM development by pravastatin (15). However, it remains unknown whether lower doses of pravastatin or other statins also exert the preventive effects on DM development independently of their lipid-lowering effects.

The plasma levels of leptin and TNF-α were elevated in the untreated-OLETF rats in our study as previously reported (20). Both TNF-α (21) and leptin (22) are secreted from adipocytes, and TNF-α increases the expression and secretion of leptin from adipocytes (23). Leptin was shown in a previous study to attenuate insulin-induced tyrosine phospholyration of the insulin receptor substrate-1 (24). The TNF-α is known to induce serine phospholyration of the insulin receptor substrate-1, which inhibited insulin receptor tyrosine kinase and attenuated insulin receptor signaling (25). Therefore, it is speculated that pravastatin acted on adipocytes and reduced the oversecretion of these cytokines that cause insulin resistance.

Centrally mediated effects of leptin reduce food intake and enhance glucose metabolism (26), unless leptin resistance exists (27). Although the leptin level was higher in the OLETF rats than in the LETO rats, food consumption and body weight were significantly greater in the OLETF rats in our experiment, which implies that the leptin resistance existed in this model (28). However, pravastatin reduced the food intake and body weight in the OLETF rats, which was accompanied by a significant reduction of leptin concentration in our study. Moreover, pravastatin did not reduce the food intake or body weight of control LETO rats, which are free from leptin resistance. Therefore, pravastatin might possibly improve leptin resistance in the OLETF rats, which might partly contribute to the improved glucose tolerance in this model, although the characteristically enhanced appetite and overeating of this nervous animal

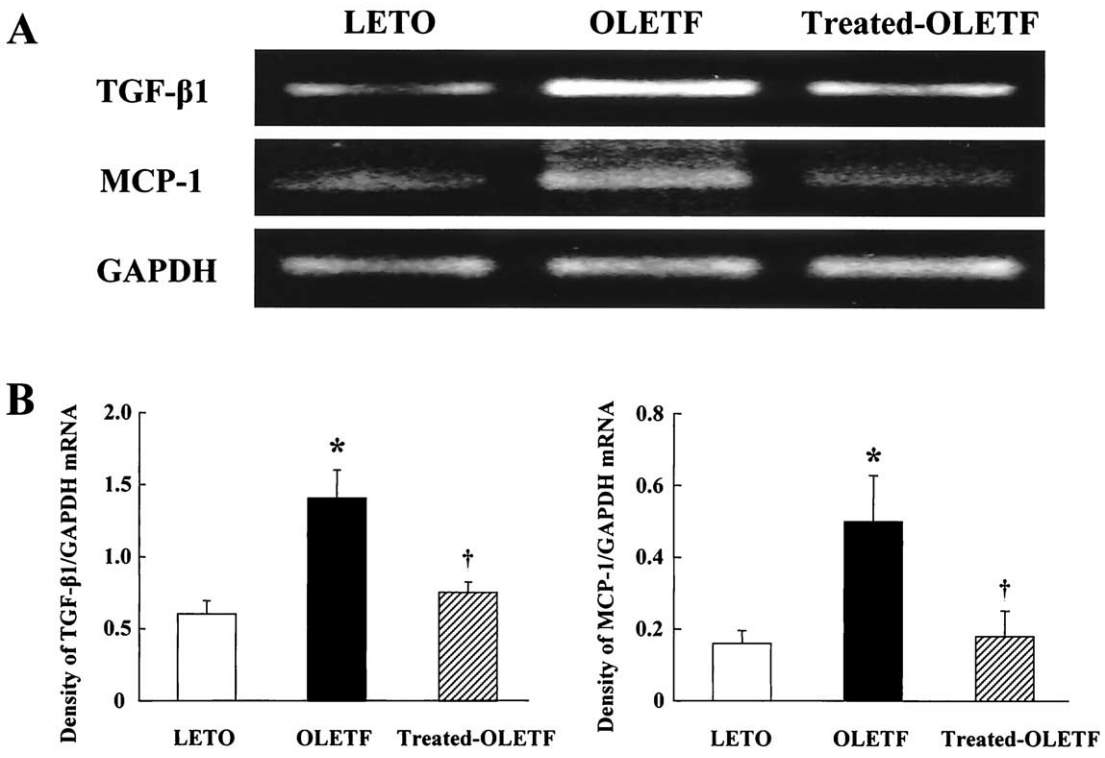

Figure 3. A. Representative RT-PCR photographs for TGF-β1, MCP-1, and GAPDH mRNA expression in the left ventricle of age-matched LETO, untreated-OLETF, and treated-OLETF rats at 30 weeks. **B.** Quantitative comparison of the mRNA expression for TGF-β1 **(left)** and MCP-1 **(right)** in the left ventricle among the groups. Values are the percentage of GAPDH as the control and expressed as mean ± SD (n = 5 for each group). *$p < 0.05$ versus LETO rats; †$p < 0.05$ versus untreated-OLETF rats. Abbreviations as in Figure 1.

may have been suppressed also by any subtle changes in atmosphere. Thus, although the precise mechanisms remain unknown, the present results may provide a basis for extending the preventive effect of pravastatin on DM development in hypercholesterolemic patients, reported in a subanalysis of a statin human trial (15), to the population prone to develop DM regardless of complication of hypercholesterolemia.

Prevention of remodeling of coronary arteries and diastolic dysfunction. In this study, we confirmed that the OLETF rats manifest small coronary arterial remodeling including thickening of the arterial wall and perivascular

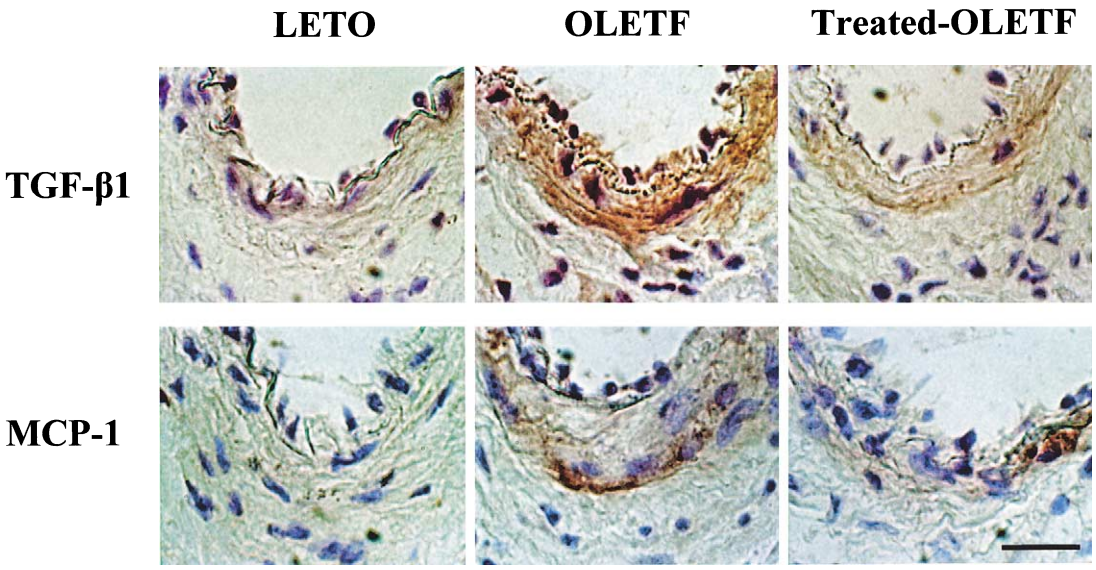

Figure 4. Representative micrographs of immunohistochemistry for transforming growth factor (TGF)-β1 and monocyte chemoattractant protein (MCP)-1 in the coronary arteries of age-matched LETO **(left),** untreated-OLETF **(middle),** and treated-OLETF **(right)** rats at 30 weeks. Scale bar is 20 μm. Abbreviations as in Figure 1.

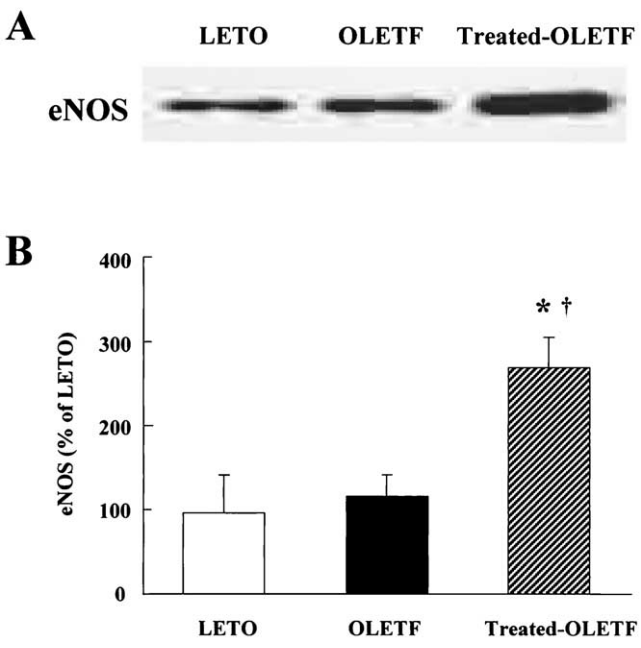

Figure 5. A. Representative Western blot photograph for endothelial nitric oxide synthase (eNOS) protein induction in the left ventricle of LETO, untreated-OLETF, and treated-OLETF rats at 30 weeks. **B.** Quantitative comparison of eNOS protein expression in the left ventricle among the groups. Values are the percentages of the average value of the LETO rats and are expressed as mean ± SD (n = 5 for each group). *$p < 0.05$ versus LETO rats; †$p < 0.05$ versus untreated-OLETF rats. Abbreviations as in Figure 1.

interstitial fibrosis (17). Such features were shown to be responsible for impaired coronary flow reserve (17) and diabetic cardiomyopathy (8–10) manifesting diastolic dysfunction in this model and also in patients with DM (29). We showed for the first time in this study that early treatment with pravastatin suppresses the increase in the wall-to-lumen ratio and the degree of perivascular fibrosis of coronary artery and restores LV diastolic function in the OLETF rats.

In the present study, cardiac expressions of TGF-β1 and MCP-1 were significantly increased in the untreated-OLETF rats compared with those in the control LETO rats. The TGF-β1 is a growth factor that is involved in fibrous tissue formation, up-regulates collagen expression by stimulating extracellular matrix synthesis (18), and mediates perivascular as well as myocardial fibrosis. In fact, increased TGF-β1 expression was correlated with coronary perivascular fibrosis in this model at 20 weeks of age in a previous study (30). Also, MCP-1 is induced by increased oxidative stress, which leads to activation of redox-sensitive transcription factors (5). The MCP-1 regulates migration and infiltration of monocytes/macrophages, leading to chronic vascular inflammation, and facilitates proliferation/migration of vascular smooth muscle cells (31).

Both overexpressions of MCP-1 and TGF-β1 were observed in the rat model of endothelial dysfunction induced by chronic inhibition of eNOS by N^{ω}-nitro-L-arginine methyl ester which resulted in coronary arterial remodeling (12) similar to that observed in our study. Thus, local shortage of NO may also be responsible for overexpression of these cytokines in DM. However, in our untreated-OLETF rats that were diabetic at 30 weeks of age, the eNOS protein expression was not reduced but was preserved at the same levels as that of age-matched LETO rats. This result is consistent with previous reports that hyperglycemia did not reduce myocardial eNOS protein expression (6), or eNOS mRNA expression was preserved until endothelial cells were lost at a very advanced stage of DM (3). In this regard, Jesmin et al. (30) reported that expressions of eNOS protein and mRNA were rather increased in the same model at 20 weeks of age. It may be possible that the eNOS expression might have also been enhanced in our OLETF rats at 20 weeks but was reduced to the similar level to that in the LETO rats as the glucose intolerance progressed by 30 weeks. Both hyperglycemia (3) and the increased advanced glycation end products (5), which are known to accumulate in the heart in this model (6), induce production of reactive oxygen intermediates in endothelial cells that inactivate NO and stimulate redox-sensitive transcription factors. In fact, our previous study showed that oxidative stress in endothelial cells of the OLETF rats was elevated (4). In addition, leptin that was found elevated in the untreated-OLETF is known to induce oxidative stress in endothelial cells in association with activation of the NH_2-terminal c-Jun kinase/stress-activated protein kinase pathway (32). Therefore, in the OLETF rats, cardiovascular remodeling may progress ow-

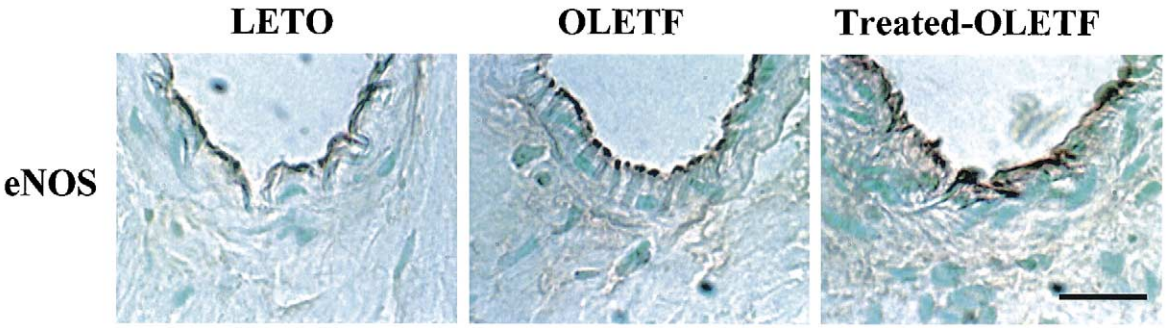

Figure 6. Representative micrographs of immunohistochemistry for eNOS in the coronary arteries of LETO **(left),** untreated-OLETF **(middle),** and treated-OLETF **(right)** rats at 30 weeks. Scale bar is 20 μm. Abbreviations as in Figures 1 and 5.

ing to the enhanced inactivation of NO by increased reactive oxygen intermediates despite the preserved eNOS activity in the endothelium.

The preventive effects of pravastatin on diastolic dysfunction and coronary arteriosclerosis in the OLETF rats were accompanied by the suppression of gene and protein overexpressions of TGF-β1 and MCP-1 in the myocardium and coronary arterial wall in this study. Of note, pravastatin enhanced the expression of eNOS to an even higher level than that of normal LETO rats, as was seen in a study that reported the direct up-regulation of eNOS expression by pravastatin in the chronic NOS inhibition model (12). Thus, it can be speculated that the direct augmentation of eNOS expression and the suppression of reactive oxygen production through the improvement of glucose tolerance and leptin resistance would restore NO, and thereby may contribute to the suppression of the overexpression of TGF-β1 and MCP-1 in the OLETF rats. In addition, because pravastatin retarded the progression of DM, it is possible that some of the other interrelated mechanisms involved in the pathogenesis of cardiovascular remodeling in DM including increased polyol pathway (33), excessive nonenzymatic glycation (34), and increased protein kinase C activity (35) might also be favorably suppressed by pravastatin.

Study limitations. The LV diastolic dysfunction is caused not only by histological alterations of myocardium on which we focused in the previous (8–10) and present studies using this model, but also by end-systolic and end-diastolic LV wall stresses and HR, especially when systolic dysfunction accompanies (36). Although blood pressure and heart rate as well as systolic function assessed as LV fractional shortening were comparable among the groups, other factors determining LV loading conditions or their effects on Doppler recordings were not examined in this study. Therefore, further studies are necessary to determine whether the preserved transmitral flow velocity pattern in the treated-OLETF rats might be partly attributable to any possible reduction of LV overload by pravastatin.

Second, we tested only a single dose of pravastatin in this study (100 mg/kg/day). The dose was chosen according to the previous report that the same oral dose yielded a serum concentration similar to that seen in patients taking clinical doses of pravastatin, presumably due to the more rapid metabolism of statin in rats (12). Besides the improvement of leptin resistance, a gastrointestinal disturbance due to the oral administration of the high dose might be a possible mechanism for the slight reductions in food intake and body weight seen in the treated-OLETF rats. However, as the oral administration of the same dose to normal LETO rats did not reduce food intake or body weight, this dose unlikely affected gastrointestinal function. Because the untreated-OLETF rats that exhibited significant vascular remodeling did not manifest cholesterol abnormalities throughout treatment, the cardiovascular remodeling in the OLETF rats seems to be independent of hypercholesterolemia. However, because the administration of such a high dose of pravastatin lowered serum cholesterol below that of LETO rats at 10 and 20 weeks slightly but significantly, the possibility remains that the lipid-lowering effect may have played a role in the prevention of DM development or cardiovascular remodeling in this model. Future studies are necessary to determine whether any lower doses that do not affect the lipid levels are also effective. Similarly, we tested only a single statin. There are controversies regarding the effect of statins on the insulin sensitivity (37,38) and on the endothelial function in DM patients among the statins (39–41). Therefore, further studies comparing different agents, especially hydrophilic and hydrophobic agents, may be necessary in the future.

Third, although this study has clearly shown that pravastatin can improve the progression of insulin resistance and can retard development of DM, the mechanism(s) for these effects remain(s) unknown. Because elevations of plasma-level TNF-α and leptin were both limited by pravastatin, and because adipose tissue is a major source of these cytokines, it is speculated that pravastatin may alter the behavior of adipocytes on which future studies should focus.

Finally, although we have shown the effect of early administration of pravastatin before manifestation of the insulin resistance, the efficacy of the statin therapy begun after the manifestation of glucose intolerance or DM on arteriosclerosis and whether statin can regress arteriosclerosis in DM remain unanswered.

Conclusions. Early treatment with pravastatin in a rat model of spontaneously developing type II DM retarded the new onset of DM and prevented coronary arterial remodeling and LV diastolic dysfunction. The favorable effects were associated with the reduction of the elevated plasma leptin and TNF-α levels, the supernormal enhancement of endothelial NO synthesis, and the inhibition of cardiac overexpressions of TGF-β1 and MCP-1. These data in the animal model may provide the basis for pravastatin therapy to prevent cardiovascular complications in a population prone to develop DM.

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