Cilostazol, a Phosphodiesterase Inhibitor, Reduces Microalbuminuria in the Insulin-Resistant Otsuka Long-Evans Tokushima Fatty Rat

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We evaluated association beween hyperinsulinemia/insulin resistance and microalbuminuria in the insulin-resistant Otsuka Long-Evans Tokushima Fatty (OLETF) rat. OLETF rats showed glomerular hyperfiltration (an increase in creatinine clearance and a decrease in fractional excretion of Na) and microalbuminuria at the insulin-resistant prediabetic stage, and both were related to expression of transforming growth factor (TGF)-β_1 and extracellular matrix protein such as fibronectin and collagen (a_1) IV. Cilostazol, a selective type III cyclic nucleotide phosphodiesterase (PDE) inhibitor, normalized glomerular hyperfiltration and microalbuminuria with a parallel decline of TGF-β_1 and extracellular matrix protein mRNA expression. Cilostazol may be beneficial to lessen early glomerular nephropathy in a state of hyperinsulinemia/insulin resistance.

THE Otsuka Long-Evans Tokushima Fatty (OLETF) rat develops insulin resistance/hyperinsulinemia at an early stage, and later suffers from hyperglycemia, similar to type 2 diabetic patients.[1] The pathophysiologic characteristics of glomerulosclerosis in OLETF rats also resemble to those in type 2 diabetics.[1-3] Yagi et al reported that urinary albumin excretion (UAE) was significantly increased at an early stage (22 weeks) before hyperglycemia become more prominent at a later stage (>38 weeks).[3] An increase in UAE is also observed in patients with obesity[4] and hyperinsulinemia[5-7] as in OLETF rats, but the mechanisms of microalbuminuria during the insulin-resistant state remain unclear.

Cilostazol, a selective type III cyclic nucleotide phosphodiesterase (PDE) inhibitor, is an antiplatelet and vasodilating agent.[8,9] Cilostazol is also known to increase peripheral blood flow and improve insulin sensitivity in OLETF rats.[10] The present study was undertaken to determine whether the insulin-resistant state would affect UAE in OLETF rats or not. We expected that cilostazol could decrease UAE by improving insulin resistance.

MATERIALS AND METHODS

Animals and Treatment

Male OLETF rats and rats of the age-matched nondiabetic control strain Long-Evans Tokushima Otsuka (LETO) were obtained from Tokushima Research Institute (Otsuka Pharmaceutical, Tokushima, Japan) at 6 weeks of age. OLETF and LETO rats received either 0.1 % (vol/vol) cilostazol chow or a standard chow from 7 to 16 weeks of age. Control rats were given free access to standard chow or pair-fed to 0.1% cilostazol chow groups. Rats were housed individually in metabolic cages for monitoring food intake, urine volume, and body weight. Plasma levels of glucose and insulin and UAE were determined periodically. Creatinine clearance (Ccr, mL/min) was calculated as [urine creatinine (mmol/L)] · [urine volume (mL/min)]/[plasma creatinine (mmol/L)]; and fractional excretion of Na (FE_{Na}, %) as [urine Na (mEq/L)/plasma Na (mEq/L)] · 100/[urine creatinine (mmol/L)/plasma creatinine (mmol/L)]. When rats were killed at the age of 16 weeks, a cortical portion of the kidney was collected for total RNA extraction (β-actin, transforming growth factor [TGF]-β_1, fibronectin, and collagen type [a_1] IV) and histologic evaluation. As described elsewhere,[11] blood pressure was monitored by a 22-gauge catheter inserted to the carotid artery using a pressure introducer system (AP601G, Nihon Kohden, Tokyo, Japan). All rats were kept under a specific pathogen-free facility with a 12-hour light and dark cycle, and given tap water ad libitum. All procedures were performed in accordance with the guidelines of University of the Ryukyus Committee on Animal Care and Handling.

Steady-State Plasma Glucose

Steady-state plasma glucose (SSPG) levels were determined according to the method of Mondon and Reaven[12] with modifications.[13] Briefly, rats fasted overnight were anesthetized with an intraperitoneal injection of pentobarbital sodium (50 mg/kg). Rats were cannulated in the right jugular vein for blood sampling, and in the left femoral vein for infusion. Somatostatin analogue (octreotide acetate, Sandoz, Basel, Switzerland) was infused at 30 ng/kg/min for 30 minutes to suppress endogenous insulin secretion. Then, a mixed solution of octreotide acetate 30 ng, insulin 2.7 mU, and glucose 8 mg/kg body weight/min was infused at the rate of 1 mL/h. Blood samples were obtained every 30 minutes during −30 to 180 minutes.

Measurement of Blood Flow

A catheter was positioned in the abdominal aorta via the femoral artery for a withdrawal of reference blood samples, and another catheter was inserted into the left ventricle via the right carotid artery for a infusion of microspheres. Colored microspheres (150,000, 15 μm in diameter, Dye-Trak, Triton Technology, San Diego, CA) were injected into the left atrium, a saline flush infused for 50 seconds at a rate of 0.6 mL/min, and the reference blood withdrawals continued for another 15 seconds (total of 75 seconds). After weighing, biceps muscle and the left kidney were dissolved in 16 mol/L KOH. The suspension was filtered (10 μm pore size, Triton) to collect microspheres, which were dissolved by dimethyl formamide (Wako Pure Chemical Industries, Osaka, Japan) to extract color dyes. The color spectra were measured by a spectrophotometer (U-2000, Hitachi, Tokyo, Japan), and the tissue blood flow was calculated as described in the manufacturer's protocol.

Biochemical Measurements

Plasma glucose levels were determined by the glucose oxidase method using a Glucose Analyzer II (Beckman Coulter, Brea, CA).

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Immunoreactive insulin concentrations were determined by a radioimmunoassay kit (RI-13K, Linco Research, St Charles, MO) standardized against rat insulin. Twenty-four–hour UAE was determined using the bromcresol green method (albumin-HR II, Wako).[14]

Reverse Transcriptase–Polymerase Chain Reaction

Total RNA was extracted from cortical portions of the kidney using the RNeasy Mini Kit (QIAGEN K.K., Tokyo, Japan) and then treated with RNase-free DNase (Boehringer Ingelheim, GmbH, Germany) for 15 minutes at 37°C to remove any contaminating DNA.[15] First-strand cDNA was performed by oligo$(dT)_{12\text{-}18}$ primed reverse transcription of 2 μg of each total RNA using first strand cDNA synthesis kit (GIBCO BRL, Rockville, MD). Primers for cDNA synthesis were designed to span introns in respective genes. Primers used were as follows: β-actin, 5′-TTG TAA CCA ACT GGG ACG ATA TGG-3′ and 5′-GAT CTT GAT CTT CAT GGT GCT AGG-3′ (764 bp); TGF-β_1, 5′-AAG AAC TGC TGT GTG CGG-3′ and 5′-GCA CTT GCA GGA GCG CAC AA-3′ (296 bp); fibronectin, 5′-GGA CAA GGT AGT GGC CAC TTA ACG-3′ and 5′-GCG GCT GAG CCC CAA GAG CAG AGG-3′; and collagen (β_1) IV, 5′-GTG CGG TTT GTA AAG CAC CG-3′ and 5′-GTT CTT CTC ATG CAC ACT T-3′ (363 bp).

Polymerase chain reaction (PCR) amplification consisted of a 5-minute hot start at 94°C, followed by 30 cycles (β-actin) and 35 cycles (others) of amplification (94°C for 1 minute; 53°C [β-actin); 54°C (TGF-β_1] and collagen [a_1) IV; 56°C [fibronectin] for 45 seconds; and 72°C for 45 seconds]. Linearity of the PCR reaction was tested by amplification of 100 ng first-strand cDNA per reaction from 10 to 50 cycles for all genes, and found to be linear for 10 to 40 cycles. In no case did the amount of the first-strand cDNA used for the PCR reaction exceed 100 ng per reaction. The amplification products were run on a 1.5% agarose gel, and the relative amount of band intensity was semiquantitated by the correction of β-actin intensity (NIH Image 1.61, National Insittues of Health, Bethesda, MD).

Histologic Examination

A cortical portion of the kidney was fixed in Bouin's solution and processed to hematoxylin-eosin staining as described.[13] In series of 5-μm thick sections, the maximum diameter of 50 randomly selected glomeruli was measured on photographs. The glomerular volume was determined from the mean glomerular diameter (d) using the formula: $4\pi(d/2)^3/3$.

Statistical Analysis

Group means were compared using Mann-Whitney's rank-sum test or Kruskal-Wallis rank test. Values are presented as the mean ± SEM, and $P < .05$ was considered to indicate statistical significance.

RESULTS

General Characteristics

Body weight and food intake of free-access, pair-fed, and cilostazol-treated OLETF rats were increased largely with age as compared to those of LETO rats (Fig 1A and B and Table 1). Plasma glucose levels were comparable among 5 groups between 6 to 16 weeks (Fig 1C). Plasma insulin levels were higher in free-access or pair-fed OLETF rats at 16 weeks ($P < .05$ *v* pair-fed LETO rats), but lower in cilostazol-treated OLETF rats ($P < .05$ *v* free-access or pair-fed OLETF) (Fig 1D). Plasma levels of cholesterol, triglyceride, and free fatty acid were also higher in OLETF rats at 16 weeks and cilostazol treatment did not change the levels (Table 1). UAE levels tended to be increased at 10 weeks and were significantly increased at 12 and 16 weeks ($P < .05$, free-access or pair-fed OLETF *v* LETO rats) (Fig 1E). UAE was decreased by approximately 30% at 16 weeks in cilostazol-treated OLETF rats ($P < .05$ *v* free-access or pair-fed OLETF rats).

Pathophysiologic Characteristics of the Kidney

At 16 weeks, kidney weight and kidney weight to body weight ratio were greater in OLETF rats, and cilostazol did not affect the values (Table 1). Blood pressure was comparable between OLETF and LETO rats, and cilostazol did not change the value. Ccr was increased by 32% in OLETF rats, and cilostazol treatment decreased Ccr to the level of normal LETO rats. FE_{Na} in OLETF was almost half of LETO rats, and cilostazol treatment increased FE_{Na} to levels of LETO rats. Fractional albumin clearance was as follows: pair-fed-LETO, 0.08% ± 0.01%; cilostazol-treated LETO, 0.08% ± .01%; pair-fed OLETF, 1.17% ± 0.48%; and cilostazol-treated OLETF rats, 0.76% ± 0.15%. The difference in fractional albumin clearance was not significant between pair-fed and cilostazol-treated OLETF rats. Cilostazol did not influence systemic hemodynamics but increased renal and muscle blood flow in OLETF rats. Histologically, glomerular volume was 2 times higher in OLETF rats but the volume was not changed by cilostazol treatment (Fig 2). There were no significant changes in tubular-interstitial morphology among pair-fed LETO, cilostazol-treated LETO, pair-fed OLETF, and cilostazol-treated OLETF rats.

Reverse Transcriptase–Polymerase Chain Reaction

Renal mRNA levels of TGF-β_1, fibronectin, and collagen (a_1) IV were significantly higher in OLETF rats at 16 weeks compared to those of LETO rats (Fig 3). Cilostazol treatment significantly decreased the values in OLETF rats, while it did not alter those in LETO rats.

Steady-State Plasma Glucose

During an adequate suppression of endogenous insulin secretion by octreotide, a continuous infusion of insulin made a constant level at approximately 2.5 μU/mL in all groups of rats (Fig 4). Plasma glucose levels were higher in OLETF rats than in LETO rats ($P < .05$) during 120 to 180 minutes, but cilostazol treatment partially decreased SSPG levels (Fig 4) ($P < .05$ *v* pair-fed OLETF). There was a positive correlation between SSPG levels and UAE (Fig 4B).

DISCUSSION

Hyperinsulinemia/insulin resistance has been proposed as an underlying mechanism for microalbuminuria at an early stage of diabetes.[4-6] We evaluated the relationship between hyperinsulinemia/insulin resistance and microalbuminuria in a type 2 diabetic model, the OLETF rat.[1-3] OLETF rats showed hyperinsulinemia and insulin resistance with modest hyperglycemia between 14 to 16 weeks of age. Even in the early stage, OLETF rats showed glomerular hyperfiltration and an increase in UAE. Thus, early renal involvement in OLETF rats could be related to hyperinsulinemia/insulin resistance.[16,17]

In OLETF rats, Ccr was increased by 32%, and FE_{Na} was decreased to half of LETO rats. Since the tubular-interstitial morphology was intact in OLETF rats, the decrease in FE_{Na} could

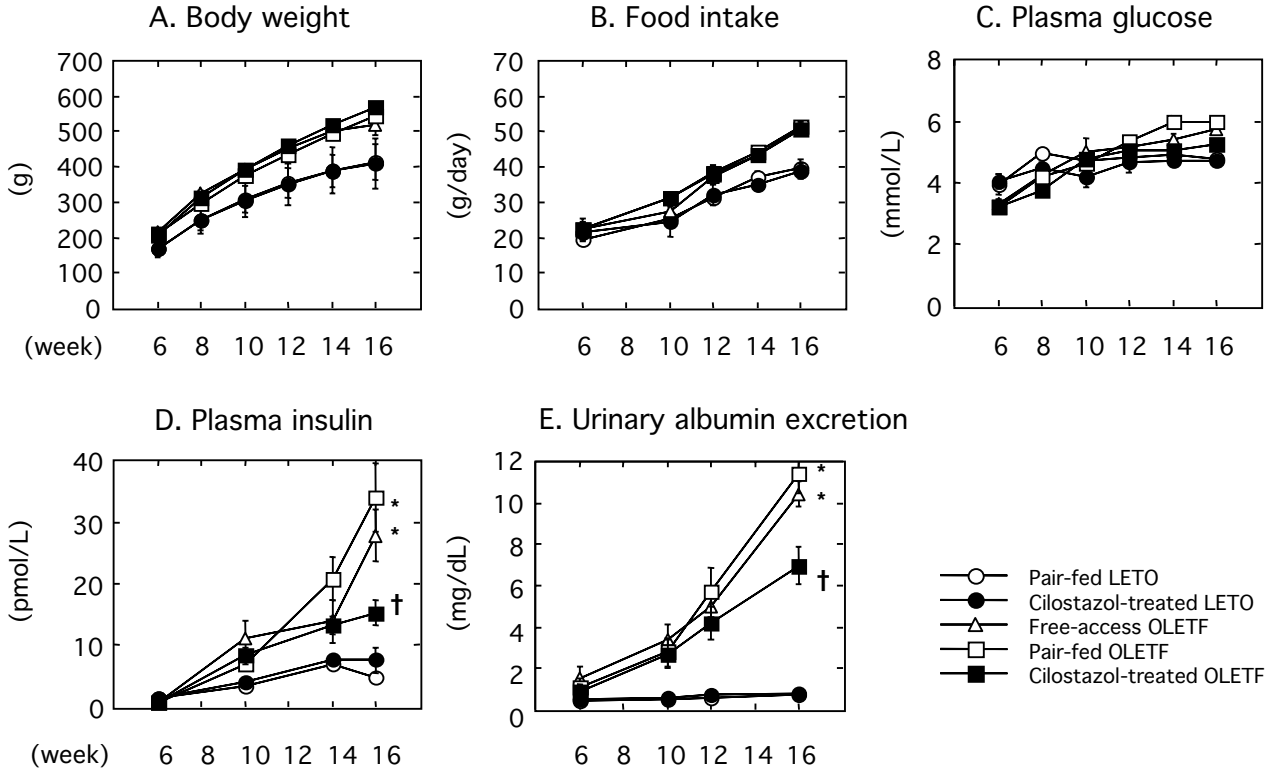

Fig 1. Changes in (A) body weight, (B) food intake, (C) plasma glucose, (D) plasma insulin, and (E) urinary albumin excretion (UAE), in pair-fed LETO (n = 5), cilostazol-treated LETO (n = 5), free-access OLETF (n = 4), pair-fed OLETF (n = 6), and cilostazol-treated OLETF (n = 6) rats. Values represent the mean ± SEM. *$P < .05$ *v* pair-fed LETO; $P < .05$ *v* pair-fed OLETF.

reflect a functional increase in Na^+ reuptake at the thick ascending limb. Actually, an increase in Na^+ reuptake at the thick ascending limb of Henle's loop is suggested to be one mechanism for glomerular hyperfiltration in early stage of diabetic nephropathy.[18] In OLETF rats, UAE reached 10 times that of LETO rats at 16 weeks of age. Fractional albumin clearance was 14 times higher in OLETF rats than that in age-matched LETO rats. Glomerular hyperfiltration and albumin permselectivity could be responsible for microalbuminuria. Overexpression of TGF-β_1 and extracellular matrix protein is thought to be one molecular mechanism of

Table 1. General Characteristics of Animals at 16 Weeks of Age

Characteristic	Pair-Fed LETO	Cilostazol-Treated LETO	Free-Access OLETF	Pair-Fed OLETF	Cilostazol-Treated OLETF
Body weight (g)	437 ± 4	391 ± 25	578 ± 15*	582 ± 14*	598 ± 13*
Kidney weight (right, g)	1.21 ± 0.03	1.20 ± 0.02	1.81 ± 0.13*	1.76 ± 0.07*	1.87 ± 0.05*
Kidney weight/body weight ($10^3 \cdot$ g/g)	2.76 ± 0.07	3.11 ± 0.14	3.10 ± 0.16	3.00 ± 0.08	3.10 ± 0.10
Systolic blood pressure (mm Hg)	105 ± 5	103 ± 2	-	108 ± 5	105 ± 5
Diastolic blood pressure (mm Hg)	73 ± 5	73 ± 4	-	72 ± 4	70 ± 7
Renal blood flow (mL/min/g)	3.03 ± 0.15	3.27 ± 0.56	-	1.85 ± 0.19*	3.48 ± 0.14†
Biceps muscle blood flow (mL/min/g)	1.03 ± 0.22	0.91 ± 0.18	-	0.28 ± 0.05*	1.14 ± 0.15†
Urinary volume (mL/24 h)	13 ± 1	14 ± 3	23 ± 6*	18 ± 3*	15 ± 2†
Cleatinine clearance (mL/min)	2.63 ± 0.44	2.93 ± 0.67	3.63 ± 0.23*	3.91 ± 0.55*	2.65 ± 0.36†
Fractional excretion of Na (%)	0.12 ± 0.02	0.13 ± 0.03	0.06 ± 0.01*	0.07 ± 0.02*	0.15 ± 0.02†
Total cholesterol (mmol/L)	1.92 ± 0.20	2.28 ± 0.33	-	3.68 ± 0.19*	3.39 ± 0.17
Triglycerides (mmol/L)	0.68 ± 0.06	0.50 ± 0.09	-	0.85 ± 0.14*	0.78 ± 0.08
High-density lipoprotein-cholesterol (mmol/L)	0.98 ± 0.04	0.95 ± 0.08	-	0.73 ± 0.03*	0.80 ± 0.05
Free fatty acid (mmol/L)	0.74 ± 0.04	0.77 ± 0.03	-	2.31 ± 0.26*	1.68 ± 0.19

NOTE. Values are means ± SEM of 12 to 14 (6 to 8 for blood flow and lipids) rats.

*$P < .05$ *v.* pair-fed LETO.

†$P < .05$ *v.* pair-fed OLETF.

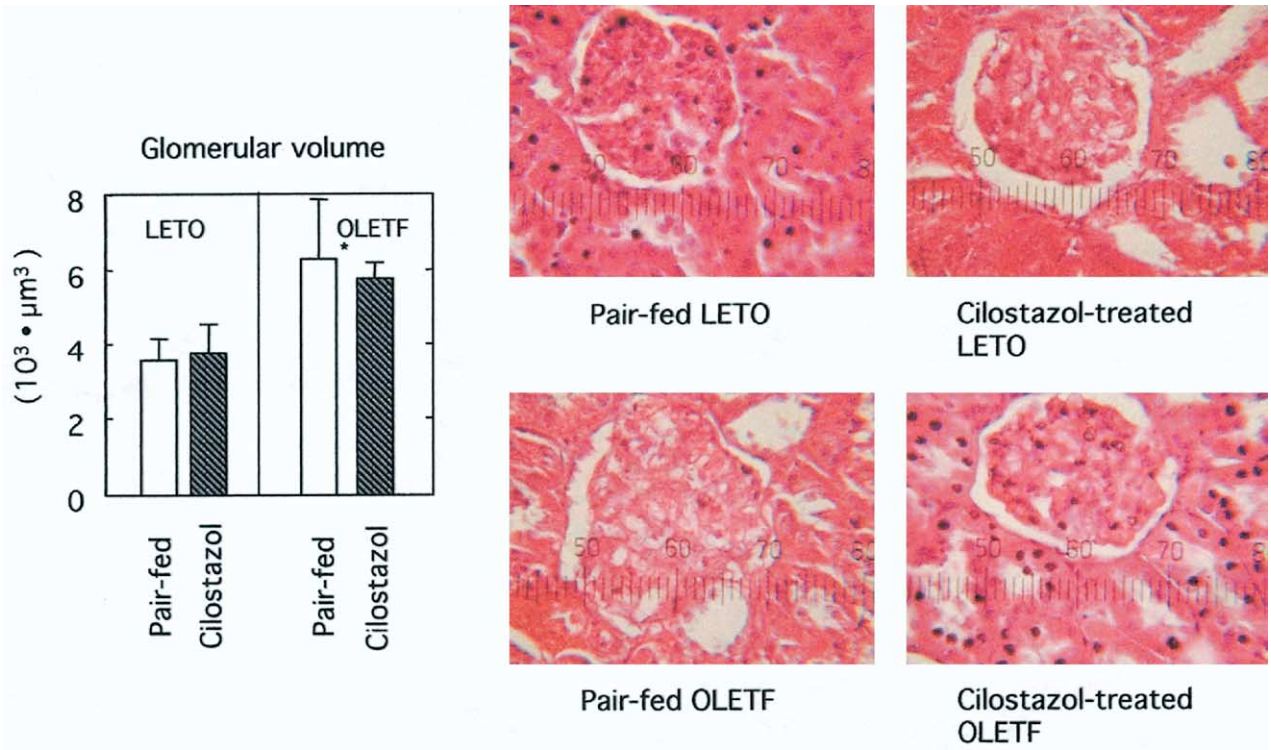

Fig 2. Microscopic findings of glomerulus and calculated glomerular volume of pair-fed LETO, cilostazol-treated LETO, pair-fed OLETF, and cilostazol-treated OLETF rats. Five-micron thick sections of the renal cortex were processed by hematoxylin-eosin staining; 1 scale = 5 μm. The glomerular volume was calculated from the mean glomerular diameter (d) using the formula: $4\pi(d/2)^3/3$. Values represent means ± SEM of 50 glomeruli of each group. *$P < .05$ *v* pair-fed LETO.

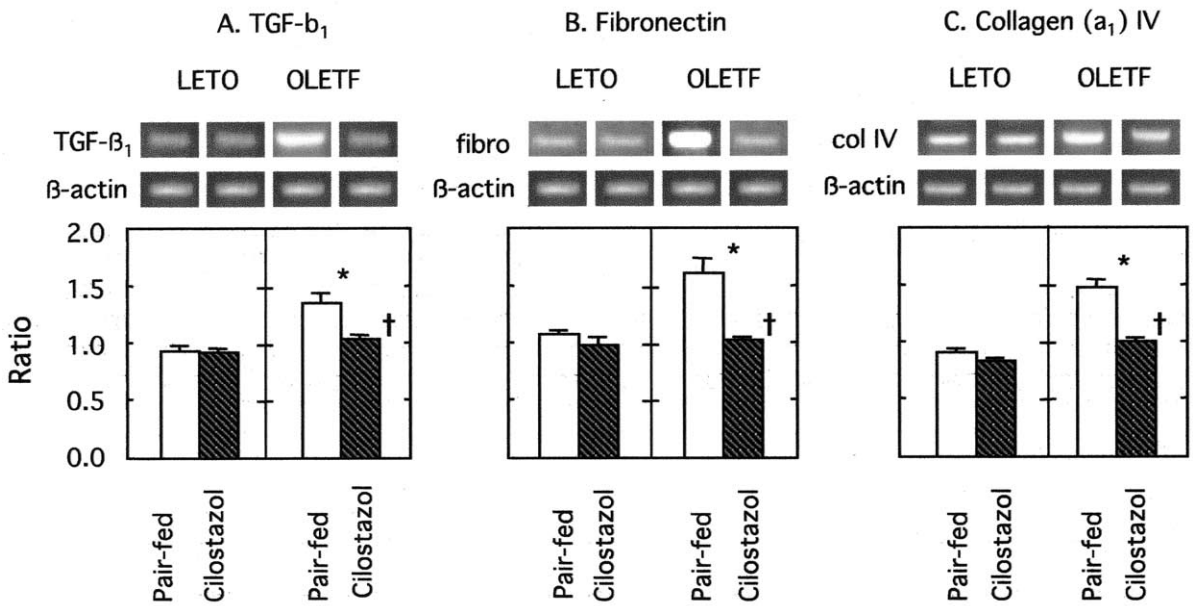

Fig 3. Ratio of mRNA of fibronectin, collagen (a_1) IV, and TGF-β_1 to that of β-actin in the renal cortex of pair-fed LETO (n = 5), cilostazol-treated LETO (n = 5), pair-fed OLETF (n = 5), and cilostazol-treated OLETF (n = 5) rats. Values represent means ± SEM of 5 rats. *$P < .05$ *v* pair-fed LETO. *P v* pair-fed OLETF.

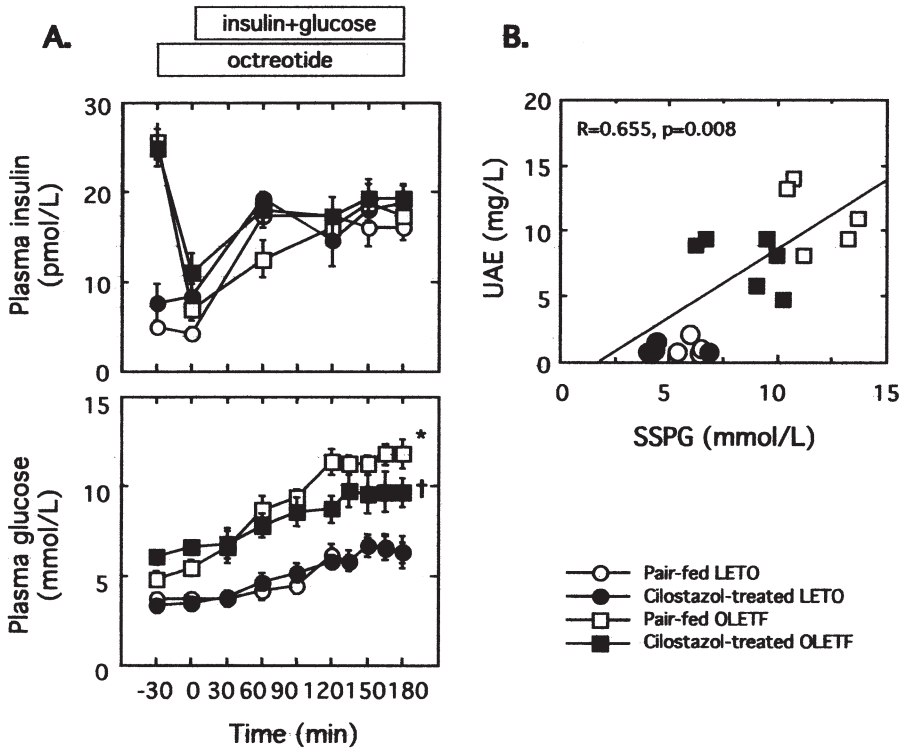

Fig 4. (A) Steady-state plasma insulin and glucose (SSPG) levels and (B) relationship between urinary albumin excretion (UAE) and SSPG in pair-fed LETO (n = 5), cilostazol-treated LETO (n = 5), pair-fed OLETF (n = 5), and cilostazol-treated OLETF (n = 5) rats. Values represent means ± SEM. *$P < .05$ *v* pair-fed LETO. $P < .05$ *v* pair-fed OLETF.

diabetic microalbuminuria.[19,20] In OLETF rats, TGF-β_1 and extracellular matrix proteins such as fibronectin and collagen (a_1) IV were overexpressed in the renal cortex at a prediabetic insulin-resistant state. Reportedly, TGF-β_1 overexpression and excess of extracellular matrix protein could cause selective albumin permeability.[19,20] A stimulation in renin-angiotensin axis, which can be caused by glomerular hyperfiltration or action of excess circulatory insulin, has been proposed as a mechanism for TGF-β_1 production and extracellular matrix protein accumulation in mesangial cells.[21]

In the present study, we demonstrated that cilostazol, a selective PDE inhibitor, could lessen glomerular nephropathy, much as glomerular hyperfiltration (higher Ccr and a lower FE_{Na}) and microalbuminuria, with a parallel enhancement of mRNA of TGF-β_1 and extracellular matrix proteins, at the prediabetic insulin-resistant stage of OLETF rats. Cilostazol normalized glomerular hyperfiltration without changing kidney weight or glomerular mass. Fractional albumin clearance was not statistically different between cilostazol-treated and pair-fed OLETF rats. Thus cilostazol may have lowered UAE in OLETF rats at least partly by decreasing glomerular filtration rate. Since there was a significant correlation between steady-state glucose levels and UAE, cilostazol may decrease microalbuminuria at least partly via improvement of insulin sensitivity. In contrast, Dengel et al reported that glomerular filtration rate and glucose disposal rate in a hyperinsulinemic-euglycemic clamp were well correlated in insulin-resistant subjects.[22] We could not draw a conclusion as to how improvements in insulin sensitivity were related to the reduction in UAE. Cilostazol did not influence systemic hemodynamics, but it did increase renal and muscle blood flow in OLETF rats. Cilostazol may influence insulin sensitivity in OLETF rats through the increase in muscle blood flow. Change in renal blood flow could not account for the normalization of GFR by cilostazol. Cilostazol prevented an enhancement of TGF-β_1 mRNA, which is one plausible mechanism for mesangial accumulation of extracellular matrix in diabetic nephropathy.[18,19] However, it is not clear that inhibition of TGF-β_1 and extracellular matrix protein expression can normalize glomerular filtration rate and microalbuminuria. Because cilostazol has pleiotropic effects and the observed changes could be epiphenomenal, the validity of the above mechanisms is limited.

In summary, (1) OLETF rats showed glomerular hyperfiltration and microalbuminuria at the insulin-resistant prediabetic stage, and both were related to expression of TGF-β_1 and extracellular matrix proteins such as fibronectin and collagen (a_1) IV; (2) cilostazol, a selective PDE III inhibitor, reduces glomerular hyperfiltration and microalbuminuria by inhibition of TGF-β_1 and extracellular matrix protein expression; and (3) cilostazol may be beneficial to lessen early glomerular nephropathy in a state of hyperinsulinemia/insulin resistance.

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