Pioglitazone Reduces Urinary Podocyte Excretion in Type 2 Diabetes Patients With Microalbuminuria

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In various renal diseases, including diabetic nephropathy, detection of podocytes in the urine indicates severe injury to podocytes in the glomeruli. Pioglitazone is a newly developed antidiabetic agent that attenuates insulin resistance. The aim of the present study was to determine whether pioglitazone affects urinary albumin excretion (UAE) or the number of urinary podocytes or both in type 2 diabetes patients with microalbuminuria. Twenty-eight patients with normotensive type 2 diabetes and microalbuminuria (18 men and 10 women; mean age, 52.5 years) and 30 age-matched normotensive controls (20 men and 10 women; mean age, 51.5 years) were included in the study. Urinary podocytes were detected by immunofluorescence with a monoclonal antibody against podocalyxin. Patients were randomly assigned to 2 groups: a pioglitazone-treatment group (30 mg/day, n = 14) and a placebo group (n = 14). Treatment was continued for 6 months. Podocytes were absent in the urine of healthy controls, but detected in 17 of 28 diabetic patients (60.7%). UAE was reduced from 96.7 ± 50.5 μg/min to 39.7 ± 22.9 μg/min ($P < .01$) in the pioglitazone-treatment group, and the number of urinary podocytes was reduced from 0.9 ± 1.0 cells/mL to 0.1 ± 0.2 cells/mL ($P < .001$). Neither UAE nor the number of urinary podocytes was affected in the placebo group. These data indicate that pioglitazone is effective for reducing UAE and podocyte injury in early-stage diabetic nephropathy.

THE MOST SEVERE podocyte lesion occurs as a detachment of podocytes from the glomerular basement membrane, and these cells appear subsequently in the urine.[1] We previously reported the presence of podocytes in the urine sediments of patients with various glomerular diseases.[1-3] More recently, we reported that glomerular epithelial cell injury occurs in patients with early diabetic nephropathy and that trandolapril, an angiotensin-converting enzyme inhibitor, may have a beneficial effect on podocyte injury.[4]

Thiazolidinediones form a new class of oral antidiabetic agents,[5] which selectively enhance or partially mimic certain actions of insulin, causing a slowly-generated antihyperglycemic effect in type 2 diabetes patients. Other effects of thiazolidinediones that seem to be independent of the lowering of the plasma glucose level have been reported. Imano et al[6] reported that troglitazone ameliorates microalbuminuria in diabetic patients. Pioglitazone, a newly developed thiazolidinedione, was approved for use in Japan in 1999. Little is known about the effect of pioglitazone on diabetic nephropathy. Thus, we studied the effect of pioglitazone on urinary albumin excretion (UAE) and urinary podocytes in normotensive type 2 diabetes patients with microalbuminuria.

SUBJECTS AND METHODS

Twenty-eight type 2 diabetes patients with microalbuminuria (18 men and 10 women; mean age, 52.5 ± 10.2 years) and 30 healthy age-matched controls (20 men and 10 women; mean age, 51.5 ± 10.6 years) were included in this study. Patients presented to our hospitals, and control subjects were recruited. Inclusion criteria included a diagnosis of type 2 diabetes, as defined by the World Health Organization,[7,8] and no history of ketosis. Each subject was fully informed of the study aim, the medication, and the clinical procedure, and consent forms were signed by each subject. All patients had been treated by diet and/or glibenclamide; none were receiving insulin treatment or lipid-lowering drugs at time of recruitment. They stayed on this medication during the study. In the preliminary studies, we found that this treatment did not affect the present results (data not shown). No patient had a serum creatinine level in excess of 1.5 mg/dL at the time of the study. No patient had a malignancy or history of heart, cerebrovascular, liver, or collagen disease. Patients with hematuria and those who had a known history of nondiabetic renal disease were also excluded. Clinical and laboratory data for each patient were obtained at the first study examination (baseline) and at 3 and 6 months later (Table 1). After an overnight fast, blood was drawn from an antecubital vein for measurement of plasma glucose, glycated hemoglobin, serum creatinine, blood urea nitrogen, and 24-hour creatinine clearance. Blood glucose concentrations were determined by the glucose oxidase method and hemoglobin A_{1c} was measured by spectrophotometric assay (Bio-Rad, Richmond, VA; normal range, 3.5% to 6.5%). On the basis of UAE in at least 3 consecutive 4-hour morning urine collections, patients were classified as having normal (less than 20 μg/min) or increased UAE.[9] The median of 3 UAE measurements was used as the baseline UAE, and microalbuminuria was defined as a median UAE of 20 to 200 μg/min. All 28 diabetes patients were at a microalbuminuric stage in which urinary albumin levels ranged from 28 μg/min to 184 μg/min at the time of this study. Patients were randomly assigned to 2 groups. They were matched and randomized in a double-blinded design.

Pioglitazone (Actos; Takeda Pharmaceutical, Osaka, Japan) was administered orally to 14 diabetes patients at 30 mg/day for 6 months (pioglitazone-treatment group). The other 14 patients were given a placebo for 6 months (placebo group). First-voided morning urine specimens were obtained from all subjects before treatment and 3 and 6 months after treatment. Urinary podocytes were stained by immunofluorescence as reported previously.[1-4] The urine specimens were processed within 30 minutes of voiding. A total of 10 mL of freshly voided urine was centrifuged for 5 minutes at 700*g*. The supernatant was aspirated and the sediment was washed with 0.01 mol/L phosphate-buffered saline (PBS), pH 7.2. The sediment was resuspended in 10 mL of PBS, cytocentrifuged onto poly-L-lysin–coated microscope slides for 5 minutes at 700*g* in an autosmear, then air-dried for 30 minutes. Slides were fixed for 5 minutes in acetone at 4°C. The urine sediments collected on the slides were partitioned into 6 areas of 1.0 × 1.0 cm

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Table 1. Clinical and Laboratory Data Before and After Treatment

	Pioglitazone			Placebo		
	Before	3 Month	6 Month	Before	3 Month	6 Month
FBS (mg/dL)	186 ± 24	148 ± 20*	122 ± 16†	176 ± 22	168 ± 24	180 ± 28
HbA_{1C} (%)	8.4 ± 1.3	7.0 ± 1.2*	6.2 ± 0.8†	8.0 ± 1.0	7.9 ± 1.0	8.1 ± 1.3
S-Cr (mg/dL)	0.9 ± 0.2	0.8 ± 0.3	1.0 ± 0.2	1.0 ± 0.3	0.9 ± 0.4	1.1 ± 0.2
BUN (mg/dL)	18 ± 7	19 ± 6	21 ± 4	19 ± 6	21 ± 8	18 ± 4
SBP (mm Hg)	126 ± 12	128 ± 12	122 ± 14	128 ± 14	130 ± 12	134 ± 12
24-hour Ccr (mL/min)	104 ± 12	102 ± 14	98 ± 16	106 ± 16	104 ± 12	102 ± 18

NOTE. Data are shown as means ± SD.

Abbreviations: FBS, fasting blood sugar; S-Cr, serum creatinine; BUN, blood urea nitrogen; SBP, systolic blood pressure; Ccr, creatinine clearance.

*$P < .05$.

†$P < .01$.

each with a PAP pen (Dako, Tokyo, Japan). After being washed with PBS, slides were incubated for 60 minutes with 20 μL per area of antihuman podocalyxin monoclonal antibody, PHM-5 (Australian Monoclonal Development, Artamon, NSW, Australia) at a 1:200 dilution,[1] followed by further washing. The slides were then incubated with fluorescein isothiocyanate-labeled F $(ab')_2$ fragments of affinity-purified antimouse immunoglobulin G (IgG) (Cappel/ICN Biomedicals, Costa Mesa, CA). Slides were washed again and examined by immunofluorescence microscopy. Nuclei of the cells were counterstained with ethidium bromide before mounting.

The number of urinary podocytes was recorded as cells per milliliter urine. In preliminary studies, we examined urinary podocyte levels at particular times (7:30 AM, 10:30 AM, 5:30 PM, and 9:30 PM) in diabetes patients and found that urinary podocyte numbers were fairly consistent over time. Freshly voided urine was collected for 5 consecutive days, and urinary podocytes were counted every day. We reported previously that podocalyxin was present in the urine sediments of pediatric patients with glomerular diseases as casts, fine granules, and entire cells.[1] In the present study, we measured only entire cells, not cell fragments, in the urine.

Data are expressed as mean ± SD. Statistical analyses were performed with the Wilcoxon signed-rank test for paired data and the Mann-Whitney U test for unpaired data. Statistical significance was determined at a P value of less than .05.

RESULTS

Podocytes were absent in the urine of healthy controls, but were present in 17 of 28 type 2 diabetes patients with microalbuminuria (range, 0.6 cells/mL to 3.2 cells/mL; mean, 1.7 cells/mL). Significant differences were found in urinary podocyte numbers and in UAE between diabetes patients and healthy controls (podocyte: diabetes, 1.02 ± 1.03 cells/mL; controls, 0.00 ± 0.00 cells/mL; UAE: diabetes, 88.1 ± 43.2 μg/min; controls, 5.6 ± 0.8 μg/min) ($P < .001$). Table 2 shows urinary podocyte and UAE data for all diabetes patients at baseline (n = 28). There was no relationship between the degree of albuminuria and the number of urinary podocytes. There was also no relationship between fasting blood sugar levels or hemoglobin A_{1c} and the number of urinary podocytes. These data coincided with our previous report.[4] Before treatment, plasma glucose, hemoglobin A_{1c} serum creatinine, blood urea nitrogen, disease duration, blood pressure, UAE, and the number of urinary podocytes differed little between the pioglitazone-treatment and placebo groups. Plasma glucose and hemoglobin A_{1c} in diabetes patients (179 ± 23 mg/dL and 8.2% ± 1.1%, respectively) were significantly higher than those in controls (82 ± 10 mg/dL and 4.6% ± 0.6%, respectively) ($P < .001$). Serum creatinine, blood urea nitrogen, blood pressure and 24-hour creatinine clearance differed little between diabetes patients and controls. After treatment, blood glucose and hemoglobin A_{1c} were significantly reduced in the pioglitazone-treatment group. Serum creatinine, blood urea nitrogen, and blood pressure were not changed in either groups (Table 1). UAE was reduced from 96.7 ± 50.5 μg/min to 39.7 ± 22.9 μg/min ($P < .01$), and the number of urinary podocytes was also reduced from 0.9 ± 1.0 cells/mL to 0.1 ± 0.2 cells/mL ($P < .001$) in the pioglitazone-treatment group (Fig 1). Neither UAE (from 79.4 ± 34.0 μg/min to 80.4 ± 36.2 μg/min) nor the number of urinary podocytes (from 1.1 ± 1.2 cells/mL to 1.1 ± 1.0 cells/mL) was affected in the placebo group (Fig 1).

DISCUSSION

Our data demonstrated that pioglitazone is effective in reducing urinary podocyte numbers in the early stage of diabetic

Table 2. Number of Urinary Podocytes (cells/mL) and Urinary Albumin Excretion (μg/min)

Pioglitazone			Placebo		
Patient	Podocyte	UAE	Patient	Podocyte	UAE
1	0.0	44	15	1.0	120
2	0.0	86	16	1.6	74
3	0.8	38	17	0.8	86
4	1.6	126	18	0.0	32
5	0.0	96	19	1.8	38
6	1.4	184	20	3.2	110
7	2.4	162	21	0.0	86
8	0.0	86	22	1.9	94
9	1.2	92	23	2.8	134
10	1.4	88	24	0.0	26
11	0.0	28	25	0.0	40
12	2.8	34	26	0.0	92
13	1.0	128	27	0.6	76
14	0.0	162	28	2.2	104

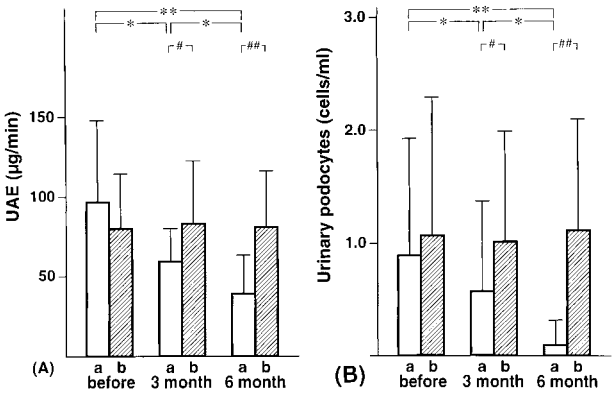

Fig 1. Changes in UAE (A) and the number of urinary podocytes (B) before and 3 months and 6 months after treatment with pioglitazone (a) (unshaded areas) or placebo (b) (shaded areas). Data are expressed as means ± SD. Pioglitazone treatment *v* placebo treatment, $P < .05$ and $P < .01$ before treatment with pioglitazone *v* after treatment with pioglitazone $*P < .05$ and $**P < .01$.

nephropathy. Disruption of podocytes contributes to the development of glomerular sclerosis.[10] Failure to replicate leads to an inappropriate response of podocytes to injury, resulting in progressive glomerular sclerosis.[11,12] Podocyte loss, or perhaps a low number of podocytes per glomerulus, contributes to the development and progression of diabetic glomerular sclerosis.[13] Meyer et al[14] reported the number of podocytes per glomerulus to be the strongest predictor of renal disease progression of glomerular morphologic characteristics analyzed. Recently, Lemley et al[15] have shown that developing podocyte insufficiency and ensuing alterations in podocyte foot processes or filtration slits could contribute to the progressive loss of glomerular size selectivity that occurs after the incipient, microalbuminuric form of nephropathy evolves to overt, macroalbuminuric nephropathy in type 2 diabetes patients. We previously reported that the detection of urinary podocytes is useful for estimation of the severity of active glomerular injury, and that urinary podocytes are detectable in early-stage diabetic nephropathy.[1,4]

Thiazolidinediones, including pioglitazone, are a particularly exciting new class of orally active drugs because they decrease insulin resistance by enhancing the action of insulin at the receptor cell level.[16] Yoshimoto et al[17] reported that pioglitazone is effective in correcting not only glucose metabolism, but also cardiovascular and renal complications in rats. Troglitazone, another thiazolidinedione, reduces microalbuminuria in patients with incipient diabetic nephropathy.[6] We recently reported that pioglitazone, but not glibenclamide or voglibose, reduces UAE and urinary endothelin (ET)-1 concentrations.[18] Some investigators have reported that podocytes can express the ET-1 gene and synthesize the protein under appropriate stimuli.[19,20] Lee et al[21] reported urinary ET-1 excretion in type 2 diabetes patients with microalbuminuria. Therefore, pioglitazone may ameliorate podocyte injury and reduce urinary ET-1 concentrations. In the preliminary studies, we found that other hypoglycemic agents, including glibenclamide and voglibose, did not reduce the number of urinary podocytes (data not shown). Thus, the reduction in urinary podocytes with pioglitazone may be due to the effect of the drug itself rather the effect of lowering blood glucose levels. The precise mechanisms are still unclear, however.

Recently, Isshiki et al[22] reported that pioglitazone prevented the activation of the diacylglycerol (DAG)-protein kinase C (PKC) pathway and activated DAG kinase in mesangial cells cultured under high glucose conditions, and that pioglitazone may be a new therapeutic agent for diabetic nephropathy. In addition to inhibitory action on PKC, an unknown action modulating the complex network of intracellular signaling pathways may ameliorate diabetes-induced glomerular dysfunction, including endothelial hyperpermeability, loss of negative charge of the glomerular basement membrane, and increased synthesis of certain growth factors.[23] However, little is known about the association between PKC and podocytes.

In the prerest study, there was no relationship between the degree of albuminuria and the number of urinary podocytes. It is likely that podocyturia and UAE are independent markers of glomerular injury in type 2 diabetes patients. However, we have previously reported that significant correlations were observed between the number of urinary podocytes and urinary protein excretion in patients with IgA nephropathy.[24] Lemley et al[25] have recently reported that as the podocyte number falls, increasing deformity of the foot processes results in progressive impairement of capillary wall hydraulic permeability, thereby accelerating the decline in glomerular filtration rate toward levels associated with end-stage renal failure. Further studies would be needed to clarify the relationship between podocyte loss and urinary protein excretion in various glomerular diseases.

In summary, pioglitazone reduced UAE and the number of urinary podocytes in type 2 diabetes patients with microalbuminuria. Thus, pioglitazone may be beneficial in the treatment of early-stage diabetic nephropathy.

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