Effect of Pioglitazone on the Early Stage of Type 2 Diabetic Nephropathy in KK/Ta Mice

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Pioglitazone (PIO) has preventive effects on impaired glucose tolerance (IGT) and urinary albumin excretion in diabetes. These effects in the early stage of diabetic nephropathy have not been fully described. Endothelial constitutive nitric oxide synthase (ecNOS) might be one of the mechanisms of glomerular hyperfiltration. The objective of the present study was to evaluate the effect of PIO, including the role of ecNOS on the early stage of diabetic nephropathy in KK/Ta mice. KK/Ta mice were given PIO (10 mg/kg/d) started at 12 or 16 weeks of age for 8 or 4 weeks, respectively. They were divided into 3 groups as follows: early treatment (n = 8), late treatment (n = 8), and control group (n = 12). The urinary albumin/creatinine ratio (ACR), fasting and casual blood glucose levels, ratio of glomerular and Bowman's capsule volume (GB ratio), and systemic blood pressure were measured as phenotypic characterizations. The ecNOS and iNOS protein expression in glomeruli were evaluated by immunofluorescence. PIO, especially early treatment, improved the ACR and the GB ratio, and ecNOS protein expression was decreased in the endothelium of glomerular vessels. The iNOS protein was not detectable. There were no significant changes in the levels of fasting and casual blood glucose and systemic blood pressure among all groups. We conclude that the effect of PIO on microalbuminuria might not be due to changing systemic blood pressure and blood glucose levels. It appears that the decrease of urinary albumin excretion might be related to improvement of glomerular enlargement, including hyperfiltration, since the levels of ecNOS protein were reduced by PIO in the glomerular vessels.

THIAZOLIDINEDIONES (TZDs), peroxisome proliferator-activated receptor (PPAR)γ agonists, such as troglitazone, pioglitazone, and rosiglitazone, are insulin-sensitizing agents.[1] It is generally considered that these drugs have preventive effects on impaired glucose tolerance (IGT) and urinary albumin excretion in diabetics.[2-4] In the kidney, PPARγ messages were found to be present in both the cortex and medulla and were localized predominantly in the inner medullary collecting ducts and renal medullary interstitial cells but not in the cortex.[5] However, previous reports have shown that TZDs ameliorate renal microcirculation, glomerular hyperfiltration, and mesangial expansion in diabetic nephropathy.[6-8] The mechanism of these effects has not been fully clarified.

The increase of renal perfusion and glomerular filtration rate (GFR) occurs early in the course of diabetic nephropathy. This feature was observed in experimental and clinical diabetes. Nitric oxide (NO) might be one of the causes of glomerular hyperfiltration.[9] NO is a paracrine mediator with a wide spectrum of physiologic actions, including the control of vascular tone such as vasodilatation, antithrombotic actions, cell cycle regulation, neurotransmission, signal transduction, and inflammation. It is produced from the conversion of L-arginine to L-citrulline by a family of enzymes known as NO synthase (NOS). NOS exists in 3 isoforms: neuronal NOS (nNOS, NOS1), inducible NOS (iNOS, NOS2), and endothelial constitutive NOS (ecNOS, NOS3).[10,11] All 3 NOS isoforms are observed in the kidney. nNOS protein and mRNA are found predominantly in the macula densa region of the distal tubules and renal nerves.[11,12] iNOS mRNA is detectable in most of tubular cells along the nephron.[13] ecNOS mRNA is typically expressed in endothelial cells along the renal vascular tree.[14] Veelken et al reported that early glomerular hyperfiltration was dependent on increased NO generation due to greater expression and activity of ecNOS in glomeruli and afferent arterioles.[15] Therefore, ecNOS might be a more important hemodynamic factor in the early stage of diabetic nephropathy.

The KK/Ta mouse is an inbred mouse strain established from Japanese native mice. This mouse spontaneously exhibits type 2 diabetes associated with hyperglycemia, glucose intolerance, hyperinsulinemia, mild obesity, and microalbuminuria.[16-18] Renal lesions in KK/Ta mice closely resemble those in human diabetic nephropathy.[19,20] The urinary albumin/creatinine ratio (ACR) in diabetic KK/Ta mice is 150 to 200 mg/g Cr at 16 weeks of age.[21] Glomeruli of KK/Ta mice show diffuse-type hyperplasia of mesangial areas with mesangial cell proliferation at 16 to 18 weeks of age.[19,21] Therefore, young KK/Ta mice are considered as a suitable model for the study of early stage of type 2 diabetic nephropathy in humans.

In the present study, we determined whether early or late treatment with pioglitazone (PIO) might improve microalbuminuria and also evaluated the role of ecNOS on morphometric changes treated with PIO, including those in the immunohistochemical analysis of ecNOS in KK/Ta mice, a spontaneous animal model for the early stage of type 2 diabetic nephropathy.

MATERIALS AND METHODS

Animal Experiments

Male hyperglycemic KK/Ta Jcl mice (7 weeks of age) were purchased from CLEA Japan (Tokyo, Japan). The mice were individually housed in plastic cages with free access to food (rodent pellet diet NMF; 348 kcal/100g, containing 5.5% crude fat) and water throughout the experimental periods. All mice were maintained in the same room under conventional conditions with a regular 12-hour light/dark cycle and controlled temperature at 24 ± 1°C.

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Reagents

Pioglitazone hydrochloride {5-[4-[2-(5-ethyl-2-pyridil)ethoxy]benzyl]-2,4-thiazolidinedione} was kindly provided by Takeda Chemical Industries (Osaka, Japan). Hyperglycemic KK/Ta mice were given PIO (10 mg/kg/d) injected intraperitoneally for 4 or 8 weeks. They were divided into 3 groups as follows: early treatment with PIO started at 12 weeks of age for 8 weeks (n = 8), late treatment with PIO started at 16 weeks of age for 4 weeks (n = 8), and saline treatment the control group (n = 12).

Phenotypic Characterizations

The ACR, ACR gain (shown by the increase in ACR from 12 to 20 weeks of age), body weight (BW), fasting and casual blood glucose levels, and serum immunoreactive insulin (IRI) levels were measured at 8, 12, 16, and 20 weeks of age. Glucose tolerance was evaluated by the intraperitoneal glucose tolerance test (IPGTT) at each age. Blood pressure was measured at 12, 16, and 20 weeks of age.

Urinary albumin and creatinine from causal samples were measured by enzyme-linked immunosorbent assay (Albuwell M and Creatinine Companion, Exocell, Inc, Philadelphia, PA). Serum IRI levels were measured by radioimmunoassay (Phadeseph Insulin RIA, Pharmacia Diagnostics, Uppsala, Sweden). IPGTT was performed by injecting glucose (2 g/kg in 20% solution) intraperitoneally in overnight-fasted mice.[22] Glucose levels in blood obtained from the retro-orbital sinus were measured at 0 (fasting blood glucose level) and 120 minutes after intraperitoneal glucose injection. Blood pressures were measured at 11 AM by a noninvasive tail cuff and pulse transducer system (Softron BP-98A, Tokyo, Japan) after the mice were externally prewarmed for 10 minutes at 38°C. At least 3 to 6 recordings were taken for each measurement. Standard deviations of less than 5.0 were defined for the blood pressure levels.[23,24]

Morphometric Analysis

All mice were sacrificed at 20 weeks of age. The kidneys were retrogradely perfused by saline via the abdominal aorta for 5 minutes at a pressure of about 150 mm Hg without prior flushing of the vasculature. After paraffin embedding, sections were cut at 3 μm and stained with periodic acid–Schiff (PAS) reagent. In PAS-stained light microscopic sections, at least 40 light microscopic midsections of glomeruli were used in this study. Glomerular (G) and Bowman's capsule (B) areas were carefully traced by hand. G areas and B areas were measured using a digitizer KS-400 Imaging System. The volume of G and B areas was calculated by the following formula: G (or B) volume = (β/k) (G area or B area)$^{3/2}$, where $\beta = 1.38$ is the shape coefficient and k = 1.1 is the size distribution coefficient for spheres based on Weibel's stereologic method.[25,26] The ratio of G/B volume was also calculated.

Immunofluorescence for ecNOS and iNOS

Indirect immunofluorescence of ecNOS and iNOS in renal tissue sections was performed with the following protocol. The 3-μm cryostat kidney sections were air-dried for 10 minutes and fixed in cold acetone for 10 minutes. To reduce the background, nonspecific binding was blocked by incubating with blocking solution (phosphate-buffered saline [PBS, pH 7.2] containing 2% bovine serum albumin, 2% fetal calf serum, and 0.2% fish gelatin) for 30 minutes. Sections were then incubated with the primary antibody diluted 1:200 in blocking solution and incubated at 4°C overnight. As the primary antibody (Ab), eNOS/NOS Type III and iNOS/NOS Type II (Transduction Laboratories, Lexington, KY) were used. The secondary Ab used the Tyramide Signal Amplification Kit with Alexa Fluor 488-tyramide (Molecular Probes, Inc, Eugene, OR). The sections were blocked by blocking solution for 30 minutes and then incubated with primary Ab diluted 1:200 in blocking solution at 4°C overnight. After washing with PBS (pH 7.2), the sections were then incubated with secondary Ab diluted 1:100 in amplification buffer with 0.0015% H_2O_2 at room temperature for 15 minutes. Intensities of fluorescence in at least 10 glomeruli from each mouse were quantitated using a digitizer KS-400 imaging system.

Statistical Analysis

All data were presented as the mean ± SE. Comparisons between 2 parameters were analyzed by Student's unpaired *t* test. Comparisons among 3 or more parameters were analyzed by 1-way analysis of variance (ANOVA). *P* values less than .05 were defined as statistically significant.

RESULTS

Phenotypic Characterizations of KK/Ta Mice Treated With PIO

The mean level of ACR at 20 weeks of age or ACR gain in the early treatment group was significantly lower than that in the controls and the late treatment group ($P \leq .05$). However, the levels of ACR or ACR gain were not significantly changed between the late treatment group and the controls (Fig 1A and B).

There were no significant changes in the levels of fasting and casual blood glucose among all groups (Fig 2A and Table 1). IGT evaluated by the IPGTT in both early and late treatment groups was significantly improved compared with that in controls ($P < .001$) (Fig 2B).

The serum IRI levels in both the early and late treatment groups were slightly decreased compared with those in the controls. However, there were no statistically significant changes in the levels of serum IRI among all groups (Table 1).

There were no significant changes in the levels of BW and systemic blood pressure among all groups (Tables 1 and 2).

Morphometric Analyses of KK/Ta Mice Treated With PIO

In the early treatment group, the G/B ratio at 20 weeks of age of KK/Ta mice was significantly lower than that in control and late treatment group ($P < .01$). This ratio in the late treatment group was significantly lower than that in the controls ($P < .01$) (Fig 3).

Immunofluorescent Stainings for ecNOS and iNOS

In immunofluorescence stainings, ecNOS protein in glomeruli appeared to be localized to the endothelium of preglomerular vessels and glomerular tufts. In the early treatment group, the intensity of ecNOS staining in KK/Ta mice at 20 weeks of age was significantly lower than that in control mouse glomeruli ($P < .001$). This intensity in the late treatment group was also significantly weaker than that in the control group ($P < .001$). However, ecNOS staining in the late treatment group was significantly stronger than that in the early treatment group ($P < .01$) (Figs 4 and 5). The iNOS protein was not detectable (data not shown).

DISCUSSION

In the present study, we demonstrated that PIO, one of the TZDs, ameliorates urinary ACR and IGT in diabetic KK/Ta mice without changing systemic blood pressure and blood glucose levels. One possible mechanism for these effects is that

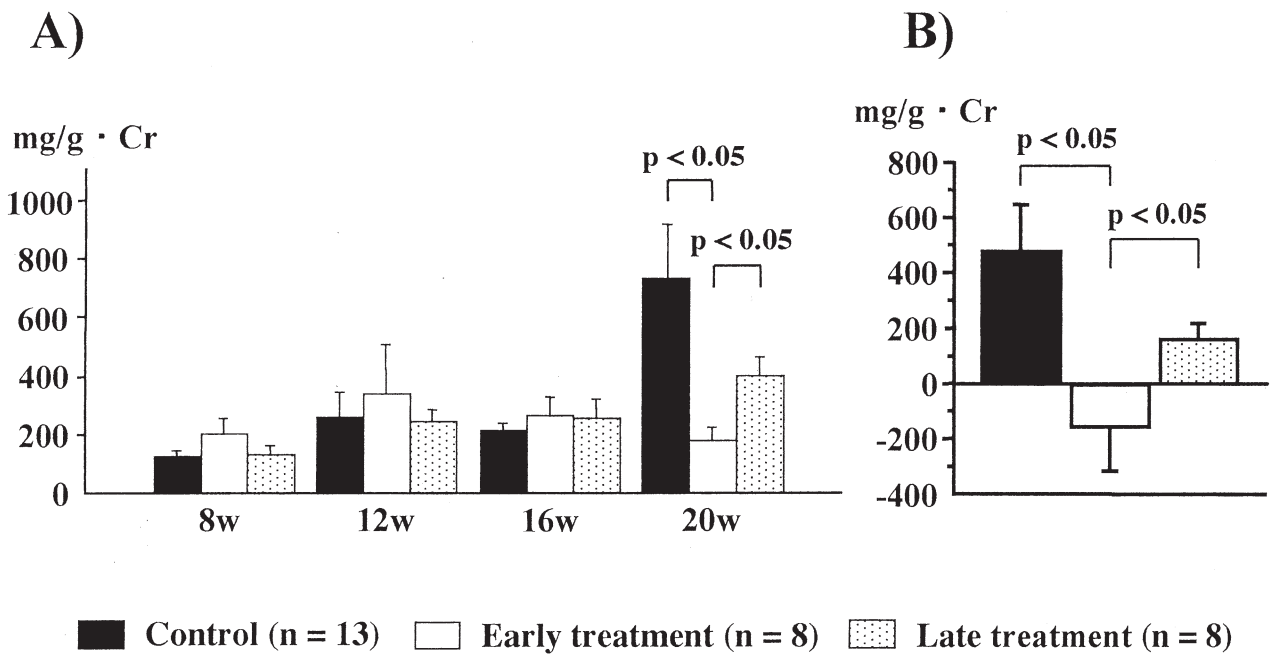

Fig 1. Mean level of (A) urinary ACR and (B) ACR gain in KK/Ta mice treated with PIO. ACR gain shows a increase of urinary ACR from 12 to 20 weeks of age. Data expressed as means ± SE.

TZDs prevent glomerular alterations such as mesangial expansion due to inhibition of the diacylglycerol (DAG)–protein kinase C (PKC)–extracellular regulated protein kinase (ERK) pathway.[6,7] Troglitazone (TRO) [(±)-5-[4-(6-hydroxy-2,5,7,8-tetramethylchroman-2-yl-methoxy)benzyl]-2,4-thiazolidinedione], another TZD, has not only the TZD moiety but also

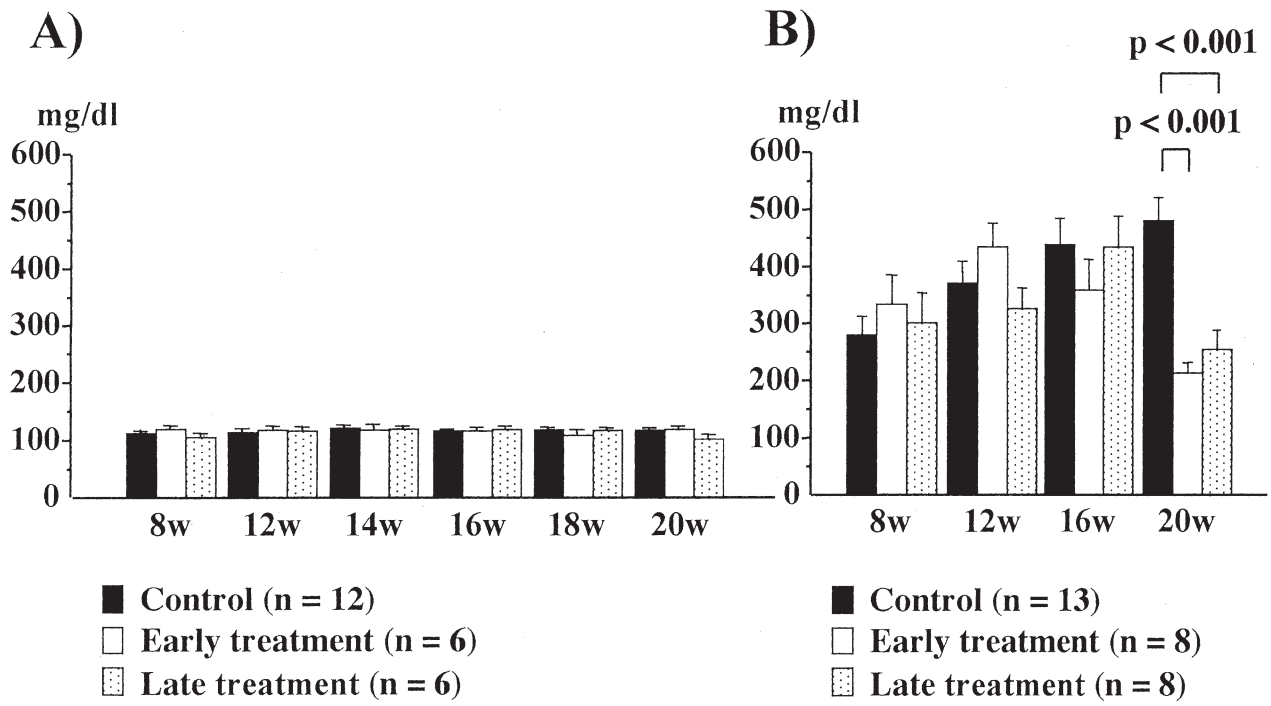

Fig 2. Mean level of blood glucose in KK/Ta mice treated with PIO. (A) Fasting blood glucose levels. (B) Impaired glucose tolerance (glucose levels at 120 minutes after intraperitoneal glucose injection). Glucose tolerance was evaluated by the IPGTT. Data expressed as means ± SE.

Table 1. Phenotypic Values of KK/Ta Mice Treated With PIO (I)

	Age (wk)	Control (n = 12-13)	Early Treatment (n = 6-8)	Late Treatment (n = 6-8)
Body weight (g)	8	31.0 ± 0.4	31.0 ± 0.5	31.0 ± 0.6
	12	35.8 ± 0.6	35.5 ± 0.5	35.4 ± 0.9
	16	39.1 ± 0.7	39.4 ± 0.8	39.4 ± 1.1
	20	41.3 ± 0.8	41.3 ± 0.9	42.1 ± 1.1
Casual blood glucose levels (mg/dL)	12	185.1 ± 12.4	194.5 ± 15.2	215.8 ± 20.9
	14	201.4 ± 18.3	204.7 ± 20.6	186.2 ± 16.1
	16	200.1 ± 17.3	183.5 ± 16.0	185.5 ± 18.2
	18	178.6 ± 14.0	139.7 ± 12.4	171.2 ± 9.6
	20	170.8 ± 11.0	180.8 ± 18.3	161.7 ± 9.3
Serum IRI levels before IPGTT* (μIU/mL)	8	10.0 ± 0.8	8.5 ± 0.6	9.5 ± 0.8
	12	15.0 ± 1.1	11.5 ± 1.3	15.9 ± 3.2
	16	12.7 ± 1.0	11.8 ± 1.9	11.0 ± 1.6
	20	12.3 ± 0.8	10.5 ± 1.0	9.6 ± 1.6
Serum IRI levels after IPGTT* (μIU/mL)	8	10.9 ± 0.7	10.9 ± 0.8	11.9 ± 1.6
	12	16.2 ± 1.3	15.4 ± 2.8	16.6 ± 3.0
	16	19.5 ± 5.7	13.3 ± 1.6	16.1 ± 4.3
	20	28.7 ± 10.1	14.7 ± 1.1	13.2 ± 0.8

NOTE. Data expressed as means ± SE.

*IRI was measured at 0 and 120 minutes after glucose administration.

α-tocopherol moiety, a chromane ring, in its structure.[27] α-Tocopherol was reported to be capable of preventing the activation of the DAG-PKC-ERK pathway and to improve early glomelular dysfunction in diabetic rats.[27] PIO does not have an α-tocopherol moiety. Recently, we compared gene expressions related to proliferating and glycating factors such as DAG, PKC, transforming growth factor (TGF)-β, fibronectin, and receptor for advanced glycation end products (RAGE) between treatment groups and controls in KK/Ta mouse kidneys using reverse-transcriptase–polymerase chain reaction (RT-PCR). There were no differences in changes in these mRNA levels between treatment groups and the controls (data not shown). Therefore, this suggests that amelioration of urinary ACR is independent of inhibition of the DAG-PKC-ERK pathway.

Systemic blood pressure is an important factor associated with progression of diabetic nephropathy.[28] Several reports have shown that TZDs decreased systemic blood pressure.[29,30] On the other hand, Ma et al[31] reported that TZDs have a renoprotective effect independent of systemic hypertension. Mesangial expansion is a structural change thought to occur later in the progression of diabetic nephropathy, relative to the volume of the glomerular capillary tuft and the gradual abrogation of the discrete organization of extracellular matrix domains in the glomerulus.[8,32] In this study, we observed blockade of glomerular expansion without changes in systemic blood pressure and inhibition of proliferating factors by morphometric analyses. Early stages of diabetic nephropathy are associated with increases in GFR and variable increases in renal plasma flow (RPF) and filtration fraction, both clinically and experimentally.[33] Several studies using the microperfusion,

Table 2. Phenotypic Values of KK/Ta Mice Treated With PIO (II)

	Age (wk)	Control (n = 12-13)	Early Treatment (n = 6-8)	Late Treatment (n = 6-8)
Heart rate (/min)	12	661.5 ± 21.1	647.2 ± 28.7	660.3 ± 26.9
	16	699.0 ± 18.8	673.1 ± 38.1	688.7 ± 33.7
	20	671.1 ± 15.0	658.4 ± 25.1	661.6 ± 44.3
Systolic blood pressure (mm Hg)	12	112.8 ± 3.2	108.8 ± 4.2	106.6 ± 3.1
	16	102.3 ± 5.2	95.5 ± 14.2	94.2 ± 8.3
	20	110.9 ± 3.6	107.1 ± 2.6	115.0 ± 5.1
Diastolic blood pressure (mm Hg)	12	73.5 ± 2.2	76.1 ± 3.8	70.5 ± 3.0
	16	78.0 ± 1.5	73.2 ± 4.8	69.2 ± 3.9
	20	71.1 ± 2.5	75.4 ± 2.3	78.0 ± 3.8
Mean blood pressure (mm Hg)	12	86.5 ± 2.3	87.0 ± 3.7	82.4 ± 2.6
	16	89.4 ± 1.6	85.0 ± 5.1	82.1 ± 4.2
	20	84.2 ± 2.7	85.9 ± 2.2	89.4 ± 3.8

NOTE. Data expressed as means ± SE.

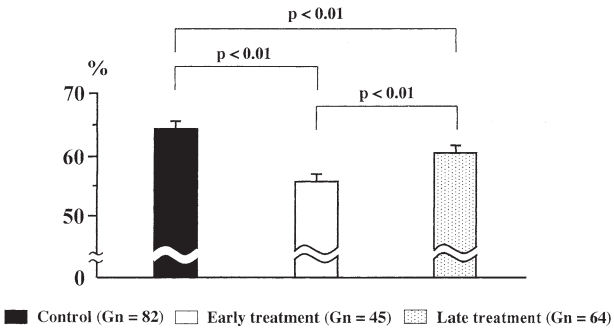

Fig 3. G/B ratio at 20 weeks of age. Gn, number of glomeruli. Data expressed as means ± SE.

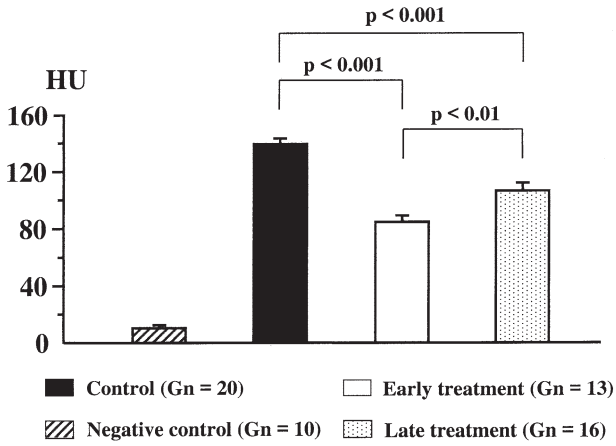

Fig 5. Mean intensities of fluorescence for ecNOS in glomeruli at 20 weeks of age. Gn, number of glomeruli; HU, Hounsfield unit. Data expressed as means ± SE.

GFR, and RPF have shown that the renal microcirculation and mesangial expansion are correlated to the glomerular enlargement in the early stage of diabetic nephropathy.[6,8,15] It was postulated that glomerular enlargement, including hyperfiltration, might be related to our morphometric analysis. Multiple factors have been reported to be involved in the development of glomerular hyperfiltration,[34] some of which might be associated with ecNOS activity. Komers and Anderson[11] noted that during the early phase of diabetic nephropathy, the balance of NO bioavailability is shifted toward NO. This plays a role in the development of characteristic hemodynamic changes and may contribute to subsequent structural alterations in glomeruli. Veelken et al[15] reported that early glomerular hyperfiltration was dependent on increased NO generation due to greater expression and activity of ecNOS in glomeruli and afferent

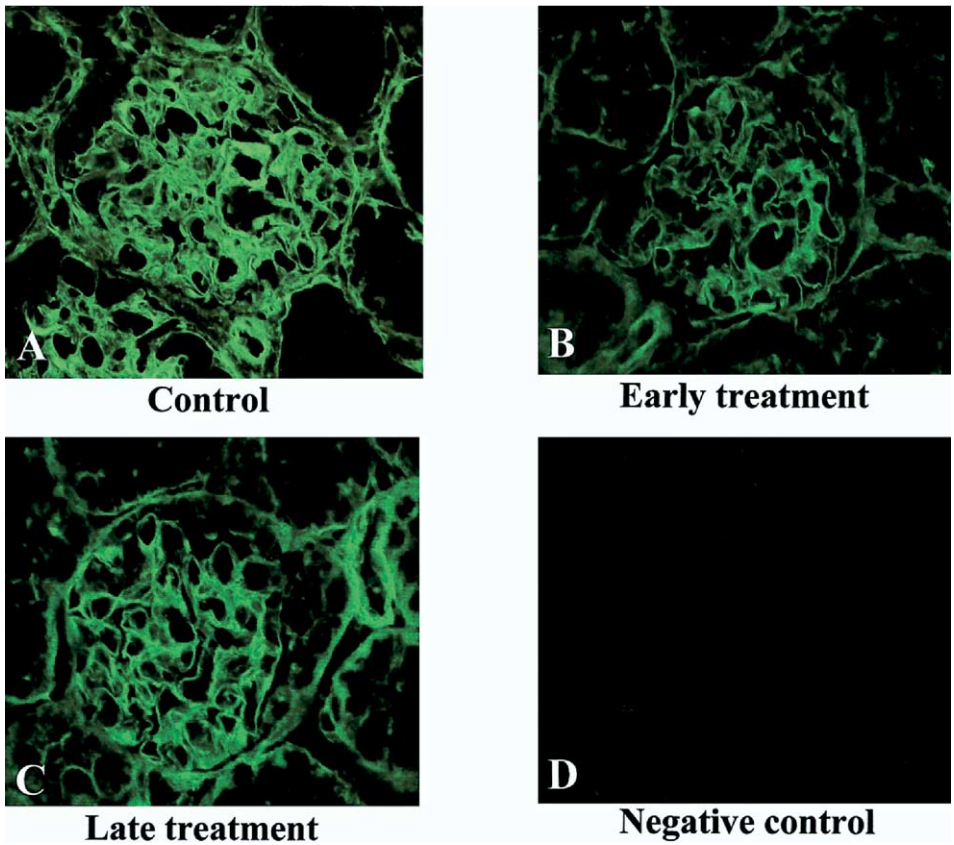

Fig 4. Immunofluorescent stainings for ecNOS in renal tissue sections. (A) Control group. (B) Early treatment group. (C) Late treatment group. (D) Negative control (control group without the primary antibody). Original magnification, ×400.

arterioles in untreated hyperfiltering diabetic rats. Choi et al[35] reported that renal cortical expression of all NOS isoforms were markedly increased in streptozotocin (STZ)-induced diabetic rats and medullary expression of NOS did not different from controls. Renal cortical expression of iNOS was reported to show no differences or to be barely detectable during the hyperfilteration stage of diabetic nephropathy.[15,36,37] The present study also showed that iNOS protein was not detectable, and there were no differences in changes at the levels of iNOS mRNA among all groups (data not shown).

In immunohistochemistry, we localized ecNOS protein in the endothelium of preglomerular arteries, arterioles, and glomerular tufts. Moreover, this positive staining in KK/Ta mice treated with PIO was less than that in control mice. The tubuloglomerular feedback mechanism is an important factor to evaluate glomerular hyperfiltration. Thomson et al[38] reviewed that kidney growth causes a primary increase in proximal reabsorption that is sufficient to classify diabetic hyperfiltration as a primary tubular event in early diabetes. Shear stress is one of the induced factors for ecNOS. Therefore, this global staining of ecNOS protein in glomeruli might be related to the shear stress. Calnek et al[39] reported that ciglitazone, another TZD, increased the levels of ecNOS mRNA in vitro. This result is in conflict with our findings. However, their investigation was not performed under high glucose conditions. In the present study, control KK/Ta mice exhibited hyperinsulinemia and glucose intolerance. In the early stage of diabetic nephropathy, a variety of receptor signaling pathways such as phosphatidylinositol 3-kinase (PI 3-kinase), Akt kinase (downstream effector of PI 3-kinase), and ERK1/2-type mitogen-activated protein (MAP) kinase are activated in the renal cortex.[40] Akt kinase has been identified as the kinase responsible for Ca^{2+}-independent activation of ecNOS and implicated in vascular endothelial cellular signaling of factors relevant to diabetic complications such as hyperinsulinemia and glucose intolerance.[41,42] Therefore, the effect of TZDs on ecNOS is complex and correlated with many important mechanisms in vivo. In vitro approaches only may miss some mechanisms that comodulate ecNOS activity. Our findings suggested that ecNOS has a key role in the early stage of type 2 diabetic nephropathy, and that PIO might improve glomerular enlargement including hyperfiltration due to a partial decrease in ecNOS expression in the early stage of this disease.

In conclusion, it appears that PIO, especially in early treatment, improved urinary albumin excretion. This effect of PIO might not be due to changes in systemic blood pressure and blood glucose levels. It is postulated that the decrease of urinary albumin excretion might be related to improvement of glomerular enlargement including hyperfiltration since the G/B volume ratio was improved and the levels of ecNOS protein in the glomerular vessels were reduced by PIO.

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