Transcriptional Regulation of Nephrin Gene by Peroxisome Proliferator–Activated Receptor-γ Agonist: Molecular Mechanism of the Antiproteinuric Effect of Pioglitazone

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The renoprotective potential of the peroxisome proliferator–activated receptor-γ (PPAR-γ) agonist pioglitazone was explored in an immune model of progressive nephropathy, passive Heymann nephritis (PHN), compared with that of an angiotensin II receptor antagonist, taken as standard therapy for renoprotection. PHN rats received orally vehicle, pioglitazone (10 mg/kg twice daily), or candesartan (1 mg/kg twice daily) from months 2 to 8. Pioglitazone reduced proteinuria as effectively as candesartan and limited renal functional and structural changes. Kidneys from untreated PHN rats showed lower nephrin mRNA and protein than controls, both restored by pioglitazone. The effect was seen both early and late during the course of the disease. Whether the antiproteinuric effect of pioglitazone could be due to its effect on nephrin gene transcription also was investigated. HK-2 cells were transfected with plasmids that harbor the luciferase gene under portions (2-kb or 325-bp) of human nephrin gene promoter that contain putative peroxisome proliferator–responsive elements (PPRE) and incubated with pioglitazone (10 μM). Transcriptional activity of luciferase gene was highly increased by pioglitazone, with the strongest expression achieved with the 325-bp fragment. Increase in luciferase activity was prevented by bisphenol A diglycidyl ether, a PPAR-γ synthetic antagonist. Electrophoretic mobility shift assay experiments showed a direct interaction of PPAR/retinoid X receptor heterodimers to PPRE present in the enhancer region of the nephrin promoter. In conclusion, pioglitazone exerts an antiproteinuric effect in immune-mediated glomerulonephritis as angiotensin II receptor antagonist does. Enhancement of nephrin gene transcription through specific PPRE in its promoter discloses a novel mechanism of renoprotection for PPAR-γ agonists.

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Peroxisome proliferator–activated receptors (PPAR) belong to the nuclear receptor superfamily of ligand-dependent transcriptional factors and consist of three isoforms—PPAR-α, PPAR-β, and PPAR-γ—that have been cloned and are differentially expressed in several tissues, including the kidney (1). PPAR play a key role in regulating adipogenesis, lipid metabolism, insulin sensitivity, immune response, and cell growth (1) and participate in the pathogenesis of a cluster of human diseases that are designated the metabolic syndrome, which includes insulin resistance, glucose intolerance, obesity, dyslipidemia, hypertension, atherosclerosis, and microalbuminuria (2). Two classes of synthetic PPAR activators have been developed and used in clinical practice. The PPAR-α activators, fibrates, effectively lower elevated plasma triglyceride and cholesterol levels and are first-line drugs for the treatment of primary hypertriglyceridemia and combined hyperlipidemia (3), whereas the PPAR-γ ligands thiazolidinediones improve insulin sensitivity and have great therapeutic potential for the control of type 2 diabetes (4). Preclinical studies showed that PPAR-γ ligands also reduced microalbuminuria and prevented glomerular hyperfiltration and extracellular matrix protein accumulation in streptozotocin-induced diabetic rats independent of their insulin-sensitizing action (5). The antiproteinuric effect was associated with protection from the development of glomerulosclerosis and tubular injury in obese Zucker rats with type 2 diabetes (6) and even in the remnant kidney model (7).

An antiproteinuric activity of pioglitazone also was documented in humans. PPAR-γ agonists were better than other oral antidiabetic agents in lowering microalbuminuria in patients with type 2 diabetes (8,9). Similar findings are available in patients with type 2 diabetics and overt nephropathy (10).

Some unique features of PPAR-γ ligands could explain renoprotection: the vasodilatory action on the renal microcirculation (11) and the direct influence on intracellular signaling systems that are involved in cell proliferation, inflammation, and extracellular matrix remodeling. In the model of renal mass reduction, PPAR-γ ligands' beneficial effect was found to be independent of insulin/glucose effects but instead associated with a

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multiplicity of actions on several transcription factors. Such effects included decreased plasminogen activator protein-1 (PAI-1) and TGF-β, which translated into less infiltration of interstitial cells of monocyte/macrophage type (7). None of the above mechanisms, however, explains the observed effect of PPAR-γ ligands' ameliorating glomerular permeability function observed in animal experiments and human studies.

Here we sought to investigate *in vivo* the pathophysiology of pioglitazone's renoprotection in an immune model of progressive nephropathy and elucidate *in vitro* the molecular basis of its property of ameliorating glomerular permeability to macromolecules. To the purpose, in *in vivo* studies, we used an accelerated model of passive Heymann nephritis (PHN). Rats were given pioglitazone in an advanced phase of the disease, when they already had proteinuria and renal structural injury. Transfection experiments of HK-2 cells that express PPAR-γ (12) with constructs that harbor a reporter gene under the control of portions of nephrin promoter that contain PPAR-γ putative consensus sequences were instrumental in unraveling the mechanism(s) underlying the effects of pioglitazone on the molecular barrier.

Materials and Methods

In Vivo *Studies*

Male Sprague-Dawley rats (Charles River Italia s.p.a., Calco, Italy; 275 to 330 g) were used. Animal care and treatment were conducted in accordance with the institutional guidelines that are in compliance with national (Decreto Legislativo n.116, Gazzetta Ufficiale suppl 40, 18 febbraio 1992, Circolare n.8, Gazzetta Ufficiale 14 luglio 1994) and international laws and policies (EEC Council Directive 86/609, OJL358-1, December 1987; Guide for the Care and Use of Laboratory Animals, US National Research Council, 1996). Animals were housed in a room with constant temperature and a 12-h dark/light cycle and had free access to standard diet (20% protein) and tap water. PHN was induced in nonanesthetized rats by a single intravenous injection of 0.4 ml/100 g body wt rabbit anti-Fx1A antibody. Unilateral nephrectomy at day 7, when animals were proteinuric, was performed to accelerate the onset of renal histologic damage (13). Two months later, rats were divided into three groups ($n = 8$ each) and treated daily up to 8 mo as follows: vehicle, 0.5% carboxymethylcellulose; pioglitazone (10 mg/kg twice daily by gavage; Takeda Chemical Industries, LTD, Osaka, Japan); or the angiotensin II (AngII) receptor antagonist candesartan (1 mg/kg twice daily by gavage; Takeda Chemical Industries, LTD) taken as standard therapy for renoprotection. Normal rats served as controls ($n = 6$). Doses of pioglitazone and candesartan were selected on the basis of previous reports in other models of renal disease (14,15).

Proteinuria was determined in 24-h urine samples by modified Coomassie blue G dye-binding assay for proteins with BSA as standard (16). Serum creatinine was measured using an autoanalyzer (CX5; Beckman Instruments Inc., Fullerton, CA).

Renal Histology. Kidneys were fixed overnight in Duboscq-Brazil, dehydrated in alcohol, and embedded in paraffin. Kidney sections (3 μm) were stained with hematoxylin and eosin and periodic acid-Schiff reagent. The extent of glomerular damage evaluated on 100 glomeruli was expressed as the percentage of glomeruli that presented sclerotic lesions. Tubular changes (atrophy, casts, and dilation) and interstitial inflammation and fibrosis were graded from 0 to 4+ (0, no changes; 1+, changes affecting <25% of the sample; 2+, changes affecting 25 to 50% of the sample; 3+, changes affecting 50 to 75% of the sample; 4+, changes affecting 75 to 100% of the sample). Data were expressed as mean score values for tubular and interstitial lesions, respectively, for each animal. All renal biopsies were analyzed by the same pathologist, who was unaware of the nature of the experimental groups.

Interstitial Inflammation. Mouse mAb was used for the immunohistochemical detection of ED-1 antigen on rat monocytes/macrophages (Chemicon, Temecula, CA). Detection of ED-1 antigen was performed on paraffin sections using an alkaline phosphatase–Fast Red technique. Positive cells were counted in at least 20 randomly selected high-power microscopic fields (×400) per each animal.

Quantitative Real-Time PCR. Whole-kidney RNA was isolated using TRIzol Reagent (Invitrogen, San Diego, CA). Contaminating genomic DNA was removed by RNase-free DNAse (Promega, Ingelheim, Germany) for 1 h at 37°C. Two micrograms of purified RNA was reverse-transcribed using random hexamers (50 ng) and 200 U of SuperScript II RT (Life Technologies, San Giuliano Milanese, Italy) for 1 h at 42°C. No enzyme was added for reverse transcriptase–negative controls. Real-time PCR amplification was performed with ABI PRISM 5700 Sequence Detection System (PE Biosystems, Warrington, UK) using SYBR Green PCR Master Mix (PE Biosystems) as described previously (17).

The following specific rat primers were used: TGF-β1 (300 nM), forward 5′-GCTGAACCAAGGAGACGGAAT-3′, reverse 5′-AAGAGCAGTGAGCACTGAAGC-3′; MCP-1 (300 nM), forward 5′-CAGATGCAGTTAATGCCCCA-3′, reverse 5′-TCTTGCCAGTGAATGAGTAGCAG-3′; nephrin (300 nM), forward 5′-GGAGGACAGGATCAGGAATGAA-3′, reverse 5′-CCCGGTCCCCAGTCCA-3′; zonula occludens (ZO-1; 300 nM), forward 5′-ACACTGTGACCCTAAAACTTGGC-3′, reverse 5′-TTCGGATCTCCAGGAAGACACT-3′; and 18S (50 nM), forward 5′-ACGGCTACCACATCCAAGGA-3′, reverse 5′-CGGGAGTGGGTAATTTGCG-3′.

Evaluation of Nephrin and ZO-1 Expression. Nephrin and ZO-1 expression was detected by immunoperoxidase analysis as described previously (18). Primary goat polyclonal anti-nephrin antibody that recognizes the amino terminus of human nephrin (Santa Cruz Biotechnology, Santa Cruz, CA; 1:100 dilution) and rabbit anti-human ZO-1 antiserum (Zymed Laboratories Inc., San Francisco, CA; 1:25 dilution) were used. Negative controls were obtained by omitting the primary antibody on adjacent sections. The glomerular signal intensity was graded on a scale from 0 to 3 (0, no staining; 1, weak; 2, moderate; 3, strong intensity). The final scores ($S_{nephrin}$ and $S_{ZO\text{-}1}$) then were calculated as the weighted mean:

$$S=\frac{(N_1\times 0+N_2\times 1+N_3\times 2+N_4\times 3)}{(N_1+N_2+N_3+N_4)}$$

where Ni (i = 1 to 4) is the number of glomeruli in each category. Seventy glomeruli per section on average were evaluated by an observer who was blind to the nature of the experimental groups.

In Vitro *Studies*

Cell Culture. HK-2 is a permanent, well-characterized human proximal tubular cell line (19) (American Type Culture Collection, Rockville, MD). They were grown at 37°C in a humidified 5% CO_2 incubator in DMEM/F-12 as described (19,20).

Transfection Experiments and Luciferase Assay. Cells were seeded on 12-well plates and transiently transfected in complete DMEM/F-12 for 3 h by SuperFect Transfection Reagent (Qiagen, Milan, Italy) according to the manufacturer's protocol. To evaluate transfection efficiency, we transfected cells with 1 μg/well pCMVβ-gal. β-Galactosidase (β-gal) activity was evaluated 48 h after transfection in cell lysate by a chemiluminescent assay (Clontech, Milan, Italy) or on intact cells by *in situ* β-gal staining (Stratagene, Amsterdam, The Nether-

lands). For luciferase assay, the following *Firefly* luciferase reporter plasmids were used: the pGL2-promoter vector that harbors the 2-kb promoter region of the human nephrin gene (1 μg/well) or the 325-bp region (1 μg/well) (21). The pRL-TK vector (20 ng/well) encoding the *Renilla* luciferase gene was co-transfected simultaneously to correct the transfection efficiency. After co-transfection, the cells were maintained in DMEM/F-12/0.5% FCS for 24 h followed by stimulation with 10 μM pioglitazone for 3, 6, and 24 h. For inhibition of the PPAR-γ activity, cells were preincubated with 25 μM bisphenol A diglycidyl ether (BADGE; Sigma-Aldrich, Milan, Italy) for 24 h followed by 3 h of incubation with 10 μM pioglitazone. Cells then were lysed in the Passive Lysis Buffer (Promega, Madison, WI) according to the manufacturer's instruction. *Firefly* and *Renilla* luciferase activities were measured using Dual-Luciferase reporter assay system (Promega) and Lumat LB9507 (Berthhold Technologies, Wildbad, Germany). The luciferase activity was calculated as the ratio of *Firefly* luciferase activity to *Renilla* luciferase, and data are expressed as percentage of control.

Preparation of Nuclear Extracts and Electrophoretic Mobility Shift Assay. Nuclear extracts were prepared from HK-2 cells with the NE-PER nuclear and cytoplasmic extraction reagents kit (Pierce/Celbio, Pero, Italy). For minimization of proteolysis, buffer contained protease inhibitor cocktail (Roche, Mannheim, Germany). The protein concentration was determined by Bradford assay using the Bio-Rad protein assay reagent.

Electrophoretic mobility shift assays (EMSA) were performed with a typical PPAR response element (Santa Cruz; 5′-CCGGCAAAACT*AGGTCA*A*AGGTCA*) or a putative PPAR response element derived from the nephrin gene (5′-CCGG*AGATGGATCACCT*G*AGGTCA*GG*AGTTCG*). Nuclear extracts (3 μg) were incubated with 50 kcpm of ^{32}P-labeled oligonucleotide in a binding reaction mixture (10 mmol/L Tris-HCl [pH 7.5], 80 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L dithiothreitol, 5% glycerol, and 1.5 μg of poly [dI-dC]) for 30 min on ice. Then, the DNA–protein complexes were electrophoresed on a nondenaturing 5% polyacrylamide gel. In competition studies, a 100-fold molar excess of unlabeled oligonucleotide was added to the binding mixture before the addition of labeled probes.

Statistical Analyses

Data are expressed as mean ± SE. The analysis of covariance was used for log-transformed proteinuria, which allowed the inclusion of proteinuria that was measured before treatment at month 2 (baseline) as a covariate in the model, followed by Bonferroni adjustment for multiple comparisons. All of the other data were analyzed using the nonparametric Kruskal-Wallis test for multiple comparisons or ANOVA followed by the Fisher test for multiple comparisons. The statistical significance level was defined as $P < 0.05$.

Results

Body Weight and Systemic Parameters

During the study, two PHN rats that received vehicle died. PHN rats gained less weight than controls despite comparable food intake, but body weight values at the end of the study did not differ significantly (Table 1).

Serum cholesterol and triglyceride levels were higher than controls in PHN rats that were given vehicle (Table 1). Pioglitazone reduced hypercholesterolemia at the same extent as candesartan, but values remained higher than those of controls. Neither drug affected serum triglyceride levels (Table 1).

Proteinuria, Renal Function, and Structure

Rats with PHN exhibited severe proteinuria, with values exceeding 350 mg/d in all groups before treatment, at month 2 (Table 2). In rats that were given vehicle, proteinuria levels increased over time, reaching 873 ± 101 mg/d at the end of the study. Administration of pioglitazone remarkably limited the development of proteinuria, which was reduced on average by 50%, compared with animals that received vehicle ($P < 0.01$). The antiproteinuric effect of PPAR-γ agonist was almost comparable to that of candesartan, which reduced urinary protein excretion by 46% in respect to vehicle ($P < 0.01$).

Renal function was impaired in PHN rats that were given vehicle, as indicated by a significant ($P < 0.01$) elevation in serum creatinine levels as compared with control rats (Table 1). Pioglitazone significantly limited renal function deterioration ($P < 0.05$ *versus* vehicle).

Untreated PHN rats showed focal and segmental glomerulosclerosis affecting on average 51% of glomeruli (Figure 1A). Tubulointerstitial changes consisted of interstitial fibrosis and inflammation associated with tubular atrophy and large eosinophilic casts in the tubular lumen (score 1.58 ± 0.33). Treatment with pioglitazone limited glomerulosclerosis (Figure 1A) and tubular damage (score 0.83 ± 0.16; $P < 0.05$ *versus* vehicle). Candesartan reduced glomerulosclerosis (Figure 1A) and tubular damage (0.75 ± 0.13) in a comparable manner. Biopsies from age-matched controls showed few glomeruli with sclerotic changes (1.7 ± 0.6%) and mild tubular damage (score 0.2 ± 0.1).

A massive accumulation of ED-1–positive monocytes/macrophages was present in the renal interstitium of PHN rats that were given vehicle (Figure 1B). Pioglitazone remarkably lim-

Table 1. Systemic parameters[a]

Groups	Body Weight (g)	Serum Creatinine (mg/dl)	Serum Cholesterol (mg/dl)	Serum Triglycerides (mg/dl)
PHN + vehicle	610 ± 42	2.88 ± 1.22[b]	147 ± 19[b]	239 ± 29[b]
PHN + pioglitazone	629 ± 26	0.95 ± 0.14[b,c]	123 ± 12[b]	246 ± 32[b]
PHN + candesartan	622 ± 23	0.78 ± 0.05[b,c]	124 ± 13[b]	255 ± 54[b]
Control	671 ± 44	0.57 ± 0.01	51 ± 5	167 ± 27

[a]Values are expressed as mean ± SE. PHN, passive Heymann nephritis.
[b]$P < 0.01$ *versus* control.
[c]$P < 0.05$ *versus* vehicle.

Table 2. Time course of proteinuria[a]

Group	Proteinuria (mg/d)			
	2 Mo (Before Treatment)	4 Mo	6 Mo	8 Mo
PHN + vehicle	352 ± 68	652 ± 99	841 ± 102	873 ± 101
PHN + pioglitazone	355 ± 61	324 ± 53[c]	345 ± 60[c]	436 ± 66[c]
PHN + candesartan	353 ± 61	434 ± 80[b]	396 ± 89[b]	472 ± 97[c]
Control	21 ± 1	26 ± 3	33 ± 8	34 ± 13

[a]Values are expressed as mean ± SE.
[b]$P < 0.05$ *versus* vehicle.
[c]$P < 0.01$ *versus* vehicle.

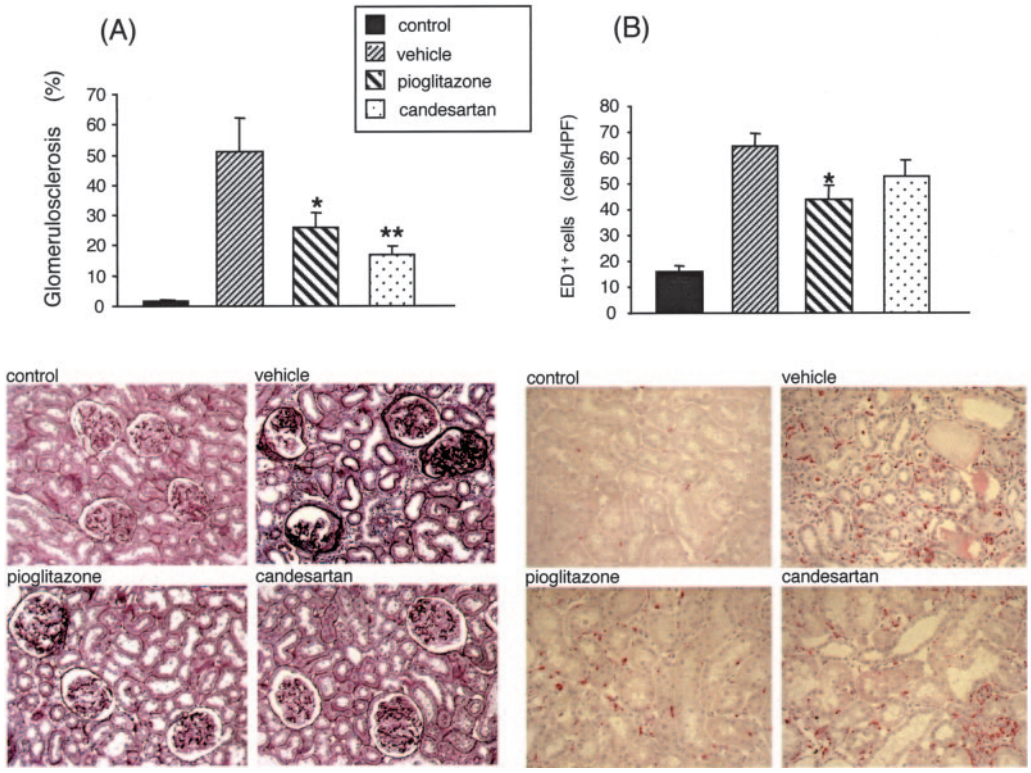

Figure 1. Effect of pioglitazone and candesartan on glomerular injury and interstitial inflammation in rats with passive Heymann nephritis (PHN). Percentage of glomeruli affected by sclerosis (A, top) and number of interstitial ED-1–positive cells counted in randomly selected high-power fields (HPF; ×400; B, top) in control animals and in PHN rats that were administered vehicle, pioglitazone, or candesartan. Values are expressed as mean ± SE. *$P < 0.05$, **$P < 0.01$ *versus* vehicle. All PHN groups *versus* control: $P < 0.01$. Representative photomicrographs of renal morphology (A, bottom) and interstitial accumulation of ED-1–positive cells (B, bottom) in control and PHN rats. Magnification, ×100.

ited the number of infiltrating monocyte/macrophages ($P < 0.05$ *versus* vehicle) to values that were numerically lower than those of candesartan (Figure 1B).

Renal Expression of Monocyte Chemoattractant Protein-1 and TGF-β mRNA

Upregulation of monocyte chemoattractant protein-1 (MCP-1) mRNA was observed in the kidney of PHN rats that were given vehicle (six-fold increased over age-matched controls; $P < 0.01$; Figure 2). MCP-1 overexpression was significantly ($P < 0.01$ *versus* vehicle) reduced by pioglitazone (Figure 2). The same effect was observed when animals were treated with candesartan. As previously reported (22), renal expression of TGF-β significantly increased in PHN rats that were given vehicle ($P < 0.01$ *versus* controls; Figure 2). Treatment with pioglitazone or candesartan limited the exaggerated expression of TGF-β in PHN rats ($P < 0.01$ and $P < 0.05$ *versus* vehicle, respectively).

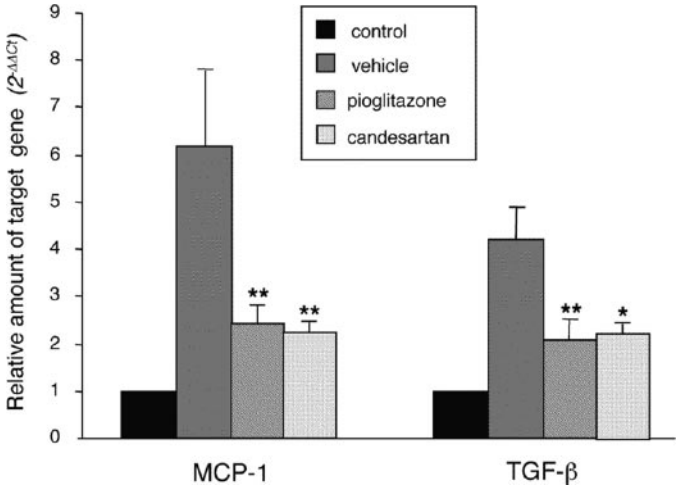

Figure 2. Modulation of inflammatory and fibrotic genes by pioglitazone and candesartan. Expression of monocyte chemoattractant protein-1 (MCP-1) and TGF-β mRNA evaluated by quantitative real-time PCR in control rats and in untreated and treated PHN rats. Values are expressed as mean ± SE. *$P <$ 0.05, **$P < 0.01$ *versus* vehicle. All PHN groups *versus* control: $P < 0.01$.

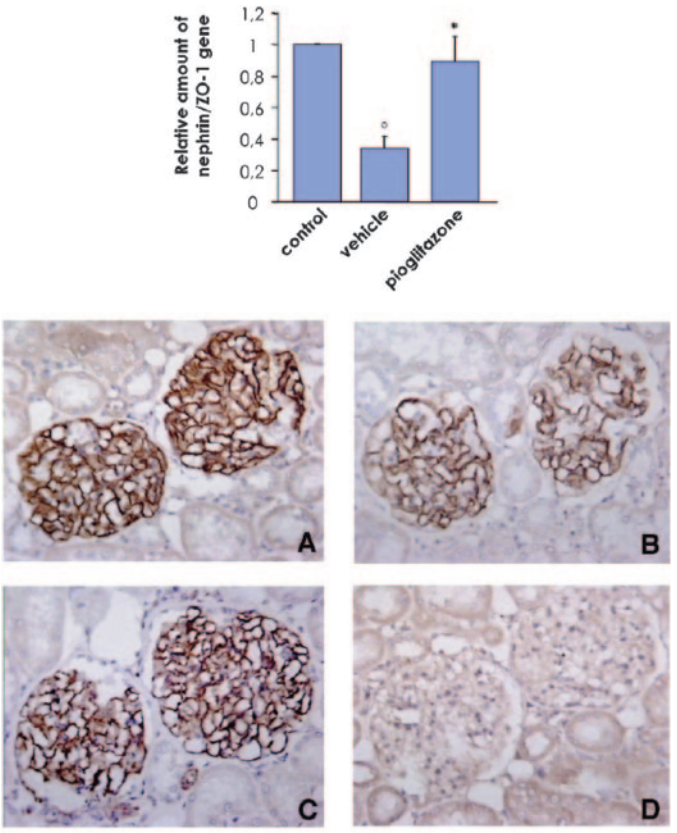

Figure 3. Changes of nephrin mRNA and protein expression by pioglitazone. Nephrin mRNA expression evaluated by quantitative real-time PCR in control, untreated, and treated PHN rats (top). °$P < 0.05$ *versus* controls; *$P < 0.05$ *versus* vehicle. Representative photomicrographs of nephrin localization by immunoperoxidase methods (bottom). Nephrin staining (brown signal) was localized in epithelial glomerular cells with a linear-like pattern in control rat (A). A reduced and discontinuous signal was observed in PHN rats that were given vehicle (B). Nephrin expression was increased by treatment with pioglitazone (C). No specific signal was obtained when the primary antibody was omitted (D). Magnification, ×250.

Pioglitazone Selectively Affects Glomerular Nephrin Expression

Consistent with previous data (18), immunohistochemistry showed a significant ($P < 0.01$) reduction of nephrin protein staining in glomeruli of PHN rats that were given vehicle (score 1.3 ± 0.23; Figure 3B) with respect to controls (score 2.87 ± 0.02; Figure 3A). A discontinuous signal along the capillary wall characterized their glomeruli. Pioglitazone (score 2.25 ± 0.21; Figure 3C) effectively restored nephrin protein expression ($P <$ 0.05 *versus* vehicle). Nephrin staining was completely abrogated when the primary antibody was omitted, indicating staining specificity (Figure 3D). At variance with nephrin, no significant changes in the podocyte protein ZO-1 was found in rats with PHN as compared with controls (score 1.99 ± 0.18 *versus* 2.36 ± 0.12). In pioglitazone-treated rats, the score for ZO-1 averaged 2.27 ± 0.11.

Nephrin mRNA expression then was assessed by quantitative real-time PCR. Because the relative amount of nephrin mRNA in whole kidney might be affected by morphologic alterations, expression of nephrin was factored for ZO-1 mRNA taken as the standard for the amount of podocyte mRNA. Consistent with protein expression, PHN rats that were given vehicle showed a reduction in nephrin mRNA with respect to controls ($P < 0.05$; Figure 3, top). Pioglitazone was effective ($P < 0.05$ *versus* vehicle) in restoring nephrin mRNA to almost control levels (Figure 3, top).

In additional experiments that were carried out at an early stage of PHN (7 d after disease induction), the intensity of nephrin staining was reduced in PHN by 16% as compared with controls (score 2.44 ± 0.08 *versus* 2.9 ± 0.09). Pioglitazone treatment restored nephrin protein expression to a level similar to controls (score 2.79 ± 0.09).

Transcriptional Activity of Promoter Region of Human Nephrin Gene

We first searched for the presence of putative consensus sequence for PPAR/retinoid X receptor (RXR) heterodimers in the recently identified nephrin gene promoter (23). Plasmids that harbored the 2-kb of the 5′-genomic sequence flanking the transcription start site of human nephrin gene and the 325-bp fragment covering the region from #-1060 through #-735 (the number of the nucleotides are referred to the sequence reported by Wong *et al.* [23]) contained nine and one putative consensus sequences for PPAR/RXR heterodimers, respectively. We therefore evaluated the possible effect of pioglitazone on nephrin gene transcription. To this purpose, HK-2 cells that express PPAR-γ were transduced with both plasmids, and the transfection efficiency was assessed detecting both the β-gal activity in the cell lysate through a chemiluminescent assay and the X-gal staining. β-gal activity was on average 5×10^6 relative light

units/mg protein in mock cells, and it increased to 1×10^9 relative light units/mg protein in transfected cells. X-gal staining revealed that almost 50% of cells were transduced.

Next, we evaluated the effect of various concentrations (5, 10, and 20 μM) of pioglitazone on the nephrin promoter. The transcriptional activity of the 325-bp promoter region was augmented by pioglitazone in a dose-dependent manner and was maximal with 10 μM after 3 h of exposure (data not shown). Therefore, the subsequent experiments were performed using 10 μM pioglitazone.

Time-course experiments showed that pioglitazone induced a significant increase in transcriptional activity that peaked at 3 h and remained high at 6 and 24 h. The strongest expression was achieved in the presence of the 325-bp fragment of the nephrin promoter ($P < 0.001$; Figure 4A), although significantly ($P < 0.05$) increased luciferase activity also was found with the 2-kb plasmid at 3 h. To evaluate whether the effect of pioglitazone was specific, cells were incubated with the PPAR-γ inhibitor BADGE (25 μM) for 24 h, before induction by pioglitazone. As shown in Figure 4B, BADGE significantly ($P < 0.0001$) prevented the enhanced transcriptional activity that was induced by pioglitazone.

To demonstrate that pioglitazone-induced activation of PPAR/RXR resulted in the binding of heterodimers to peroxisome proliferator–responsive elements (PPRE) present in the enhancer region of the nephrin promoter, we performed EMSA experiments with two different consensus sequences for PPRE in nuclear extracts from HK-2 cells. As shown in Figure 5, pioglitazone activated the binding of PPAR-γ to PPRE in a time-dependent manner. PPAR/RXR heterodimers that were activated by pioglitazone were able to bind both the typical consensus sequence of PPRE (5′-CCGGCAAAACT*AGGTCA*A*AGGTCA*) and a putative consensus sequence that was detected in the human nephrin promoter (5′-CCGG*AGATGGATCACCT*G*AGGTCA*GG*AGTTCG*).

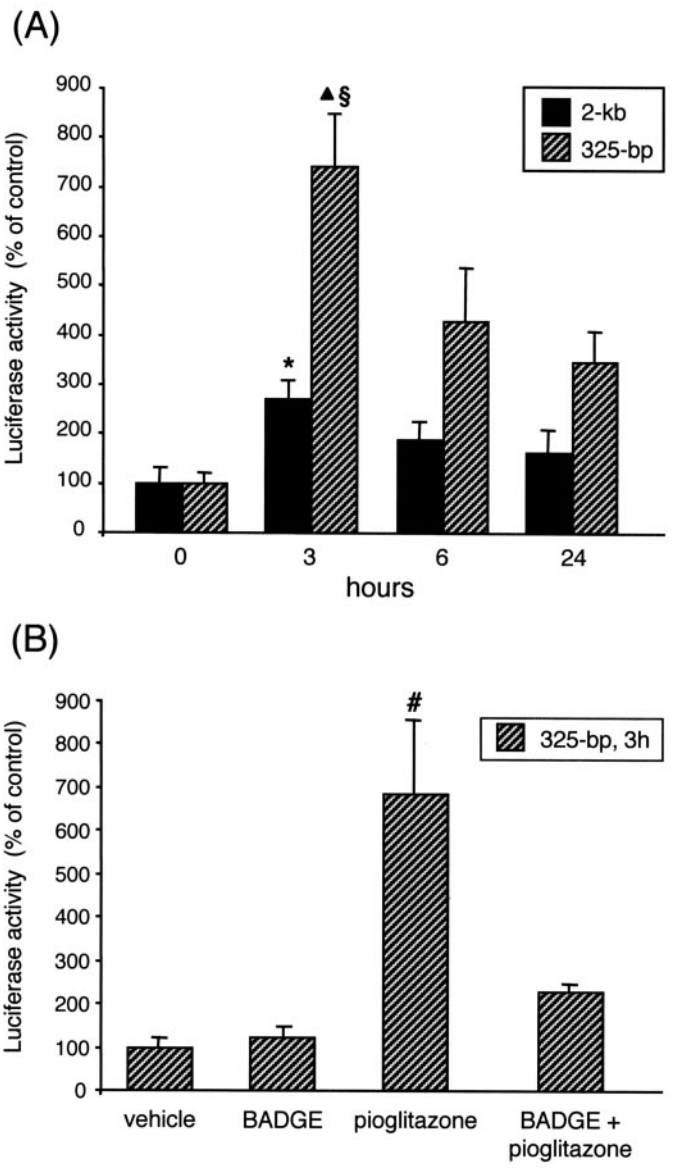

Figure 4. Luciferase activity induced by the 2-kb and the 325-bp regions of the human nephrin promoter. (A) HK2 cells that were transfected with the *Firefly* luciferase reporter plasmid that harbors the 2-kb or the 325-bp region were stimulated with 10 μM pioglitazone. The pioglitazone-induced transcriptional activity was evaluated at various times. (B) Luciferase assay was performed on HK-2 cells that were transfected with the plasmid that harbors the 325-bp promoter region in the presence of 25 μM bisphenol A diglycidyl ether (BADGE). Transfection efficiency was adjusted by the activity of the co-transfected *Renilla* luciferase. The results are shown as the ratio of *Firefly* to *Renilla* luciferase activity and expressed as percentage of control (mean ± SE; $n = 3$; $^{\S}P < 0.05$ *versus* 24 h; $^{*}P < 0.05$ *versus* vehicle; $\Delta P < 0.001$ *versus* vehicle; $^{\#}P < 0.0001$ *versus* vehicle, BADGE, and BADGE + pioglitazone).

Discussion

In this study, we found that in the model of accelerated PHN in the rat, which resembles late stages of membranous nephropathy in humans, where the disease has a rapidly progressive course, pioglitazone limited proteinuria and prevented renal function deterioration. The renoprotective effect of pioglitazone was comparable to that of the AngII blocker candesartan. Both treatments started at 2 mo, when rats had overt nephropathy that mimics the clinical condition of patients who are referred late in the course of the disease and are less susceptible to therapeutic interventions.

Pioglitazone's renoprotection in PHN rats somehow was independent of the effect on the lipids, to the extent that only a mild reduction of hypercholesterolemia was observed during treatment. Previous studies by us documented that in PHN, increased urinary protein excretion over time was associated with a remarkable increase in renal NF-κB activity, which was almost completely suppressed by reducing proteinuria with lisinopril (24). NF-κB activation was paralleled by upregulation of MCP-1 mRNA and interstitial accumulation of monocytes/macrophages and CD8-positive T cells. Here we found that pioglitazone reduced renal overexpression of MCP-1 and TGF-β mRNA, which suggests the possibility that PPAR-γ agonists, by reducing urinary protein excretion, also limit the

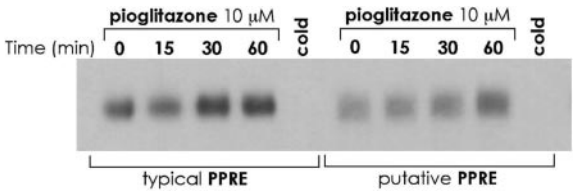

Figure 5. Effect of pioglitazone on peroxisome proliferator–activated receptor-γ (PPAR-γ) DNA-binding activity. Electrophoretic mobility shift assay determination of PPAR-γ binding activity was performed in nuclear extracts from HK2 cells that were exposed for various times to pioglitazone 10 μM, using a typical PPAR responsive element or a putative peroxisome proliferator–responsive element (PPRE) derived from nephrin promoter gene. For confirmation of the specificity of binding reaction, a 100-fold molar excess of the typical or putative PPRE-unlabeled (cold) nucleotides was used to compete with the labeled probes for binding to nuclear protein.

deleterious sequence of events that are elicited by proteinuria at the tubulointerstitial level. A similar mechanism has been proposed for rapamycin, which ameliorated the tubulointerstitial disease that was associated with chronic proteinuria by lowering the expression of proinflammatory and profibrotic genes, including MCP-1 and TGF-β (25). That thiazolidinedione can limit inflammatory responses rests on the observation in a different experimental setting that rosiglitazone significantly diminished stretch-induced NF-κB activation, MCP-1 production, and monocyte chemotaxis (26). Debate exists, however, on whether PPAR-γ ligands are indeed consistently capable of lowering cytokine and chemokine expression and reducing inflammation, because other studies rather found a proinflammatory effect of this class of molecules in models of experimental glomerulonephritis (27).

In a previous study in rats with PHN, we documented a progressive decrease of renal nephrin mRNA expression with time, which was paralleled by reduced immunostaining of the corresponding protein (18). Results of our study confirmed that rats with accelerated PHN and progressive disease had less renal nephrin mRNA and protein than controls. Pioglitazone's renoprotection was associated with almost normalization of nephrin, a crucial component of the slit diaphragm ultrafiltration barrier, which opposes to protein trafficking throughout. Compared with AngII receptor antagonist, pioglitazone was equally potent on both parameters. This is the first demonstration, to the best of our knowledge, linking pioglitazone's renoprotection to its capacity to restore slit diaphragm integrity *via* nephrin re-expression. That pioglitazone also could interfere with components of the glomerular barrier cannot be excluded in view of the observation that pioglitazone prevented the loss of anionic sites of the glomerular basement membrane in streptozotocin-induced diabetic spontaneous hypertensive rats (28). These data are particularly robust in that they were obtained when the disease was already established, which is at variance with most of the studies that have been reported so far (7,29). The finding that pioglitazone restored nephrin expression at an early stage of the disease, *i.e.*, in the acute model, supports the possibility that the drug may exert renoprotective action through a direct effect on the slit diaphragm protein nephrin.

We next addressed the mode of action that is exerted by pioglitazone at the level of podocyte nephrin and its molecular target. Previous studies showed that nephrin expression can be regulated directly by all-trans retinoic acid after binding to the nuclear receptor retinoic acid receptor–RXR complex, which recognizes putative retinoic acid response elements in the promoter region of nephrin gene (21). RXR do not function alone but rather serve as master regulators of other regulatory pathways, including that induced by PPAR (30). Transcription of target genes indeed can be accomplished through the PPAR/RXR heterodimers after activation by either of specific ligands (31). Therefore, we hypothesized that pioglitazone could activate nuclear receptors that recognize specific PPRE in the promoter of the nephrin gene that regulates its expression. A large portion of the human nephrin promoter that favors the identification of putative *cis*-binding elements for transcription factors that regulate nephrin expression at the podocyte level has been described (23). PPRE typically are composed of two degenerate hexanucleotides, AGGTCA, arranged in tandem as direct repeats spaced by one nucleotide (32). Analyzing the existing database (23), we found the presence of putative PPRE in the promoter of human nephrin gene. That PPAR/RXR heterodimers activated by pioglitazone could effectively bind putative PPRE has been documented by our *in vitro* experiments in HK-2 cells, showing that pioglitazone, acting on nephrin promoter, induced luciferase transcription. Because of the low transfection efficiency of cultured podocytes with plasmid vectors, we elected to use HK-2 that constitutively express PPAR-γ to test the transcriptional regulation of the nephrin gene by pioglitazone. Much stronger transcriptional activity was found with cells that were transduced with the 325-bp than the 2-kb region in the presence of pioglitazone, which can be taken to suggest that the 325-bp region contains the PPRE as the enhancer. EMSA experiments further confirmed the direct binding of PPAR/RXR heterodimers to imperfect PPRE in the human nephrin promoter. Consistent with human, the rat nephrin promoter possesses in the 5′-flanking region putative PPRE that do not completely match the typical PPRE ones, but they are functional *in vivo*. Therefore, modulation of nephrin expression that is induced by pioglitazone seems to be mediated by imperfect PPRE that are present in the promoter of both species. Here we propose a novel mechanism by which PPAR-γ agonists can repair the injured slit diaphragm and restore the permeability properties of the glomerular barrier to macromolecules. PPAR-γ agonists indeed are capable of modulating a central component of the podocyte slit diaphragm whose integrity is instrumental to glomerular size selective function to the extent that mice with nephrin deficiency or mutations have nephrotic syndrome (33,34).

Conclusion

This study shows that a PPAR-γ agonist that is given to rats with progressive nephropathy is renoprotective even when the treatment started late during the course of the disease. The beneficial effect was independent of changes in lipid metabo-

lism but instead related to the reduction of proteinuria and preservation of renal structure and function. Amelioration of glomerular permselectivity was associated with restoration of nephrin expression that was induced by pioglitazone in PHN rats. The molecular mechanism that is responsible for this effect resides in the intrinsic capability of pioglitazone to enhance nephrin gene expression, acting on specific responsive elements in the nephrin gene promoter. These findings unveil a novel mechanism of renoprotection for PPAR-γ agonists that may have broad therapeutic implications in human renal medicine.

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