Amelioration of anti-Thy1-glomerulonephritis by PPAR-γ agonism without increase of endothelial progenitor cell homing

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Westerweel PE, den Ouden K, Nguyen TQ, Goldschmeding R, Joles JA, Verhaar MC. Amelioration of anti-Thy1-glomerulonephritis by PPAR-γ agonism without increase of endothelial progenitor cell homing. Am J Physiol Renal Physiol 294: F379–F384, 2008. First published December 12, 2007; doi:10.1152/ajprenal.00019.2007.—Impaired glomerular endothelial integrity is pivotal in various renal diseases and depends on both the degree of glomerular endothelial injury and the effectiveness of glomerular endothelial repair. Glomerular endothelial repair is, in part, mediated by bone marrow-derived endothelial progenitor cells. Peroxisome proliferator activated receptor-γ (PPAR-γ) agonists have therapeutic actions independent of their insulin-sensitizing effects, including enhancement of endothelial progenitor cell function and differentiation. We evaluated the effect of PPAR-γ agonist rosiglitazone (4 mg·kg⁻¹·day⁻¹) on the course of anti-Thy1-glomerulonephritis in rats. Rosiglitazone limited the development of proteinuria and prevented plasma urea elevation (8.1 vs. 12.5 ± 1.1 mmol/l, P = 0.002). Histologically, inflammatory cell influx was not affected, but rosiglitazone-treated rats did show fewer microaneurysmatic glomeruli on day 7 (26 ± 3 vs. 41 ± 5%, P = 0.01) and reduced activation of matrix production with reduced renal cortical transforming growth factor-β, plasminogen activator inhibitor type I, and fibronectin-1 mRNA expression. However, bone marrow-derived endothelial cell glomerular incorporation was not enhanced (3.1 ± 0.4 vs. 3.6 ± 0.3 cells/glomerular cross section; P = 0.31). Rosiglitazone treatment in nonnephritic rats did not influence proteinuria, urea, or renal histology. In conclusion, treatment with PPAR-γ agonist rosiglitazone ameliorates the course of experimental glomerulonephritis (10) and involves angiogenesis of glomerular capillaries (15), stimulated by the release of angiogenic factors VEGF and basic fibroblast growth factor (10, 13, 14). Consistently, the limited upregulation of angiogenic factors in uninephrectomized rats injected with anti-Thy1.1 antibody is associated with a progressive course of the glomerulonephritis (30). Our laboratory has shown that glomerular endothelial repair after anti-Thy1-glomerulonephritis is in part dependent on bone marrow-derived EPC (21).

Recently, it was observed that peroxisome proliferator activated receptor-γ (PPAR-γ) agonist rosiglitazone, a thiazolidinedione, can promote EPC differentiation and accelerate EPC-mediated reendothelialization of a denuded segment of the femoral artery in mice (31). Furthermore, the number and function of EPC cultured from human blood were enhanced by rosiglitazone in vitro (19, 31). We, therefore, hypothesized that rosiglitazone treatment would ameliorate the course of anti-Thy1 glomerulonephritis due to enhanced contribution of bone marrow-derived EPC. Here we demonstrate that rosiglitazone indeed improves the course of anti-Thy1 glomerulonephritis. However, this favorable effect on renal function could not be explained by enhanced EPC-mediated replacement of damaged glomerular endothelium.

METHODS

Animals. Male 11-wk-old Brown Norway (BN)/RijHsd and Wag/RijHsd rats, weighing 280–300 g (Harlan, Horst, the Netherlands), housed in a 12:12-h light-dark cycle, and receiving food and acidified water ad libitum, were used for all experiments. The animal ethics committee of our institution approved all protocols.

Experimental design. For the first study, 16 BN rats were treated with PPAR-γ agonist rosiglitazone (GlaxoSmithKline), starting 3

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days before anti-Thy1 injection (day −3) and compared with 12 BN controls. Rosiglitazone was mixed through powdered chow, and food intake was measured. The average dose received during the study was 4 mg·kg body wt ·day −1. Anti-rat Thy1 monoclonal antibody (ER4, 1 mg/kg body wt) was injected intravenously on day 0 in all animals, except for four rosiglitazone-treated rats, who served as nonnephritic controls. Rats were placed in metabolic cages with free access to food and water at several time points to collect 24-h urine samples. Systolic blood pressure was measured in conscious animals by tail cuff sphygmomanometry (IITC, San Diego, CA). The animals were killed at days 7 and 28, and kidneys were excised after perfusion at 120 mmHg with ice-cold 0.9% saline. Kidney specimens were transversely cut in 1-mm slices and snap-frozen in liquid nitrogen or fixed in 4% buffered formaldehyde and embedded in paraffin.

For the second study, BN rats underwent an allogenic bone marrow transplantation with WagRij bone marrow (WagRijBM → BN), as previously described (21), allowing identification of bone marrow-derived cells in the rat chimeras based on the expression of WagRij-specific major histocompatibility complex (MHC) I haplotype. A minimal chimerism of 90% was required to enter the WagRij-specific major histocompatibility complex (MHC) I haplotype. For real-time (RT) PCR, 3 μg of total RNA were reverse transcribed using random hexamers and the TaqMan Reverse Transcription Reagents (Applied Biosystems; Nieuwerkerk a/d IJssel, the Netherlands). RT-PCRs for rat transforming growth factor-β (TGF-β), plasminogen activator inhibitor type 1, fibronectin-1, nophon, and housekeeping gene thiorredoxin-binding protein were performed using mRNA-specific Taqman primert/probe assay mixes and universal PCR mastermix (Applied Biosystems). PCR products were amplified during 45 cycles (95°C for 15 s followed by 60°C for 1 min for each cycle) and analyzed on a RT-PCR cycler (ABI Prism 7900; Applied Biosystems). mRNA expression was quantified using the comparative threshold cycle method with the first control sample as calibrator sample. All samples were assayed in duplicate and averaged for analysis.

Immunohistochemistry. Inflammatory cell influx into the glomerulus was assessed by ED-1 immunohistochemical staining on 5-μm paraffin sections using mouse-anti-rat ED-1 antibody (kindly provided by Ed Dub, Amsterdam, the Netherlands), rabbit-anti-mouse-peroxi-
dase (Dako Cytomation, Campergna, CA), and swine-anti-rabbit-peroxi-
dase (Dako Cytomation). ED-1 staining was visualized using 3,3-diaminobenzidine (Sigma, Zwijndrecht, the Netherlands). ED-1-positive cells were counted in at least 20 glomeruli per kidney. Fibronectin staining was performed on pepsin-pretreated paraffin sections using horseradish peroxidase-conjugated rabbit-anti-human fibronectin antibody known to cross-react with rat fibronectin (Dako Cytomation) and visualized with Nova Red peroxidase substrate (Vector Laboratories, Burlingame, CA). Endothelial cells were identified on frozen sections using anti-RECA-1 (rat endothelial cell antigen-1; Serotec, Hilversum, the Netherlands) and Nova Red peroxi-
dase substrate (Vector Laboratories).

To identify donor bone marrow (WagRij)-derived endothelial cells in bone marrow transplanted animals, 6-μm cryostat sections were incubated with WagRij-specific anti-MHC I U9F4 (kindly provided by J. Rozing, Groningen, the Netherlands) and anti-RECA-1 (Serotec) antibodies, which were biotinylated and visualized using the DAKO-Animal Research Kit (DAKO, Carpenteria, CA) and tyramide-FITC and TRITC, respectively (TSA systems, PerkinElmer Life Sciences, Boston, MA), after blocking sections using the avidin and biotin blocking kit (Vector Laboratories). Nuclei were stained with 4,6-diaminido-2-phenylinde, and sections were mounted with Vectashield (Vector Laboratories). The total number of glomerular U9F4 positive and U9F4/RECA double-positive cells were counted in at least 10 glomeruli per kidney section.

To assess endothelial cell proliferation, animals were injected with bromodeoxyuridine (BrdU) 16 h before termination. Cryostat sections (6 μm) were stained for RECA-1, as described above, and then incubated with swine-anti-BrdU-horseradish peroxidase (Abcam, Cambridge, UK), tyramide-FITC, and 4,6-diaminido-2-phenylinde. The number of RECA-1/BrdU double-positive cells was counted in at least 10 glomeruli per kidney section. An additional staining for RECA-1 and Ki-67 was performed using biotinylated mouse-anti-rat-Ki-67 (clone MIB-5, DAKO) and tyramide-FITC to confirm the frequency of proliferating endothelial cells identified based on BrdU incorporation.

Two blinded observers performed quantification of stains. Immunofluorescent double-stainings were quantified at 630-fold magnification using a Leica DMR microscope (Leica, Wetzlar, Germany). Confocal laser-scanning microscopy confirmed the specificity of the fluorescent staining and was used to photograph the sections. Non-fluorescent staining was quantified at 400-fold magnification using a light microscope (Olympus BX40, Zoeterwoude, the Netherlands).

For all stains, isotype-stained sections served as controls.

Statistical analysis. All values are expressed as means ± SE. Data were analyzed using SPSS version 11.0 software. Differences between groups were analyzed using the two-tailed Student’s t-test, one-way or two-way ANOVA where appropriate. A P value of <0.05 was considered significant.

RESULTS

Rosiglitazone was well tolerated. No adverse effects were observed with rosiglitazone treatment, notably no edema or hyperphagia. Food intake and weight gain were not affected by rosiglitazone treatment. All nephritic rats experienced a transient decrease in body weight after induction of anti-Thy1-glomerulonephritis, but rosiglitazone intake remained stable. Systolic blood pressure tended to be slightly reduced with rosiglitazone treatment (111 ± 3 vs. 118 ± 2 mmHg, \( P = 0.08 \)).

Rosiglitazone ameliorates renal function impairment in anti-Thy1-glomerulonephritis. Induction of glomerulonephritis caused an increase of proteinuria in all nephritic rats, peaking at day 7 (Table 1). Rosiglitazone treatment ameliorated the increase in proteinuria and lead to an earlier recovery to baseline levels, with proteinuria returning to normal values at day 14 vs. day 21 in controls. Rosiglitazone did not affect proteinuria in nonnephritic rats (data not shown). Rosiglitazone prevented the significant increase in plasma urea observed in nephritic control rats on day 7 after anti-Thy1 injection (12.5 ± 1.1 vs. 8.1 ± 0.4 mmol/l on day 7, \( P = 0.002 \), Fig. 1). On day 28, plasma urea levels of all nephritic controls had normalized to baseline levels and were not significantly different between nephritic rats with or without rosiglitazone treatment and nonnephritic rats (9.4 ± 0.8 vs. 9.7 ± 0.2, and 8.8 ± 1.0 mmol/l, respectively, \( P = 0.67 \), Fig. 1).
Rosiglitazone reduces microaneurysm formation and activation of extracellular matrix production. Inflammatory cell influx is an early key event in the development of anti-Thy1-glomerulonephritis. Rosiglitazone treatment did not reduce the number of ED-1-positive cells in the glomeruli at day 7 (6.2 ± 0.5 vs. 7.3 ± 0.5, P = 0.15). Also, glomerular endothelial cell surface by semiquantitative assessment of RECA staining was not significantly influenced by rosiglitazone treatment (1.62 ± 0.30 vs. 1.38 ± 0.22 on a semiquantitative score between 0 and 4; P = 0.52). However, rosiglitazone-treated animals did have a significantly lower percentage of microaneurysmatic glomeruli at day 7 after anti-Thy1 injection than controls (26 ± 3 vs. 40 ± 5%, P = 0.01). Rosiglitazone-treated animals also had reduced activation of matrix production as renal cortex mRNA expression of TGF-β was reduced (1.5-fold; P = 0.006, Fig. 2A), together with reduced expression of TGF-β target gene plasminogen activator inhibitor type 1 and fibronectin-1 (2.4- and 1.8-fold downregulation; P < 0.001 and P = 0.02, respectively, Fig. 2, B and C). Immunohistochemical staining for fibronectin confirmed that rosiglitazone reduced extracellular matrix production at the protein level (2.01 ± 0.35 vs. 1.31 ± 0.11 on a semiquantitative score between 0 and 4 for extent and intensity of glomerular fibronectin staining; P = 0.04, Fig. 2, D and E). The number of BrdU/RECA double-positive cells was not different between rosiglitazone-treated and control animals and relatively low (0.065 ± 0.046 vs. 0.058 ± 0.038/high-power field; P = 0.90). Double-staining for Ki-67 and RECA confirmed the low incidence of proliferating endothelial cells in both groups, regardless of treatment. Urinary excretion of NOx, metabolites of nitric oxide (NO), tended to be higher in rosiglitazone-treated animals than in controls (12.0 ± 0.5 vs. 9.9 ± 1.0 μmol/24 h, P = 0.07).

Renal nephrin expression in anti-Thy1-glomerulonephritis is not attenuated by rosiglitazone treatment. As treatment with PPAR-γ agonist pioglitazone was previously shown to attenuate the development of proteinuria through downregulation of renal nephrin in passive Heymann nephritis (2), we measured renal cortical nephrin mRNA levels, but did not find a statistically significant effect of rosiglitazone treatment (1.44 ± 0.32 vs. 1.17 ± 1.18 relative quotient; P = 0.45).

Rosiglitazone does not affect the glomerular incorporation of bone marrow-derived endothelial cells after anti-Thy1-glomerulonephritis. To evaluate a possible modulating effect of rosiglitazone on the incorporation and differentiation of bone marrow-derived progenitor cells, we performed immunofluorescent stainings on kidney sections from BN rats with anti-Thy1-glomerulonephritis that had previously undergone bone marrow transplantation from WagRij rats. Rats were killed 7 days after injection with anti-Thy1, at which time point our laboratory has previously seen the highest number of incorporated bone marrow-derived endothelial cells (21). Double-staining with WagRij-specific anti-MHC I U9F4 antibody and endothelial-specific RECA antibody confirmed the presence of bone marrow-derived endothelial cells in the glomeruli (Fig. 3), but the number of these cells per glomerulus was not influenced by rosiglitazone treatment (3.1 ± 0.4 vs. 3.6 ± 0.3 U9F4/RECA double-positive cells per glomerular cross section; P = 0.31, Fig. 3). The total number of bone marrow-derived cells was also equal (6.0 ± 0.5 vs. 6.8 ± 0.5 U9F4-positive cells per glomerular cross section; P = 0.27, Fig. 3).

DISCUSSION

Our data show that treatment with the PPAR-γ agonist rosiglitazone ameliorates the impairment of renal function following induction of anti-Thy1-glomerulonephritis. Rosiglitazone partly prevented the development of proteinuria, led to an earlier normalization of the proteinuria, fully prevented plasma urea elevation, attenuated microaneurysm-formation, and limited activation of matrix production after induction of the glomerulonephritis. However, rosiglitazone did not increase the increase of bone marrow-derived progenitor cells in glomerular endothelial repair.
PPAR-γ agonists are widely used in the treatment of Type 2 diabetes mellitus, but may have therapeutic potential based on drug actions beyond the metabolic effects. Several experimental studies in rats showed favorable effects of PPAR-γ agonism on nondiabetic renal pathology (24), including anti-glomerular basement membrane antibody-induced crescentic glomerulonephritis (8), passive Heymann nephritis (2), the development of glomerulosclerosis after 5/6 nephrectomy (12), and renal ischemia-reperfusion induced damage (26). The course of anti-Thy1-glomerulonephritis is determined by the degree of inflammatory cell invasion and inflammatory damage (4), as well as by the degree of endothelial repair (15). Although PPAR-γ agonism may have anti-inflammatory effects (7), we did not observe a significant effect of rosiglitazone on the glomerular influx of ED-1-positive monocytes/macrophages in

![Fig. 1. Plasma urea levels in rats with and without rosiglitazone treatment at day 7 and 28 after anti-Thy1 injection and in nonnephritic rats with rosiglitazone treatment. *P < 0.05 vs. baseline; †P < 0.05 vs. control at the same time point. Values are means ± SE; n = 4 nonnephritic controls, 4 nephritic controls, and 6 rosiglitazone-treated nephritic animals on day 7, and n = 6 controls and 6 rosiglitazone-treated nephritic animals on day 28.]
In other animal glomerulonephritis models, the consequences of PPAR-γ agonist treatment on inflammatory cell glomerular influx have been variable, as both enhancement [in anti-thymocyte-induced glomerulonephritis (16)] and inhibition [in crescentic (8) and passive Heymann (2) glomerulonephritis] have been reported. The lack of effect of rosiglitazone on the influx of ED-1-positive monocytes/macrophages or endothelial cell loss in our model suggests that, in our study, the inflammatory stimulus from anti-Thy1 binding to the mesangium and subsequent loss of endothelial cells was not influenced by rosiglitazone treatment. However, we cannot exclude some effect of rosiglitazone on the induction of glomerular/endothelial damage, as damage and repair of mesangial and endothelial cells occur simultaneously in this model. Furthermore, even in the absence of structural differences, functional effects on inflammatory or glomerular cells may underlie the observed attenuating effects.

As endothelial repair also involves glomerular endothelial cell proliferation (13), we assessed the effect of rosiglitazone on endothelial cell proliferation in our study, but we did not observe an enhancement of glomerular endothelial proliferation and could not detect a difference in glomerular endothelial cell content. This is in line with in vitro studies performed by others, in which rosiglitazone inhibited rather than stimulated endothelial cell proliferation (25). Endothelial integrity does not rely only on the number of cells, but also on their function. A hallmark feature of endothelial function is the capacity of the endothelium to produce NO (29). EPC are also capable of producing NO (9). Several studies have shown that enhanced NO availability improves the clinical course of anti-Thy1-glomerulonephritis (17, 28), presumably via stimulation of endothelial NO synthase (eNOS). Consistently, enhancement of NO-stimulated cGMP production by soluble guanylate cyclase slows progression of anti-Thy1-glomerulonephritis (32). Rosiglitazone has been shown to enhance NO production by increasing eNOS transcription in isolated endothelial cells and eNOS phosphorylation in mice hearts (6) and isolated endothelial cells (20). In human Type 2 diabetic patients, rosiglitazone treatment was shown to increase intrarenal NO levels, which was associated with improvement of renal hemodynamics and reduction of proteinuria (18). This suggests a renoprotective effect of rosiglitazone mediated by improvement of renal endothelial function. A similar beneficial effect of rosiglitazone on endothelial function may have contributed to ameliorating the course of anti-Thy1-glomerulonephritis in our study, as rosiglitazone treatment was associated with increased...
urinary levels of NO metabolites (NOx), although this did not reach statistical significance. Increased glomerular NO levels by rosiglitazone may have contributed to vasodilation of the afferent arterioles (3), thereby limiting the development of microaneurysmata.

Our laboratory previously demonstrated that the regenerated glomerular endothelium during recovery from anti-Thy1-glomerulonephritis originates in part from bone marrow-derived EPC (21). Although we observed no significant differences in endothelial cell content between the groups, this may have been caused by a high variability in RECA-positive cell quantification, probably due to the irregular RECA staining pattern in the injured glomerulus. Thus this does not exclude a potential effect on endothelial cell proliferation or incorporation of bone marrow-derived endothelial cells, particularly since the number of bone marrow-derived cells in untreated nephritic rats is modest [3 cells per glomerular section in this study, which is in accordance with our laboratory’s previous observations (21)]. Rosiglitazone is known to stimulate EPC function and differentiation in mice (31) and humans (19, 31), which appears to be a drug class effect, as PPAR-γ agonist pioglitazone has similar effects in mice (5) and humans (33). We, therefore, specifically investigated the effect of rosiglitazone on the contribution of EPC to restoration of glomerular endothelium in our model. Using a rat allogenic bone marrow transplantation model, we show that treatment with rosiglitazone does not enhance the number of incorporated bone marrow-derived glomerular endothelial cells in the recovering glomerulus. We cannot exclude that rosiglitazone may have enhanced the incorporation/proliferation of bone marrow-derived endothelial cells because circulating EPC levels do not increase in rats. However, as an increase in circulating EPC with PPAR-γ agonist treatment occurs both in mice (5, 31) and humans (19, 31, 33), we think this is not likely.

In conclusion, we have shown that PPAR-γ agonism with rosiglitazone ameliorates the course of anti-Thy1-glomerulonephritis, which may be consistent with enhanced regeneration of glomerular endothelium. However, this could not be attributed to stimulation of the incorporation of bone marrow-derived EPC to glomerular recovery. Our study does bring further evidence that thiazolidinediones may have beneficial effects on nondiabetic renal disease, including attenuation of the course of acute glomerulonephritis.

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Fig. 3. Identification and quantification of bone marrow-derived endothelial cells in the glomeruli. Immunofluorescent images show U9F4-positive bone
marrow-derived cells (FITC channel), rat endothelial cell antigen-1 (RECA)-positive endothelial cells (TRITC channel), and a partial overlap in the overlay,
identifying U9F4/RECA double-positive bone marrow-derived endothelial cells. Quantifications of the total number of U9F4-positive bone marrow-derived cells
and U9F4/RECA double-positive bone marrow-derived endothelial cells, averaged per glomerular cross section at day 7 after anti-Thy1 injection, do not show
an effect of rosiglitazone treatment. Values are means ± SE. ns, Nonsignificant.
REFERENCES


