The peroxisome proliferator-activated receptor γ (PPARγ) is a member of the family of nuclear receptors of ligand-activated transcription factors that include steroid, retinoid, and thyroid hormone receptors [1]. The PPARs form heterodimers with the 9-cis retinoic acid receptor, RXRa, and bind to characteristic DNA sequences termed peroxisome proliferator response elements (PPRE) located in the promoter region of target genes. Three isoforms of PPAR (PPARα, PPARβ and PPARγ) have been described [2]. PPARγ is expressed at high levels in adipose tissues and plays a crucial role in lipid metabolism and glucose homeostasis [3]. A structurally diverse group of compounds, naturally occurring fatty acid derivatives, eicosanoids (such as 15d-PGJ₂) and synthetic molecules [such as thiazolidinediones (TZD), for example, troglitazone and ciglitazone] can bind and activate these receptors [4, 5]. PPARγ ligands of the TZD group lower blood glucose levels and are in use as potent anti-diabetic drugs in humans [6]. They protect against the development and progression of diabetic nephropathy [7, 8] and non-diabetic glomerulosclerosis in rats [9]. Recent reports show that PPARγ is expressed in monocytes/macrophages (M/M) and that activators of PPARγ can modulate their activation and down-regulate their expression of inflammatory cytokines and chemokines [10–12]. Controversy exists, however, since other studies demonstrated pro-inflammatory rather than anti-inflammatory effect of PPARγ ligands on the expression of cytokines and chemokines in M/M [13, 14]. Furthermore, several studies demonstrated differences between the effects of the synthetic PPARγ agonists of the TZD group and the natural PPARγ ligand 15d-PGJ₂ on nuclear factor-κB (NF-κB) and activator protein-1 (AP-1) dependent gene expression [15, 16]. Thus, the role of PPARγ activators in the setting of inflammation remains controversial.
In several forms of glomerulonephritis, the activation and infiltration of M/M in glomeruli and the tubulointerstitium is a typical pathological feature closely correlated to disease progression. The recruitment of M/M into injured tissues is largely dependent on a group of cytokines called chemokines [17]. Among these chemokines, monocyte chemoattractant protein-1 (MCP-1) [18] is an important mediator for M/M infiltration in several forms of experimental glomerulonephritis [19–21].

To investigate a possible role of different PPARγ activators in the modulation of M/M attraction into glomeruli and on the course of GN, the effects of troglitazone, ciglitazone and 15d-PGJ2 were studied in a rat model of mesangial immune GN.

Our results demonstrate that stimulation of PPARγ through troglitazone and ciglitazone increases MCP-1 expression and M/M recruitment in the early stage of a rat model of mesangial immune glomerulonephritis, and suggest that these effects are partly mediated through an enhanced binding activity of the nuclear transcription factor AP-1. In contrast, application of 15d-PGJ2 reduces NF-κB activation and did not exert pro-inflammatory effects. These results demonstrate the complex role of PPARγ activation in the setting of inflammatory disease and underscore the need of a careful evaluation of possible deleterious effects of PPARγ activators in GN.

**METHODS**

**Induction of glomerulonephritis and experimental design**

Immune-mediated mesangial cell injury was induced in male Wistar rats (180 to 200 g/BW) by IV injection of an anti-rat-thymocyte antiserum (ATS). ATS was induced in rabbits by repeated immunization with thymocytes from Lewis rats as described earlier [22].

Eight different groups of animals (total N = 140) were studied at 24 hours after nephritis induction. For each experimental setup groups of four animals were compared, the experimental setup was repeated at least two times. Experiments with troglitazone were performed four times. Control rats (N = 32) received 0.5 mL/100 g BW of non-antibody IgG intravenously. Nephritic rats (N = 32) received 0.5 mL/100 g BW of ATS intravenously. Control and nephritic rats treated with troglitazone (N = 52) received 20, 50, 200 and 500 mg/kg BW of troglitazone per day in a 1% methylcellulose suspension orally. Control and nephritic rats treated with 15d-PGJ2 (N = 12) received 100 mg/kg BW ciglitazone orally per day in a 1% methylcellulose suspension orally. Control and nephritic rats treated with 15d-PGJ2 (N = 12) received 1.5 mg/kg BW 15d-PGJ2 daily in normal saline
subcutaneously. In all groups medication was started 24 hours before the induction of the disease and continued until sacrifice.

**Histology**

Renal tissue was fixed in 4% buffered formaldehyde. Paraffin embedded sections (2 μm) were either stained with an antibody directed against the monocyte specific marker ED-1 (Chemicon International, Temecula, CA, USA) to evaluate glomerular M/M infiltration or with an antibody against the proliferative cell nuclear antigen (PCNA; Dakopatts M879, Hamburg, Germany). Tissue sections were developed with the alkaline phosphatase anti-alkaline phosphatase (APAAP) technique. ED-1 and PCNA positive cells in 50 glomeruli per kidney were counted by light microscopy in a blinded fashion where the tissue source was unknown. Four different kidneys of each group were evaluated at 24 hours. The cell numbers of ED-1 and PCNA positive cells are given per glomerular cross section as means ± SEM.

**Isolation of glomeruli, preparation of total RNA, and Northern blot hybridization**

Glomeruli were isolated by a fractional sieving technique using 90 μm and 53 μm mesh wire sieves, cellular RNA from glomeruli was isolated and 10 to 20 μg of total RNA was used for Northern blot analysis. A rat MCP-1 cDNA fragment was used for hybridization. Membranes were rehybridized with a cDNA probe for human 18 S RNA to account for small loading and transfer variabilities. Densities on exposed films were quantified using a phosphoimager Bio-Rad-GS-363 System (multi-analyst software; Bio-Rad, Munich, Germany). Data analysis of Northern blot experiments was performed using three independent sets of experiments, and the results are shown as mean ± SEM.
Fig. 4. Immunohistologic staining of glomerular monocytes/macrophages (M/M) infiltration. Immunohistologic staining of glomerular M/M infiltration with an ED-1 antibody revealed a marked increase of glomerular ED-1 positive cells 24 hours after induction of the ATS-nephritis (B) in comparison to non-nephritic animals (A) and the troglitazone treated animals (C). Treatment of nephritic animals with troglitazone (D) further enhanced glomerular M/M recruitment (×1000).

Fig. 8. Immunohistologic staining against PCNA. Compared with controls (A) and the troglitazone treated control group (C) nephritic animals (B) and troglitazone treated nephritic rats (D) revealed a markedly increase in PCNA positive cells 5 days after nephritis induction (×1000).
RT-PCR

For reverse transcription-polymerase chain reaction (RT-PCR) 1 μg of total RNA, 4 μL 5× First strand buffer, 1 μL dNTPs (20 mmol/L), 2 μL dithiothreitol (DTT; 0.1 mol/L), 0.4 μL poly(dT) primer (1 mg/mL; Pharmacia, Freiburg, Germany), 0.4 μL RNasin (Promega, Madison, WI, USA), and 1 μL Moloney-murine leukemia virus (M-MLV) reverse transcriptase (2 U/mL; Gibco BRL, Eggenstein, Germany) per sample were mixed. The RT reaction was carried out for two hours at 37°C. Exact primer sequences for CCR-2 and MCP-1 are available from the authors. Five microliters of each RT reaction sample was added to 29.5 μL H₂O, 5 μL 10× PCR buffer, 3 μL MgCl₂ (25 mmol/L), 4 μL dNTPs (2.5 mmol/L), 1.5 μL forward primer (50 ng/mL), 1.5 μL reverse primer (50 ng/mL), and 0.5 μL Taq DNA polymerase (5 U/mL; Promega). The PCR was run for 28 cycles without reaching the plateau phase (data not shown) using the following temperature profile: 95°C for 10 seconds, 57°C for 20 seconds, and 72°C for 30 seconds. RNA samples were also analyzed for the expression of the housekeeping gene GAPDH as described earlier [23]. Amplified cDNA fragments were separated on a 1.5% agarose gel containing 1 mg/mL ethidium bromide, and the gel was photographed under UV light using a Polaroid 655 film. The amplified cDNA of the predicted size was isolated, subcloned, and sequenced to confirm specificity of the reaction. To control for genomic DNA artifacts, a control PCR without RT was performed. Exposed films were quantified using a phosphoimager Bio-Rad-GS-363 (multi-analyser software).

Glomerular nuclear mini-extracts and EMSA

Kidneys were harvested after in situ perfusion with 100 mL of sterile phosphate buffered saline (PBS). We adapted a method described by Sakurai et al to prepare the nuclear mini-extracts and run the electrophoretic mobility shift assay (EMSA) [24]. For nuclear proteins, isolated glomeruli were resuspended in 400 μL buffer A [10 mmol/L HEPES (pH 7.9), 10 mmol/L KCl, 0.1 mmol/L ethylenediaminetetraacetic acid (EDTA), 0.1 mmol/L egtazic acid (EGTA), 1 mmol/L DTT, 0.5 mmol/L phenylmethylsulfonyl fluoride (PMSF), 10 μg/mL aprotinin, 10 μg/mL leupeptin]. After homogenization with 50 strokes in a 7 mL glass-homogenizer (Wheaton) the suspension was chilled on ice for 15 minutes. Twenty-five microliters of 10% Noniodet P-40 were added, the homogenate vigorously mixed for 10 seconds and centrifuged at 15,000 × g for five minutes. The pellet was resuspended in 100 μL buffer B (20 mmol/L HEPES, 0.4 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L EGTA, 1 mmol/L DTT, 1 mmol/L PMSF, 10 μg/mL aprotinin, 10 μg/mL leupeptin) and placed on ice on a shaking platform at 400 rpm for 15 minutes. Nuclear protein extracts were obtained after centrifugation at 15,000 × g for five minutes. Protein concentration was determined using DC protein assay (Bio-Rad Laboratories), aliquots were stored at −80°C. EMSA was performed using a PPARγ (5′-GGG CCA CAA AGG TCA CGT TC 3′) [25], NF-κB (5′-AGT TGA GGG GAC TTT CCC AGG C 3′) (Promega), and AP-1 (5′ CGC TTG ATG AGT CAG CCG GAA 3′) (Promega) consensus oligonucleotide probe that was end-labeled with α32P ATP (3,000 Ci/mmol; Amersham Pharmacia Biotech, Freiburg, Germany) according to the manufacturer’s instructions. One to 5 μg of nuclear protein was incubated for 30 minutes at room temperature with 100,000 cpm of the 32P labeled probe in 20 mmol/L HEPES (pH 7.9), 0.3 mmol/L EDTA, 0.2 mmol/L EGTA, 0.4 mmol/L NaCl and 2 μg Poly(dI-dC) (Amersham Pharmacia Biotech) in a total volume of 20 μL. Competition experiments were performed by adding unlabeled PPARγ, NF-κB and AP-1 consensus oligonucleotides in 100-fold molar excess to the binding reaction. The DNA-protein complexes were subjected to electrophoresis on a 4% polyacrylamide gel containing 2.5% glycerol, 6.7 mmol/L Tris-HCl (pH 7.5), 3.3 mmol/L sodium acetate and 0.1 mmol/L EDTA for 2.5 hours at 15°C. The gel was then vacuum-dried and autoradiography was performed. Exposed EMSA films were quantified using a phosphoimager Bio-Rad-GS-363 (multi-analyser software) and corrected to the density of the probe.

Western blotting for PCNA

For Western blotting glomeruli were isolated and centrifuged in 1× PBS. The pellet was resuspended in 100 μL of Laemmli buffer [33% vol/vol 0.5 mmol/L Tris-HCl pH 6.8, 66% vol/vol sodium dodecyl sulfate (SDS) 10%]. Samples were boiled for 10 minutes and centrifuged in 1× PBS. After rinsing the membrane in washing PBS, 0.1% Tween 20) for one hour at room temperature, then incubated for another hour with an anti-rat proliferating cell nuclear assay (PCNA) antibody (Dakopatts M879) added to a concentration of 1:2000 in the same buffer. After rinsing the membrane in washing
Fig. 3. Electrophoretic mobility shift assays (EMSA) for activator protein-1 (AP-1), nuclear factor-κB (NF-κB) and peroxisome proliferator activated receptorγ (PPARγ) of nuclear extracts of isolated glomeruli. To demonstrate identical loading of radiolabeled oligos unbound probes are shown below. The competition experiments using non-radioactive labeling are shown on the right.

buffer for $3 \times 10$ minutes, the secondary antibody, a rabbit anti-goat-immunoglobulin G conjugated to horseradish-peroxidase (Southern Biotechnology, Atlanta, GA, USA) was added at a concentration of 1:1000. The luminescence detection of peroxidase was performed with the enhanced chemiluminescence (ECL) system according to the manufacturer’s recommendations (Amersham). Films were exposed 2 to 10 minutes at room temperature.
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RESULTS

Induction of glomerulonephritis and treatment with PPARγ agonists

Glomerular chemokine and chemokine receptor expression. Glomerular MCP-1 mRNA expression increased significantly in nephritic animals at 24 hours after induction of the disease when compared with control animals (Fig. 1A). Treatment with troglitazone and ciglitazone further enhanced up-regulation of MCP-1 mRNA at 24 hours (Figs. 1A and 2A). The mRNA expression of the chemokine receptor CCR-2 (receptor for MCP-1) also was up-regulated 24 hours after induction of the disease (Figs. 1B and 2B). Troglitazone and ciglitazone further enhanced the CCR-2 expression. In contrast, 15d-PGJ2 did not up-regulate MCP-1 and CCR-2 expression (Fig. 2). Application of troglitazone, ciglitazone and 15d-PGJ2 to control animals did not affect MCP-1 or CCR-2 mRNA expression.

Activation of nuclear protein binding for PPARγ, NF-κB and AP-1. Electrophoretic mobility shift assay experiments demonstrated that induction of glomerulonephritis activated glomerular nuclear protein binding of NF-κB and AP-1, whereas glomerular PPARγ DNA binding was reduced in nephritic rats at 24 hours (Fig. 3). As expected, PPARγ binding was enhanced in animals treated with troglitazone, ciglitazone and 15d-PGJ2 in control and nephritis groups. In nephritic animals troglitazone and ciglitazone treatment enhanced glomerular nuclear binding of AP-1, but did not influence glomerular NF-κB activity. In contrast, treatment of nephritic rats with 15d-PGJ2 did not lead to an increase in AP-1 binding, but reduced NF-κB binding. Application of troglitazone, ciglitazone and 15d-PGJ2 to control animals did not influence glomerular DNA binding of the transcription factors NF-κB and AP-1 in comparison to the untreated control group.

Glomerular monocyte/macrophage infiltration. Induction of glomerulonephritis resulted in a significant increase in glomerular monocyte infiltration from 0.3 ± 0.07 cells/glomerular cross section (c/gcs) in controls to 2.95 ± 0.39 cells in nephritic rats at 24 hours (P < 0.01; Fig. 4A, B and Fig. 5). Treatment of nephritic animals with troglitazone and ciglitazone further enhanced glomerular monocyte recruitment at 24 hours (nephritis + Tro, 4.51 ± 0.24 c/gcs; nephritis + Cig, 4.34 ± 0.37; P < 0.05; Figs. 4D and 5), whereas 15d-PGJ2 treatment of nephritic animals did not alter glomerular M/M recruitment (Fig. 5). Application of troglitazone, ciglitazone and 15d-PGJ2 to control animals did not influence glomerular M/M recruitment (Figs. 4C and 5). Dose response experiments with troglitazone demonstrated that...
concentrations between 20 and 500 mg/kg BW lead to an increased glomerular M/M infiltration and glomerular MCP-1 RNA expression (Fig. 6).

**Effect of troglitazone on glomerular cell proliferation.** Glomerular cell proliferation was assessed by PCNA Western blotting (Fig. 7) and immunohistologic staining of tissue for PCNA (Figs. 8 and 9). As expected, there was an increase in PCNA protein formation in nephritic animals 24 hours and 5 days after induction of nephritis when compared to the control and troglitazone treated animals (Figs. 7 to 9). Treatment of nephritic rats with troglitazone (200 mg/kg BW/day) lead to a small decrease of cell proliferation at both time points studied (Figs. 7 to 9). However the effect is not statistically significant (Fig. 9).

**DISCUSSION**

Peroxisome proliferator activated receptor γ ligands of the thiazolidinedione group (troglitazone, pioglitazone, rosiglitazone) have become an important group of anti-diabetic drugs in humans [6]. In experimental studies in diabetic rats, PPARγ activators protected against the development and progression of diabetic nephropathy [7, 8]. Troglitazone decreased microalbuminuria in patients with diabetic nephropathy [26], but it was withdrawn from the market when reports provided evidence for unexpected aggravation of hepatitis as a side effect [27]. Although the observed renal protective effect in diabetes may be an indirect effect of the antidiabetic and antihyperlipidemic effects of PPARγ activators, it is possible that it results from direct effects through PPARγ receptors in the kidney itself. Recent studies have shown that PPARγ receptors are expressed in the kidney [28–30] and, more specifically, in glomeruli [30]. Several investigators have reported that PPARγ expression can be regulated in cultured mesangial cells [30–32].

Importantly, it has been demonstrated that PPARγ is functionally expressed in monocytes/macrophages and that PPARγ ligands may modulate chemokine expression and inflammatory responses [10, 11] as well as trans-differentiate monocytes into macrophage foam cells [12]. In studies on isolated monocytes/macrophages treatment with PPARγ agonists reduced tumor necrosis factor-α (TNF-α), interleukin (IL)-1β and IL-6 synthesis induced by phorbol ester [10], and inhibited the expression of several inflammatory response genes induced by interferon-γ (IFN-γ) [11]. In a mouse model of inflammatory bowel disease PPARγ agonists attenuate cytokine gene expression and reduce colonic inflammation after seven days [33].

We were therefore interested in examining a possible role of different forms of PPARγ activators in a model of glomerulonephritis characterized by early monocyte/macrophage infiltration and predominant mesangial involvement. In support of other observations [25, 28–29] our current study demonstrates PPARγ DNA binding in isolated nuclear preparations of glomeruli from control animals. In contrast to the demonstrated up-regulation in isolated macrophages [11], the induction of ATS-nephritis reduces glomerular PPARγ DNA binding. However, treatment of nephritic animals with the PPARγ ligands troglitazone, ciglitazone and 15d-PGJ2 significantly increases the glomerular DNA binding of PPARγ, providing evidence that the PPARγ ligands in the doses used in our study activate glomerular PPARγ.

An important chemokine for the attraction of M/M in the anti-thymocyte antibody induced glomerulonephritis is monocyte-chemoattractant protein-1 (MCP-1) [19–21]. Its expression is positively correlated with the infiltration of monocytes/macrophages in glomeruli and blockade of MCP-1 action in vivo has been demonstrated to lead to a reduction of glomerular monocytes in this model [19, 21, 34]. In contrast to observations on isolated monocytes/macrophages, where cytokine expression was reduced by stimulation of PPARγ [11], in our study of glomerular inflammation the PPARγ activators troglitazone and ciglitazone enhanced the expression of a major
glomerular chemokine, MCP-1. Treatment with troglitazone and ciglitazone lead to an increased number of infiltrating monocytes/macrophages. Twenty-four hours after induction of the disease, the enhanced glomerular MCP-1 formation and monocyte recruitment was paralleled with an increased glomerular RNA expression of the MCP-1 receptor CCR-2, which is predominantly expressed on mononuclear cells. A recent in situ hybridization study provides evidence for the absence of CCR-2 on resident glomerular cells [35], suggesting that the upregulated glomerular CCR-2 RNA expression is due to the infiltration of CCR-2 positive monocytes attracted by MCP-1.

To determine which transcription factors might be regulated by PPARγ activation in this form of glomerulonephritis and, more specifically, drive the expression of the chemokine MCP-1, we investigated the glomerular DNA binding of the transcription factors NF-κB and AP-1. NF-κB and AP-1 are important regulators of MCP-1 gene expression [36, 37], and recent studies have shown that PPARγ can directly influence the activity of NF-κB and AP-1 in macrophages [11]. In addition, it seems unlikely that PPARγ activation may directly regulate the transcription of the C-C chemokine MCP-1 because no PPRE element is reported in the 5’region of the MCP-1 gene. Our results further confirm an earlier study in the ATS model of glomerulonephritis demonstrating an increased glomerular activation of NF-κB and AP-1 [38]. We show that the activity of NF-κB was unaffected by troglitazone and ciglitazone, whereas the activity of AP-1 was markedly increased. Our results suggest that PPARγ activators of the TZD activate the transcription factor AP-1, which increases the expression of MCP-1, thereby leading to an enhanced glomerular M/M recruitment in this form of GN. This seems to contrast earlier findings in isolated macrophages where treatment with PPARγ agonists reduced TNF-α, IL-1β and IL-6 synthesis induced by phorbol ester [10], and inhibited the expression of several inflammatory response genes induced by IFN-γ [11]. However, in a recent study in vascular smooth muscle cells PPARγ agonists led to enhanced AP-1 activity through activation of MEK/ERK pathways by phosphatidylinositol 3-kinase [39]. Furthermore, in mice challenged with lipopolysaccharide (LPS) PPARγ activation through TZD did not suppress cytokine production, but rather increased blood levels of TNF-α and IL-6 [13]. In this study an important difference in the effects observed when using thiazolidinediones and 15d-PGJ2 was seen, raising doubt that the beneficial effects of 15d-PGJ2 are mediated through PPARγ. The results of our study demonstrate different effects between the PPARγ agonists of the TZD group and 15d-PGJ2 in this model of experimental glomerulonephritis. In contrast to the treatment with troglitazone or ciglitazone, DNA binding of NF-κB was reduced and MCP-1 expression was not elevated in glomeruli of nephritic rats treated with 15d-PGJ2. Hence, our results support findings of other groups where 15d-PGJ2 was shown to exert complex effects beyond PPARγ activation in the NF-κB signaling pathway. In monocytes 15d-PGJ2 directly inhibited the action of IκB kinase independently of PPARγ activation [15, 16], and recently it was demonstrated in human mesangial cells that 15d-PGJ2 also inhibits the translocation of NF-κB through attenuated degradation of IκBα [40].

Because results of previous studies show possible effects of PPARγ agonists on cell proliferation [41, 42], we measured proliferation cell nuclear antigen (PCNA) as a marker of cell proliferation in our model of GN at 24 hours and five days after induction of disease. However, glomerular PCNA determined by Western blotting and immunohistology was not altered significantly in nephritic animals when they were treated with troglitazone.

In summary, our results demonstrate that treatment with the PPARγ agonists of the TZD group (troglitazone and ciglitazone) but not 15d-PGJ2 enhances MCP-1 formation and M/M recruitment in the induction phase of a rat model of mesangial proliferative glomerulonephritis. Our data suggest that these effects may be partly mediated through an increased binding activity of the nuclear transcription factor AP-1. It seems possible that PPARγ agonists of the TZD group may lead to an enhanced attraction of inflammatory cells in glomerulonephritis. The findings of this study underscore the need for a careful evaluation of possible deleterious effects of PPARγ activators in human glomerulonephritis.
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