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Comparison of Direct Action of Thiazolidinediones and Glucocorticoids on Renal Podocytes: Protection from Injury and Molecular Effects

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ABSTRACT

The U.S. Food and Drug Administration-approved thiazolidinediones pioglitazone and rosiglitazone are peroxisome proliferator-activated receptor-γ (PPARγ) agonists developed to control serum glucose in patients with diabetes. They have been found to reduce proteinuria and microalbuminuria in both diabetic nephropathy and nondiabetic glomerulosclerosis. We hypothesized that the renal protective effects of thiazolidinediones result, at least in part, from their direct action on podocytes, similar to glucocorticoids. Treatment with pioglitazone, rosiglitazone, or dexamethasone significantly protected podocytes against puromycin aminonucleoside-induced injury (designed to mimic nephrotic syndrome-related injury), as determined by both cell survival and actin cytoskeletal integrity. Furthermore, we compared the ability of these drugs to modulate key signaling pathways in podocytes that may be critical to their protective effects. Rosiglitazone deactivated the mitogen-activated protein kinases (MAPKs), extracellular signal-regulated kinases 1/2, p38 MAPK, and stress-activated protein kinase/c-Jun NH$_2$-terminal kinase, whereas pioglitazone did not, and dexamethasone deactivated to some extent. Similar to dexamethasone, both thiazolidinediones increased the glucocorticoid receptor phosphorylation, and this response to rosiglitazone and possibly to pioglitazone was PPARγ-dependent. Furthermore, both drugs mimicked or enhanced the effects of dexamethasone on glucocorticoid-responsive genes in a PPARγ- and glucocorticoid receptor-dependent manner. In addition, both thiazolidinediones mimicked dexamethasone-induced effects on calcineurin activity. In summary, thiazolidinediones are able to modulate the glucocorticoid pathway and exert direct protective effects on podocytes, similar to glucocorticoids. This suggests that thiazolidinediones may have potential clinical utility as either primary or adjunctive therapy for nephrotic syndrome or other diseases treated with glucocorticoids. These findings may also lend mechanistic insight into the well established but poorly understood renal protective effects of thiazolidinediones in diabetic nephropathy.

Introduction

Peroxisome proliferator-activated receptors γ (PPARγ) are ligand-activated transcription factors of the nuclear hormone receptor superfamily known to be involved in adipogenesis, glucose homeostasis, inflammatory responses, and apoptosis (Michalik et al., 2006). Endogenous ligands of PPARγ include fatty acids and prostaglandin-type eicosanoids such as 15-deoxy-$\Delta^{12,14}$-PGJ$_2$. However, the best known PPARγ agonists are the thiazolidinediones (TZDs) [e.g., pioglitazone (Pio),

ABBREVIATIONS: PPARγ, peroxisome proliferator-activated receptor γ; CaN, calcineurin; CORT108297, (*R*)-4a-ethoxy-1-(4-fluorophenyl)-6-(4-trifluoromethylbenzenesulfonyl)-4,4a,5,6,7,8-hexahydro-1*H*-1,2,6-triazacylopenta[*b*]naphthalene; DAPI, 4′,6-diamidino-2-phenylindole; Dex, dexamethasone; ERK1/2, extracellular signal-regulated kinase 1/2; FBS, fetal bovine serum; FDA, U.S. Food and Drug Administration; GC, glucocorticoid; GR, glucocorticoid receptor; GRE, glucocorticoid-responsive element; GW9662, 2-Chloro-5-nitro-*N*-phenylbenzamide; MAPK, mitogen-activated protein kinase; NS, nephrotic syndrome; O/N, overnight; PCR, polymerase chain reaction; RT-PCR, reverse transcription-polymerase chain reaction; PAN, puromycin aminonucleoside; PBS, phosphate-buffered saline; PBS-T, PBS containing 0.1% Tween 20; Pio, pioglitazone; Rosi, rosiglitazone; RU486, 11β-(4-dimethylamino)phenyl-17β-hydroxy-17-(1-propynyl)estra-4,9-dien-3-one; SAPK/JNK, stress activated protein kinase/c-Jun N-terminal kinase; PAGE, polyacrylamide gel electrophoresis; TZD, thiazolidinedione; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium; HEK, human embryonic kidney; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; FK506, tacrolimus; PP, protein phosphatase; GW9662, 2-chloro-5-nitrobenzanilide.

rosiglitazone (Rosi), and ciglitazone], which comprise a class of synthetic drugs that are FDA-approved and are widely used for the treatment of type II diabetes. These drugs induce a complex response involving both binding of the PPARγ to peroxisome proliferator response elements and various mechanisms that are independent of DNA binding. Moreover, structurally different PPARγ agonists are known to induce both common and selective responses (Gervois et al., 2007).

Thiazolidinediones are now also emerging as novel therapeutic agents in other diseases, including cancer, arthritis, and inflammatory and renal diseases (Guan and Breyer, 2001; Koeffler, 2003; Ialenti et al., 2005; Yang et al., 2009). They have been shown to reduce proteinuria, microalbuminuria, and podocyte injury in both diabetic nephropathy and nondiabetic glomerulosclerosis in mouse and rat models, as well as in humans (Ma et al., 2001; Yang et al., 2006; Cha et al., 2007; Sarafidis et al., 2010). In addition, Pio has been shown to provide protective effects against progression of puromycin aminonucleoside (PAN)-induced glomerulosclerosis in vivo and in an in vitro model using cultured podocytes (Yang et al., 2006; Kanjanabuch et al., 2007). Rosi has also been reported to attenuate proteinuria and glomerulosclerosis in doxorubicin-induced focal segmental glomerulosclerosis in rats (Liu et al., 2010). Based in part on this, Rosi successfully passed a phase I safety trial (Joy et al., 2009) and was scheduled for a phase II clinical trial as a treatment for this kidney disease until being withdrawn from study because of new safety concerns (Peyser et al., 2010). Despite this, in a recent meta-analysis, it was concluded that TZDs significantly decrease albuminuria and proteinuria in patients with diabetes and suggested that they may do so by providing direct renoprotective effects (Sarafidis et al., 2010).

Nephrotic syndrome (NS) is one of the most common kidney diseases seen in children and adults. It is a remitting and relapsing disease characterized by massive loss of serum proteins into the urine through a damaged glomerular filtration barrier, leading to hypoalbuminemia and swelling throughout the body (edema) (Smoyer and Mundel, 1998). Podocytes are a key component of the kidney's filtration barrier, and during NS, they undergo dramatic structural alterations in the foot processes that attach these cells to the glomerular basement membrane. The most widely accepted experimental models used to mimic the podocyte injury that occurs during NS in humans include PAN injection of rats (Pippin et al., 2009) and PAN treatment of cultured podocytes (Ransom et al., 2005). For the last 50 years the primary therapy for NS has been oral glucocorticoids (GC). Unfortunately, GCs have serious side effects, and in ~20% of patients, they are ineffective in inducing clinical remission of disease (i.e., steroid-resistant NS). Thus, it is clear that alternative therapies with greater efficacy and/or less severe side effects are critically needed (Hodson and Craig, 2008).

Based on the well established, but poorly understood, renal protective effects of thiazolidinediones, we hypothesized that their protection results, at least in part, from their direct action on podocytes, similar to GCs. To test this hypothesis, we compared the ability of Pio and Rosi to protect podocytes from PAN-induced injury to the known protective effects of dexamethasone (Dex) (Ransom et al., 2005; Xing et al., 2006). In addition, given the recently reported roles of various mitogen-activated protein kinases (MAPKs) in kidney diseases

(Grande and López-Novoa, 2008) and the known ability of PPARγ agonists to regulate MAPK activities in other cell types (Gardner et al., 2005), we also analyzed the ability of Rosi, Pio, and Dex to alter MAPK activation in podocytes. Furthermore, because TZDs have been reported recently to act as partial agonists to the glucocorticoid receptor (GR) (Ialenti et al., 2005; Matthews et al., 2009) and GCs are the primary therapy for NS, we analyzed the ability of Rosi, Pio, and/or Dex to modulate the GC signaling pathway in podocytes.

Materials and Methods

Cell Culture. The conditionally immortalized mouse podocyte cell line MPC-5 was cultured as described previously (Smoyer and Ransom, 2002). Podocytes were cultured under proliferating conditions in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, 100 μg/ml streptomycin (Invitrogen, Carlsbad, CA), and 10 U/ml mouse γ-interferon (Sigma-Aldrich, St. Louis, MO) at 33°C in a humidified atmosphere of 5% CO_2. Differentiation was induced by shifting the cells to 37°C in the same medium without γ-interferon for 10 to 14 days on culture plates (Greiner, Monroe, NC) coated with rat tail collagen type I (BD Biosciences, Bedford, MA). Human embryonic kidney (HEK) epithelial cells (293T) were maintained in Dulbecco's modified Eagle's medium supplemented with 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C in a humidified atmosphere of 5% CO_2. Podocytes were treated with puromycin aminonucleoside (Sigma-Aldrich), rosiglitazone (Fisher HealthCare, Houston, TX), pioglitazone (Enzo Life Sciences International Inc., Plymouth Meeting, PA), or dexamethasone (Sigma-Aldrich) as indicated in complete or serum-free medium, or in medium containing charcoal-stripped FBS (Invitrogen) at 8%. Pretreatments or short-term treatments were performed for 4 h followed by washing and medium change. Simultaneous or long-term treatments were performed for the entire length of the experiment until the assay or extraction.

Use of PPARγ and GR Antagonists. Where indicated, cells were preincubated 1 h before the addition of PPARγ and GR agonists, with 10 μM 2-chloro-5-nitrobenzanilide (GW9662) (a PPARγ antagonist), 10 μM 11β-(4-dimethylamino)phenyl-17β-hydroxy-17-(1-propynyl)estra-4,9-dien-3-one (RU486) (a GR antagonist), or 10 μM (R)-4α-ethoxy-1-(4-fluorophenyl)-6-(4-trifluoromethylbenzenesulfonyl)-4,4α,5,6,7,8-hexahydro-1H-1,2,6-triazacylopenta[b]naphthalene (CORT108297) (a highly selective GR antagonist).

GW9662 (Sigma-Aldrich) is an irreversible and selective PPARγ antagonist that is 10- and 600- fold less potent in binding PPARα and PPARδ, respectively. RU486 (Sigma-Aldrich) is a potent GR antagonist, although with limited specificity, because it also binds to the progesterone receptor. RU486 has a K_i = 0.4 nM in GR binding assays and a K_i = 1.5 nM in functional GR antagonist assays (Clark et al., 2008; Clark, 2008). RU486 exhibits partial agonistic activities (Matthews et al., 2009).

CORT108297 (synthesized by Ricerca Biosciences, Concord, OH, and obtained from Corcept Therapeutics, Menlo Park, CA) is a highly selective antagonist of the GR compared with the progesterone, estrogen, androgen, and mineralocorticoid receptors, as shown in binding assays (Clark et al., 2008). It has also been found inactive for PPARγ binding (R. Clark, Corcept Therapeutics, personal communication). CORT108297 has a K_i of 0.9 nM in GR binding assays and a K_i of 6.8 nM in functional GR antagonist assays (Clark et al., 2008; Clark, 2008). It also exhibits a partial agonist activity, which is higher than that of RU486 (R. Clark, personal communication).

Viability Assays. Differentiated podocytes were either pretreated for 4 h with the vehicle dimethyl sulfoxide, Rosi, Pio, and/or Dex followed by PAN treatment (5 μg/ml) or simultaneously treated with these reagents and PAN (5 μg/ml) at concentrations specified under *Results*. Viability assays were performed after 3 or 5 days of

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treatments. In brief, cells were washed with phosphate-buffered saline (PBS), incubated in medium containing 3-(4,5-dimethylthi-azol-2-yl)-2,5-diphenyl tetrazolium (MTT; Sigma-Aldrich) at 500 μg/ml for 4 h, and the MTT formazan crystals were extracted in dimethyl sulfoxide containing 0.01 M glycine and 0.01 M NaCl, pH 10.5. Absorbance was measured at 570 nm with the reference wavelength of 630 nm on a SpectraMax M2 plate reader (Molecular Devices, Sunnyvale, CA). Positive control cells (100% viable) did not receive any PAN treatment, whereas cells treated for 20 min with 0.01% Triton X-100 were used as negative controls (0% viable).

Actin Staining and Microscopy. Differentiated podocytes were cultured on coverslips and pretreated with vehicle, 100 μM Rosi, 1 μM Pio, or 1 μM Dex for 4 h. After treatment, cells were washed and cultured for another 5 days with the medium containing PAN at 5 μg/ml. Control cells did not receive any PAN. Cells were washed two times with PBS, fixed in 3.7% formaldehyde for 10 min, washed two times with PBS, permeabilized with 0.1% Triton X-100 for 5 min, washed two times with PBS, blocked with 1% bovine serum albumin for 30 min, and stained with Texas Red–X phalloidin (Invitrogen) for 20 min. Cells were washed two times with PBS, air-dried, and mounted with Prolong Gold antifade reagent containing 4′,6-di-amidino-2-phenylindole (DAPI) (Invitrogen). Images were captured using 40× objective on a Leica DMI6000B inverted fluorescence microscope (Leica Microsystems, Bannockburn, IL) equipped with a TX2 cube for visualizing Texas Red (excitation 560/40 nm, emission 645/75 nm) and with a A4 cube (excitation 360/40 nm, emission 470/40 nm) for visualizing DAPI. Digital micrographs were captured using a Retiga SRV 14-bit grayscale charge-coupled device camera (QImaging, Surrey, BC, Canada). The images were processed using Adobe Photoshop CS3 (Adobe Systems, Mountain View, CA). The DAPI (in green channel) and Texas-Red images were merged to the final images, showing actin filaments in red and cell nuclei in green.

Western Blotting. After treatments, cells were harvested, and whole-cell extracts were prepared using MPER lysis reagent (Thermo Fisher Scientific Inc., Waltham, MA) containing protease and phosphatase inhibitor cocktails (Sigma-Aldrich). Protein concentrations were determined using bicinchoninic acid (BCA) protein assay reagent (Thermo Fisher Scientific) according to the manufacturer's instructions. Cell lysates (20 μg of protein) were processed for SDS-polyacrylamide gel electrophoresis (PAGE), and the separated proteins were transferred to nitrocellulose membranes (Bio-Rad Laboratories, Hercules, CA). After transfer, nitrocellulose membranes were blocked for 1 h with 5% milk in PBS containing 0.1% Tween 20 and incubated with primary antibody O/N. Antibodies against p38 MAPK, phospho-p38 MAPK (Thr180/Tyr182), p44/42 MAPK (ERK1/2), phospho-p44/42 MAPK (Thr202/Tyr204), SAPK/JNK, phospho-SAPK/JNK (Thr183/ Tyr185), and phospho-GR (Ser211) were purchased from Cell Signaling Technology (Danvers, MA), anti-GR from Santa Cruz Biotechnology (Santa Cruz, CA), and anti-GAPDH from Millipore Corporation (Billerica, MA). Membranes were washed four times with PBS containing 0.1% Tween 20 after primary and secondary antibody incubations. Blots were probed with horseradish peroxidase-conjugated anti-mouse IgG or anti-rabbit IgG secondary antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA) for 2 h at room temperature. Immunoreactive proteins were

detected on X-ray film using the ECL chemiluminescence reagent (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK). X-ray films were scanned using a calibrated ArtixScan M1 transillumination scanner (Microtek Lab, Cerritos, CA) controlled by the ScanWizard Pro program (version 7.042) using standard settings. Densitometric analysis of the integrated band density was performed using ImageJ (version 1.39, standard settings; http://rsb.info.nih.gov/ij/).

RNA Extraction and Real-Time Polymerase Chain Reaction. After treatment, cells were harvested, and total RNA was extracted using the RNeasy kit (QIAGEN, Germantown, MD). Purity and yield were determined by measuring the absorbance at 260 and 280 nm. RNA (1 μg) was subjected to DNase (Ambion, Austin, TX) treatment at 37°C for 30 min followed by a DNase inactivation step in the presence of 5 mM EDTA at 75°C for 10 min. cDNA was prepared from 1 μg of DNase-treated RNA in a 20-μl reaction using iScript reverse transcriptase (Bio-Rad Laboratories) at reaction conditions of 5 min at 25°C, 30 min at 42°C, followed by 5 min at 85°C. FKBP51, GILZ, and β-actin mRNA levels were measured by real-time polymerase chain reaction (PCR) using SYBR green and an iQ5 thermal cycler (Bio-Rad Laboratories). PCR conditions were as follows: first cycle at 95°C for 3 min, 40 cycles at 95°C for 10 s and 55°C for 10 s, followed by a melt curve analysis. The primers used for the amplification are listed in Table 1. The amplification efficiency of each primer pair was measured by plotting the efficiency curve of serial dilutions of selected cDNA samples. Values were normalized to the housekeeping gene β-actin and plotted as the fold change relative to time-matched vehicle control treatments.

Luciferase Reporter Assays. HEK-293T cells were cotransfected with 1 μg of pGRE-Luc (Agilent Technologies Inc., Santa Clara, CA) together with 20 ng of pRL-TK (Promega, Madison, WI), which served as control to correct for transfection efficiency using FuGENE 6 (Roche Applied Science, Indianapolis, IN). pGRE-Luc is a GR-luciferase reporter gene construct that contains four glucocorticoid response element (GRE) repeats (GGTACATTTTGTTCT)$_4$. The constitutively expressing pRL-TK cassette encodes the *Renilla reniformis* reporter gene under the control of herpes simplex virus thymidine kinase promoter. After O/N incubation in charcoal-stripped FBS-containing medium, cells were treated at various concentrations and time points as indicated under *Results*, lysed after 24 h, and assayed for luciferase activity using a Firefly and *R. reniformis* luciferase reporter assay system (Biotium, Hayward, CA) according to the manufacturer's instructions.

Calcineurin Activity Assay. Differentiated podocytes were incubated O/N in medium containing charcoal-stripped FBS. Cells were treated with vehicle, 100 μM Rosi, 1 μM Pio, or 1 μM Dex for 4 h, washed with PBS, and further incubated in charcoal-stripped FBS-containing medium for 24 h. After 1 day, cells were lysed, and the calcineurin (CaN) phosphatase activity was measured using the Colorimetric CaN Cellular Activity Assay Kit (EMD Chemicals Inc., Gibbstown, NJ) according to the manufacturer's instructions. This assay used RII phosphopeptide as the substrate, which is the most well known and efficient substrate for CaN, and the detection of released phosphate is based on malachite green assay. To rule out the contribution of other competing phosphatases, phosphatase activity was measured in the total extract and in the presence of EGTA (blocks

TABLE 1
Genes and their respective primers used in this study
The sequences are in 5′ to 3′ orientation.

Gene	Gene Description (Protein)	Forward	Reverse	Amplicon
				base pairs
Fkbp5	Murine FK506 binding protein 51 (FKBP51)	cttggaccacgctatggtttt	tcccttctcttcacgatgg	171
Tsc22d3	Murine glucocorticoid induced leucine zipper (GILZ)	cgtgaagaaccacctgatgtacgc	ttacaccgcagaaccaccaggggc	235
Actb	Murine β-actin	cttcgttgccggtccacaccc	ctgggcctcgtcacccacat	212
FKBP5	Human FKBP51	tccctcgaatgcaactctct	aaacatccttccaccacagc	178
TSC22D3	Human GILZ	ggtgaagaatcatctgatgtatgc	ttacaccgcagaaccaccagggg	226
ACTB	Human β-actin	acagagcctcgcctttgccg	cttgctctgggcctcgtcgc	238

CaN) and okadaic acid (blocks PP-1 and PP-2A but not CaN). The difference in phosphatase activities [okadaic acid-(okadiac acid-EGTA)] was read as the CaN (PP-2B) activity.

Results

Rosi and Pio Prevent PAN-Induced Podocyte Injury. To test the direct protective effect of both Pio and Rosi on cultured podocytes, cells were injured with 5 μg/ml PAN for 3 or 5 days, which resulted in decreased viability to ~50 to 65% and ~20 to 25%, respectively, compared with untreated cells (Fig. 1). Pretreatment of cells for 4 h, or simultaneous treatment throughout the experiment with the PPARγ agonists Rosi or Pio, significantly increased podocyte viability in a concentration- and time-dependent manner (Fig. 1A). Specifically, pretreatment with Rosi at 100 μM increased the viability to ~75% after 3 days of PAN exposure (Fig. 1a), whereas simultaneous treatment with 0.1, 1, or 10 μM Rosi increased the viability to ~75 to 85% of that of the untreated control cells (Fig. 1b). Likewise, Pio increased the viability to ~80 to 90% with both pretreatment (0.1, 1, and 10 μM) and simultaneous treatment (0.01, 0.1, and 1 μM) after 3 days of PAN exposure (Fig 1, a and b). The glucocorticoid Dex provided almost complete protection to PAN-injured podocytes at the concentrations used (1 and 10 μM for pretreatment, 0.1 and 1 μM for simultaneous treatment) (Fig. 1, a and b). After 5 days of PAN injury, a similar pattern of protection by both TZDs was observed, although the overall extent of protection was less pronounced. Pretreatment with Rosi at 100 μM and Pio at 1 and 10 μM increased the viability to ~50%, compared with ~25% viability with PAN injury alone (Fig.

1c). Likewise, simultaneous treatment with 0.1, 1, or 10 μM Rosi or with 0.01, 0.1, or 1 μM Pio increased the viability to ~25 to 30% (Fig. 1d). Significant protection was also achieved by pretreatment with 1 and 10 μM Dex and simultaneous treatment with 0.1 and 1 μM Dex, which increased viability to ~75 and ~60%, respectively (Fig. 1, c and d).

To determine the potential of TZDs to improve the efficacy of GCs, we also determined the effects of Dex treatment combined with either Rosi or Pio on the protection of podocytes injured with PAN. Although combined treatment with Pio and Dex tended to result in increased cell viability compared with Dex treatment alone, it remained below the statistical significance (Fig. 1B). Likewise, combined treatment of Rosi with Dex also tended to provide better protection than Dex alone (data not shown). Viability values greater than 100% with some treatments indicated continued cell proliferation compared with controls. These data together suggest that both TZDs exhibit the capability to protect podocytes from PAN-induced injury.

Rosi and Pio Prevent PAN-Induced Podocyte Actin Cytoskeletal Disruption. The podocyte foot processes that are disrupted during NS are well known to contain prominent actin filaments (Smoyer and Mundel, 1998; Ransom et al., 2005). Likewise, cultured podocytes contain a distinct actin cytoskeleton with bundles of filamentous actin fibers spanning across the cell body (stress fibers), which can be visualized with Texas Red-labeled phalloidin (Fig. 2A). Podocyte injury and recovery in vitro have been well documented to involve extensive reorganization of actin cytoskeleton (Smoyer and Mundel, 1998; Ransom et al., 2005). PAN treat-
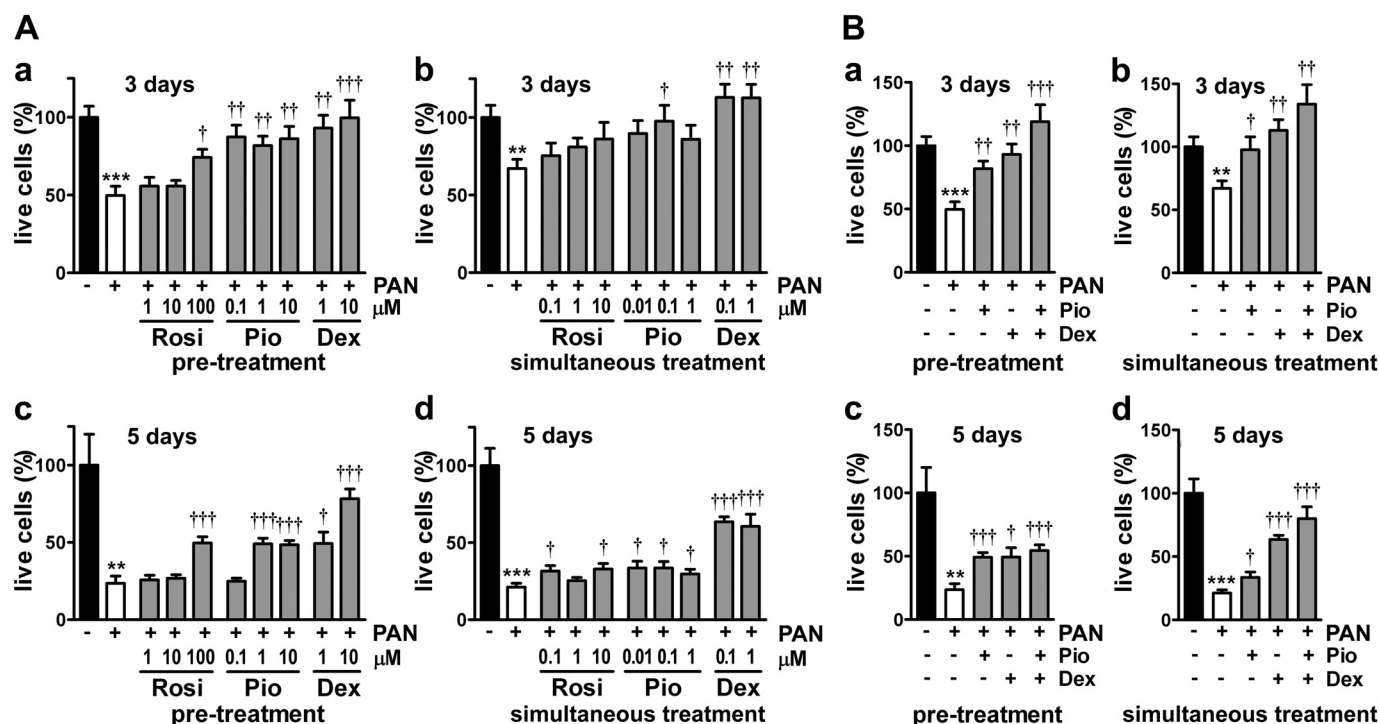
Fig. 1. Viability assays of PAN-injured podocytes treated with Rosi, Pio and/or Dex. A, podocytes were either pretreated for 4 h (a and c) or simultaneously treated until the completion of assay (b and d) with vehicle, Rosi, Pio, or Dex at indicated concentrations, injured with 5 μg/ml PAN for 3 days (a and b) or 5 days (c and d) and assayed for viability using MTT. B, podocytes were treated with vehicle, Pio, and/or Dex at 1 μM each for pretreatments (a and c), or with 0.1 μM each for simultaneous treatments (b and d), injured with 5 μg/ml PAN for 3 days (a and b) or 5 days (c and d) and assayed for viability using MTT. Viability values greater than 100% with some treatments indicate continued cell proliferation compared with controls. Significant differences from control ($*$) or PAN treatment ($\dagger$) are indicated as obtained by unpaired t test ($*/\dagger$, $P < 0.05$; $**/\dagger\dagger$, $P < 0.01$; $***/\dagger\dagger\dagger$, $P < 0.001$). Black columns, controls; white columns, PAN treatment; gray columns, PAN + TZD/Dex treatments.
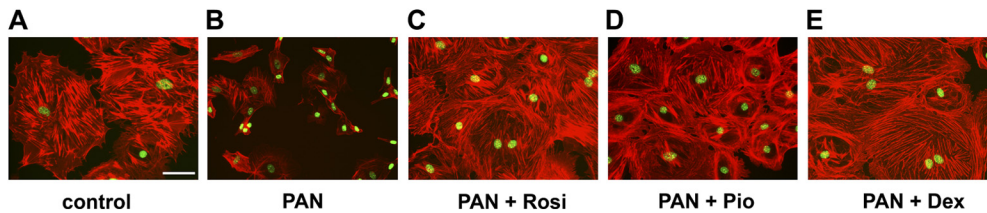
Fig. 2. Effects of Rosi, Pio, or Dex treatments on PAN-induced podocyte actin cytoskeletal injury. After pretreatment for 4 h with vehicle, 100 μM Rosi, 1 μM Pio, or 1 μM Dex, cells were injured with 5 μg/ml PAN for 5 days. After fixation, actin filaments were stained with Texas Red phalloidin (shown in red), and nuclei were stained with DAPI (shown in green). Scale bar, 25 μm.

ment (5 μg/ml for 5 days) resulted not only in a reduced cell number, but also in the substantial loss of the filamentous actin in the remaining cells (Fig. 2B). Pretreatment with Rosi (100 μM) or Pio (1 μM) preserved podocyte filamentous actin, in addition to the cell numbers, similarly as did pretreatment with Dex (1 μM) (Fig. 2, C–E). However, after pretreatment with either TZD, the organization of these actin fibers was somewhat different from control cells, with relatively more filamentous actin being located in subcortical ring-like structures. This was especially evident after pretreatment with Pio (Fig. 2D).

Rosi Attenuates MAPK Signaling in Podocytes. PPARγ agonists have been reported to elicit "nongenomic," receptor-independent effects in addition to their well documented role in inducing transcription of target genes in a PPAR-dependent manner. The rapid alteration of MAPK activities by Rosi and Pio observed previously in several cell types provides an example of such "nongenomic" effects (Gardner et al., 2005). Based on this, we tested the ability of Rosi and Pio and compared it with Dex to alter the activation of the major MAPKs, including p38 MAPK, ERK1/2, and SAPK/JNK, in podocytes. Podocytes were treated with Rosi or Pio in the presence and absence of Dex, and the relative activation of the various MAPKs was determined by immunoblotting using antibodies specific for the phosphorylated (activated) and total forms of these protein kinases (Fig. 3A). The semiquantitative densitometric evaluation of these gels is shown in the plots of Fig. 3B. Although treatment with Rosi deactivated all three MAPKs tested, Pio did not. In addition, treatment with Dex possibly resulted in a minor deactivation of ERK1/2 and p38 MAPK. Combined treatment with Rosi and Dex resulted in a further deactivation of Erk1/2 compared with individual treatments, whereas it had no major effect on the activities of the other two MAPKs.

Combined treatment with Pio and Dex resulted in generally similar MAPK activities compared with Dex treatment alone. In summary, deactivation of the various MAPKs in podocytes seemed to be a selective property of Rosi compared with Pio, although Dex also modestly deactivated ERK1/2 and p38 MAPK as well.

Rosi and Pio Increase the GR Phosphorylation in Podocytes. The GR has been shown to undergo rapid downregulation and phosphorylation on Ser211 in response to GCs, and phosphorylation at this site is believed to be associated with activation of the GR (Tasker et al., 2006). PPARγ ligands have been reported to exert "off-target" effects involving seemingly unrelated molecules and receptors, including the GR (Ialenti et al., 2005; Matthews et al., 2009). Recent studies have also shown that the PPARγ and GR may interact directly upon PPARγ activation, suggesting cross-talk between the two signaling pathways (Nie et al., 2005; Lahiri et al., 2009). To determine whether Rosi and Pio exert some of their effects in podocytes by modulating the expression or phosphorylation of the GR, we measured the total amount of the GR and its phosphorylation in podocytes treated with Rosi and Pio and compared these responses with that of Dex (Fig. 4A). As seen after 4-h treatment, Dex induced downregulation of the GR and enhanced its phosphorylation, similarly as has been reported previously (Guess et al., 2010). It is noteworthy that Rosi and Pio also increased the phosphorylation of the GR, although they had no detectable effect on its down-regulation. Combined treatments with Rosi or Pio together with Dex did not show any detectable differences from Dex treatment alone. In summary, Rosi, Pio, and Dex all resulted in increased phosphorylation of the GR in the following order: Dex > Rosi > Pio.

To dissect whether the phosphorylation of the GR by Rosi and Pio is PPARγ and/or GR-mediated effect, we performed
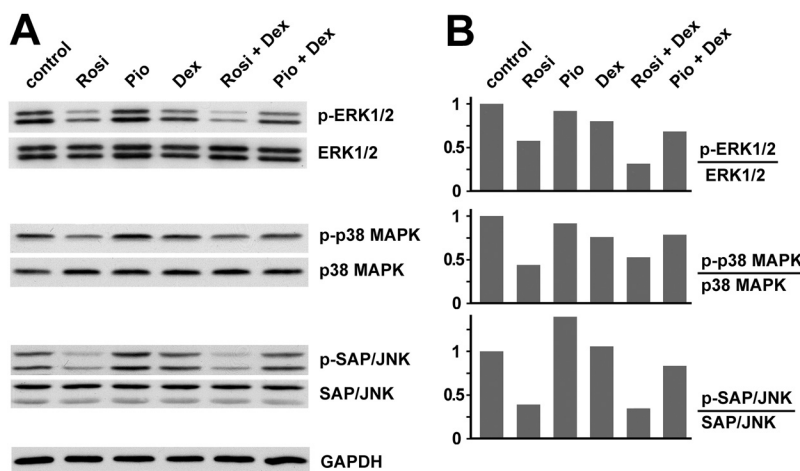
Fig. 3. Effects of Rosi, Pio, and Dex treatments on activities of MAPKs in podocytes. A, serum-starved cells were treated with vehicle, 100 μM Rosi, 1 μM Pio, and/or 10 μM Dex for 4 h, harvested, and the total protein extracts were subjected to SDS-PAGE followed by immunoblot analysis for phosphorylated (activated) and total ERK1/2, p38 MAPK, and SAPK/JNK. GAPDH served as a loading control. Representative blots from three independent experiments are shown. B, densitometric analysis of the blots shown in A was performed and the ratios of the phosphorylated (p-), and total MAPK forms were plotted as fold changes relative to the controls.

A

B
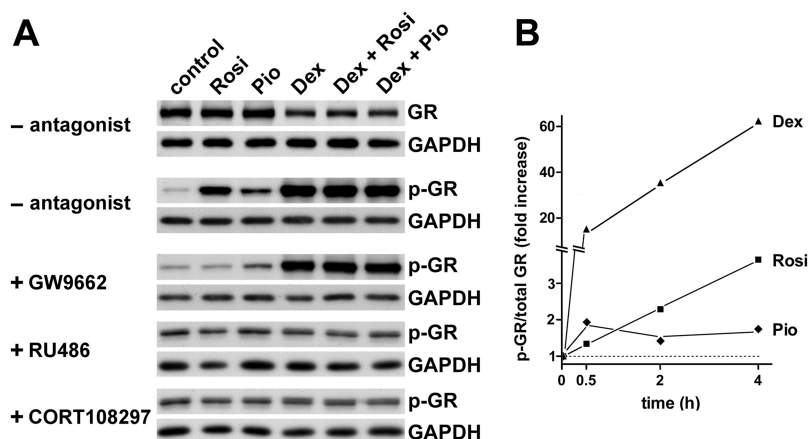
Fig. 4. Phosphorylation and expression of the GR in podocytes in response to Rosi, Pio, and Dex. A, serum-starved cells were treated for 4 h with vehicle, 100 μM Rosi, 1 μM Pio, or 10 μM Dex alone, or in combination as indicated. Where indicated, cells were preincubated with 10 μM GW9662 (PPARγ antagonist), 10 μM RU486, or 10 μM CORT108297 (GR antagonists) for 1 h before Rosi, Pio, and Dex treatments. Protein lysates were subjected to SDS-PAGE followed by immuno-blot analysis for phosphory-lated (p-GR) and total GR, and for GAPDH. Representative blots from three independent experiments are shown. B, cells were treated with Rosi, Pio, and Dex as in A, harvested at 0 (control), 0.5, 2, and 4 h, and subjected to immunoblot analysis. Densitometric analysis was performed, and the ratios of the phosphorylated and total GR were plotted as fold increases relative to the control (indicated by the dotted line).

the above experiments in the presence of antagonists specific for these receptors (Fig. 4A). In the presence of the PPARγ antagonist GW9662, Rosi treatment did not result in increased GR phosphorylation, thus suggesting PPARγ dependence. In addition, the effects of Pio were inhibited to some extent by GW9662. No differences were observed in the presence or absence of GW9662 with Dex alone or combined treatments, suggesting that PPARγ is not involved in the GR phosphorylation in response to Dex.

As expected, the GR antagonists RU486 and CORT108297 inhibited the phosphorylation of the GR in response to Dex alone or to the combination treatments (Fig. 4A). RU486 and CORT108297 by themselves increased GR phosphorylation moderately, consistent with the known partial agonistic properties of these drugs (see *Materials and Methods*). This increased GR phosphorylation in response to the GR antagonists may obscure possible effects resulting from Rosi or Pio alone treatments. Therefore, it is difficult to conclude whether phosphorylation of the GR in response to Rosi and Pio is dependent on GR signaling. In summary, our data suggest that the phosphorylation of GR in response to Rosi, and possibly Pio, is PPARγ-dependent, and that GR phosphorylation by Dex alone or in combination with Rosi and Pio requires GR signaling.

In a separate experiment, we determined the time course of the increase in phosphorylation of the GR at Ser211 in

response to the Dex, Rosi, and Pio by semiquantitative evaluation of the corresponding immunoblots (Fig. 4B). To estimate the changes in the relative degree of phosphorylation of the GR, the signals for phospho-GR were related to those of total GR. Treatment with Dex greatly increased GR phosphorylation in a time-dependent manner. Treatment with Rosi and Pio also resulted in increased GR phosphorylation, although to a lesser extent. Pio seemed to induce an early response with no further increase in the relative phosphorylation of the GR after 0.5 h.

Rosi and Pio Induce and Enhance the GR Transcriptional Activity in Podocytes. Given our observations that TZDs are able to both protect podocytes and stimulate the GR, although to a lesser extent than Dex, we examined the ability of Rosi and Pio to regulate the transcription of two well studied GC-responsive genes, FKBP51 and GILZ (Vermeer et al., 2003; Ayroldi and Riccardi, 2009). FKBB51 is commonly associated with the GR complex and tends to inhibit its activity, whereas GILZ is a transcription factor controlling the expression of many other genes in response to GCs. Dex increased the mRNA expression of FKBP51 ~10- to 15-fold and GILZ ~3- to 5-fold, as measured after 1 and 3 days of treatment, after either short-term or long-term treatments (Fig. 5, A and B). Rosi and Pio alone induced FKBP51 and GILZ mRNA expression (~2 fold) in both short-term (except for GILZ induction in response to Pio) and long-term

A FKBP51

B GILZ
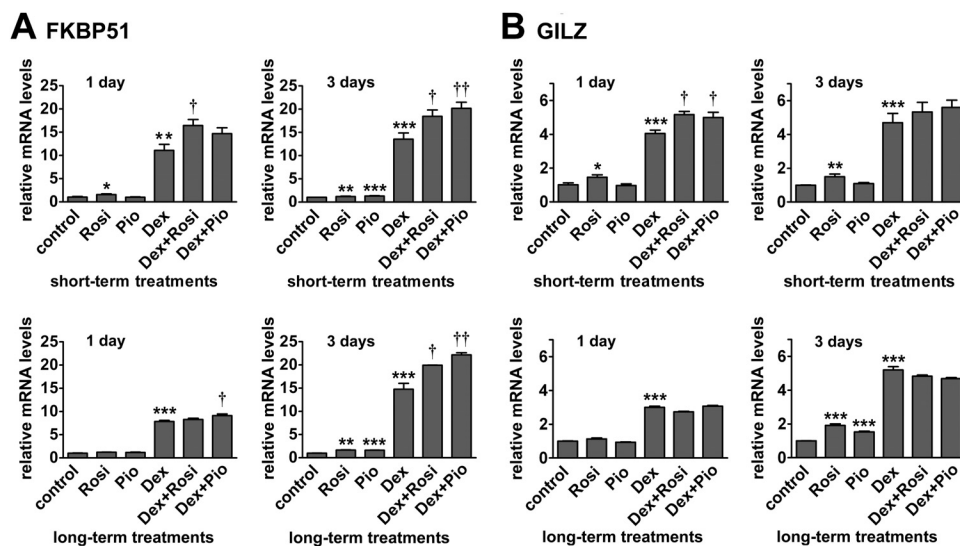
Fig. 5. mRNA expression of FKBP51 and GILZ in podocytes in response to Rosi, Pio, and/or Dex. A and B, cells incubated O/N in charcoal-stripped FBS-containing medium were subjected to short-term treatment (4 h) with 100 μM Rosi, 1 μM Pio, and/or 10 μM Dex or long-term treatment (1 or 3 days) with 10 μM Rosi, 0.1 μM Pio, and/or 1 μM Dex. Total RNA was extracted after 1 and 3 days and analyzed by real time RT-PCR using SYBR green for mRNA expression analysis of FKBP51 and GILZ, and the values were normalized to β-actin. Fold change values compared with controls (treatment with vehicle only) were plotted as mean $\pm$ S.D. from triplicates of three experiments. Significant differences of individual treatments versus controls (∗) or combined treatments versus Dex treatments (†) are indicated as obtained by unpaired t test (∗/†, $P < 0.05$; ∗∗/††, $P < 0.01$; ∗∗∗/†††, $P < 0.001$).

treatments, as measured after 3 days. Both TZDs also enhanced the induction of FKBP51 and GILZ mRNAs by Dex, as seen in both short-term and long-term treatments for FKBP51 (3 days) and in short-term treatments for GILZ (1 day). Specifically, Rosi and Pio enhanced the induction of FKBP51 from ~10- to 15-fold by Dex alone to ~20-fold when used in combination after 3 days (Fig. 5A).

Modulation of the GR Transcriptional Activity in Podocytes by Rosi and Pio Is Both GR- and PPARγ-Dependent. Given the "GR modulator effects" of the TZDs in podocytes (see above), we determined whether Rosi and Pio modulate the transcriptional activity of the GR in a GR- and/or PPARγ-dependent way. For this purpose, we measured the expression of GC-responsive genes in podocytes in response to Rosi, Pio, and Dex in the presence of the GR- and PPARγ-specific antagonists RU486, CORT108297, and GW9662 (Fig. 6). As expected, RU486 blocked the induction of FKBP51 and GILZ mRNAs by Dex, alone or in combination treatments. Moreover, there was no significant difference in FKBP51 expression between the Dex-alone treatment and combined treatments with Rosi and Pio in the presence of RU486 (unlike in the absence of the antagonist), suggesting that RU486 also inhibits any enhancing effects of Rosi and Pio on Dex-induced expression. Given the known nonspecific antagonistic effects of RU486 on other nuclear receptors (e.g., the progesterone receptor), this GR dependence was further confirmed by measuring the mRNA expression of one of the studied genes, FKBP51, using the highly specific GR antagonist CORT108297. Similar to RU486, CORT108297 blocked the induction of FKBP51 by Dex alone or in combination treatments. In addition, as observed with RU486, no significant difference was observed between the Dex-alone treatment and combined treatments with Rosi and Pio in the presence of CORT108297 (unlike in the absence of the antagonist). Although CORT108297 is a highly specific antagonist for the GR, it is also known to exert partial agonistic effects (R. Clark, Corcept Therapeutics, personal communication; also see *Materials and Methods*). This partial agonistic activity is reflected in the relatively high baseline expression of FKBP51 in the presence of CORT108297 (Fig. 6).

Finally, to determine the PPARγ dependence, the effects of Rosi and Pio in the presence of the PPARγ antagonist, GW9662, were also measured on the induction and enhancement of Dex-induced mRNA expression of FKBP51 (Fig. 6). GW9662 blocked the induction of FKBP51 mRNA by Rosi and the Rosi- and Pio-mediated enhancement of Dex-induced increase in FKBP51 expression. In addition, no significant difference was observed between the Dex-alone treatment and combined treatments with Rosi and Pio in the presence of GW9662 (unlike in the absence of the antagonist). As expected, GW9662 did not significantly reduce the expression of FKBP51 induced by Dex alone. These data suggest that both the induction of FKBP51 and the enhancement effects on Dex-induced expression by TZDs are PPARγ-dependent. Taken together, the transcriptional activity of the GR in podocytes can be modulated by both Rosi and Pio, and this modulation involves both GR and the PPARγ signaling.

Rosi and Pio Have Disparate Effects on Dex-Induced Activity of Minimal and Native Endogenous Promoters. To further examine the direct effects of Rosi and Pio on GR transcriptional activity, we analyzed the luciferase reporter activity under the control of a minimal promoter containing four GRE repeats (GGTACATTTGTTCT) in HEK-293T cells (Fig. 7, A and B). Although Dex activated the promoter strongly, we did not observe major effects by Rosi or Pio in activating the luciferase gene expression driven by the minimal GRE promoter. Likewise, both Rosi and Pio, in combination with Dex, did not exhibit major effects on Dex-induced activation of this promoter in both short- and long-term treatments.

Because we had previously seen enhancing effects on endogenous gene expression in podocytes when these drugs were used in combination (Fig. 5), we chose to explore whether the absence of enhancement effects were due to the different cell types used or if they were dependent on promoter specificity. We therefore analyzed the expression of the endogenous genes, FKBP51 and GILZ, in HEK-293T cells treated with Rosi, Pio, and/or Dex (Fig. 7, C and D). We observed that Rosi or Pio alone significantly induced the
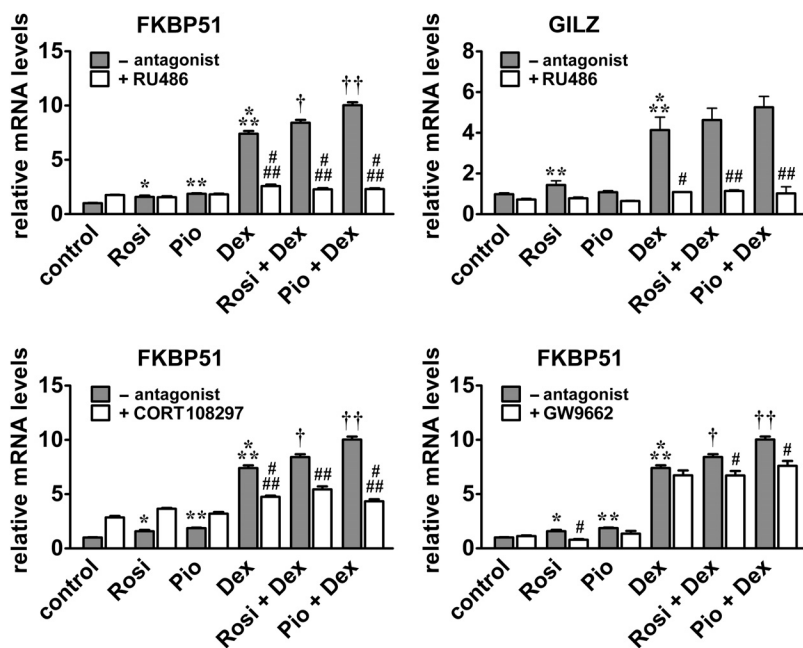
Fig. 6. mRNA expression of FKBP51 and GILZ in podocytes in response to Rosi, Pio, and/or Dex in the presence of GR and PPARγ antagonists. Serum-starved cells were preincubated for 1 h with 10 μM GW9662 (PPARγ antagonist), 10 μM RU486, or 10 μM CORT108297 (GR antagonists), followed by 4-h treatments with 100 μM Rosi, 1 μM Pio, and/or 10 μM Dex. Total RNA was extracted after 3 days and analyzed by real-time RT-PCR assay using SYBR green for mRNA expression analysis of FKBP51 and GILZ, and the values were normalized to β-actin. Fold change values compared with controls (treatment with vehicle only) were plotted as mean ± S.D. from triplicate experiments. Significant differences in the absence of antagonists of individual treatments versus controls (∗) and combined treatments versus Dex treatments (†), and significant decreases in the presence versus absence of antagonists (#) are indicated as obtained by unpaired t test (∗/†/#, $P < 0.05$; ∗∗/††/##, $P < 0.01$; ∗∗∗/†††/###, $P < 0.001$).

expression of both FKBP51 and GILZ. Dex, as expected, also induced the expression of these genes. Combined treatments with Rosi showed enhancing effects on Dex-mediated activation of these endogenous promoters, as was also shown in podocytes in Fig. 5. Combined treatment with Pio enhanced the Dex-mediated activation of FKBP51. These observations are in accordance with earlier studies in which Rosi had an inhibitory effect on Dex-mediated activation of a simple promoter (TAT3) (Matthews et al., 2009), whereas it enhanced Dex-mediated activation of the natural and more complex mouse mammary tumor virus promoter (Johnson et al., 1999).

Taken together, both TZDs exhibited disparate effects on the minimal promoter tested and endogenous promoters. Although the activity of the minimal promoter tested was not affected by either TZD, the expression of the endogenous genes (driven by native promoters) tested was clearly enhanced.

Rosi, Pio, and Dex Increase CaN Activity in Podocytes. CaN inhibitors, including tacrolimus (FK506) and cyclosporine, are widely used drugs for the treatment of NS in cases in which GCs are clinically ineffective. Previous reports have demonstrated the ability of Dex to stimulate CaN phosphatase activity in some cell types and suppress it in other cell types (Tumlin et al., 1997; Hirakawa et al., 2009). To date, the ability of neither Rosi, Pio, nor even Dex to alter CaN activity in podocytes has been studied. Therefore, we compared the CaN activity in podocytes treated with each of these compounds by measuring phosphate released by CaN phosphatase activity (Fig. 8). We found that Dex treatment significantly stimulated CaN activity in podocytes, as measured by the total phosphate released. Likewise, Rosi and Pio both exhibited similar effects to Dex by increasing the CaN

activity significantly, thus suggesting another similarity between the molecular actions of TZDs and those of GCs in podocytes.

Discussion

The TZDs Pio and Rosi are FDA-approved drugs widely used for the treatment of type II diabetes that have also been shown to reduce proteinuria and urinary podocyte loss in patients with diabetic nephropathy and nondiabetic glomerulosclerosis (Sarafidis et al., 2010). We hypothesized that these renal protective effects of TZDs result, at least in part, from a direct action on podocytes. We found that both Pio and Rosi were indeed able to directly protect podocytes, similar to Dex, against experimentally induced injury designed to mimic NS. In addition, we observed that Rosi and/or Pio were able to modulate the podocyte GR pathway and alter MAPK activation, as illustrated in Fig. 9. We also found that Rosi, Pio, and Dex induced common, as well as selective, molecular signaling effects in podocytes. Together, our findings suggest that Pio and Rosi may have potential clinical utility as either a primary or adjunctive therapy for NS or other diseases treated with GCs. Moreover, these findings may also lend new mechanistic insight into the well established but poorly understood renal protective effects of TZDs in diabetic nephropathy (Lennon et al., 2009).

Healthy podocytes contain an extensive network of actin filaments, with high content in the distal foot processes that attach to the underlying glomerular basement membrane (Smoyer and Mundel, 1998). During NS, podocyte injury results in rearrangement of the actin cytoskeleton in the foot processes (effacement), and in some cases apoptosis, cell proliferation, or dedifferentiation. Podocytes typically respond to
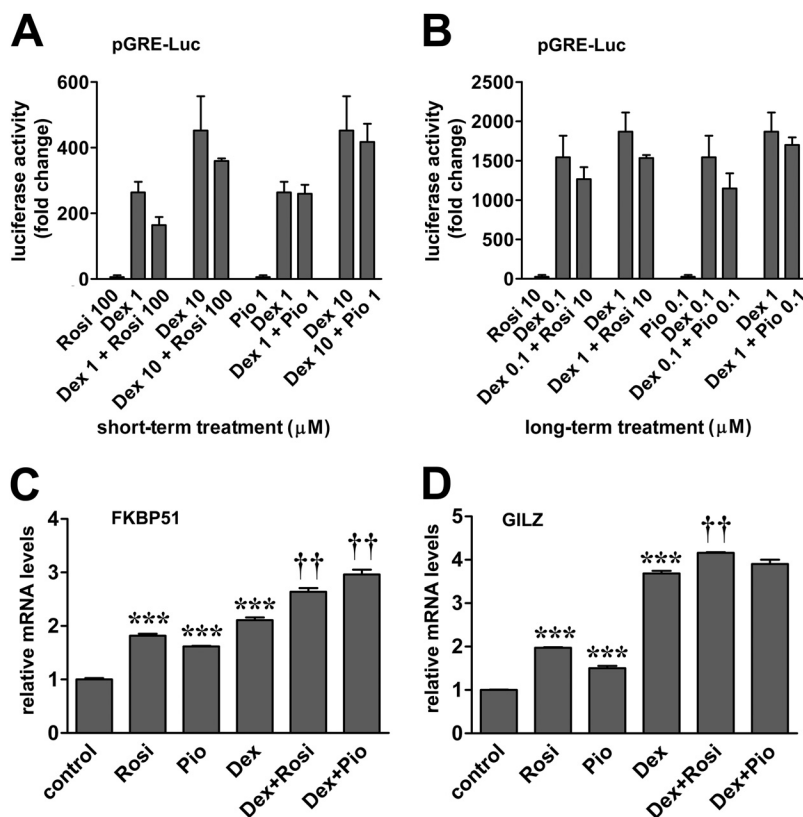
Fig. 7. Effects of Rosi and Pio on a minimal promoter containing GREs and on endogenous genes. A and B, action of Rosi and Pio on Dex-induced luciferase reporter activity driven by the minimal GRE promoter. HEK-293T cells were cotransfected with pGRE-luc (firefly luciferase reporter gene under the control of minimal GRE promoter) and pRL-TK (constitutively expressed *R. reniformis* luciferase), treated for 4 h (A, short-term) or for 24 h (B, long-term) with indicated concentrations of Dex, Rosi, and Pio after O/N incubation in charcoal-stripped FBS-containing medium. After 24 h of treatment, cells were lysed and assayed for firefly and *R. reniformis* luciferase activities. The values of firefly luciferase activity were corrected for transfection efficiency using the *R. reniformis* luciferase and plotted as mean ± S.D. from triplicate analyses and are representative of three experiments. The plotted fold changes are in comparison with vehicle-treated control cells transfected with the reporter constructs. C and D, mRNA expression of FKBP51 and GILZ in HEK-293T cells in response to Rosi, Pio, and/or Dex. Cells incubated O/N in charcoal-stripped FBS-containing medium were treated for 4 h with 100 μM Rosi, 1 μM Pio, and/or 10 μM Dex. Total RNA was extracted after 1 day and analyzed by real-time RT-PCR assay for expression of FKBP51 and GILZ, and the values were normalized to β-actin. Fold change values compared with controls (treatment with vehicle only) were plotted as mean ± S.D. from triplicates and are representative of three experiments. Significant differences of individual treatments versus controls (∗) or combined treatments versus Dex treatments (†) are indicated as obtained by unpaired *t* test (∗/†, $P < 0.05$; ∗∗/††, $P < 0.01$; ∗∗∗/†††, $P < 0.001$).

stressors (e.g., treatment with PAN, shear stress) with reorganization or loss of filamentous actin, focal adhesions, or cell-cell contacts. In cultured podocytes, these changes can be prevented by treatment with GCs or by ectopic expression of protective genes (Ransom et al., 2005). In the present study, we found that, similar to GCs, TZDs can directly protect podocytes from PAN-induced injury, both in terms of cell viability and disruption of the actin cytoskeleton.

TZDs act as PPARγ ligands, but are becoming increasingly known to mediate a portion of their actions through PPARγ-
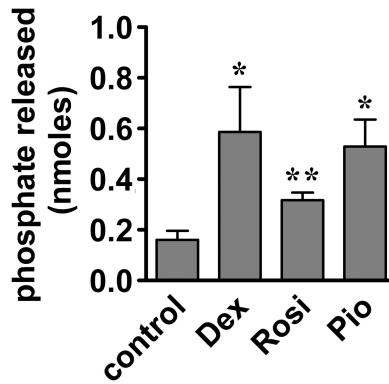
Fig. 8. CaN activity of podocytes treated with Rosi, Pio, or Dex. Cells incubated O/N in charcoal-stripped FBS-containing medium were treated with 1 μM Dex, 100 μM Rosi, or 1 μM Pio for 4 h and assayed for CaN activity after 24 h by detecting the free phosphate released from the RII phosphopeptide substrate by malachite green assay. The values plotted on the graph are the mean ± S.D. from triplicates and are representative of three experiments. Significant differences of treatments versus the control are indicated as obtained by unpaired t test ($*$, $P < 0.05$; $**$, $P < 0.01$).

independent pathways (Gardner et al., 2005; Ialenti et al., 2005). In addition, it has been reported that minor differences in the chemical structure of Rosi and Pio may result in marked and distinct differences in their molecular, biological, and pharmacological responses (Gervois et al., 2007; Kintscher, 2008). Given these selective effects of Rosi and Pio and the PPARγ-independent effects of TZDs in general, we explored the ability of Rosi and Pio to modulate two of the major signaling pathways (MAPK and GC pathways) most relevant to podocyte protection in various renal diseases, including NS (Ransom et al., 2005; Grande and López-Novoa, 2008; Guess et al., 2010).

The MAPKs have been implicated in the progression of various glomerulopathies, and their inhibition is emerging as a promising therapeutic area for renal diseases and for steroid resistance in other diseases (Bloom, 2004; Grande and López-Novoa, 2008). Given the importance of MAPK signaling in renal diseases, we were also interested in determining the ability of TZDs to modulate the various MAPKs in podocytes and in comparing these results with those of GCs. We found that Rosi deactivated ERK1/2, p38 MAPK, and SAPK/JNK. Although Dex also seemed to deactivate ERK1/2 and possibly p38 MAPK, Pio did not have any notable effects. Inhibition of p38 MAPK and possibly ERK1/2 in PAN and doxorubicin animal models of NS has been reported to suppress proteinuria and actin reorganization in podocytes (Koshikawa et al., 2005). In addition, we have shown that inhibiting either p38 MAPK or its downstream substrate MK2 prevents PAN-induced injury to podocytes (Pengal et al., 2011). Increased amounts of activated ERK and p38 MAPK
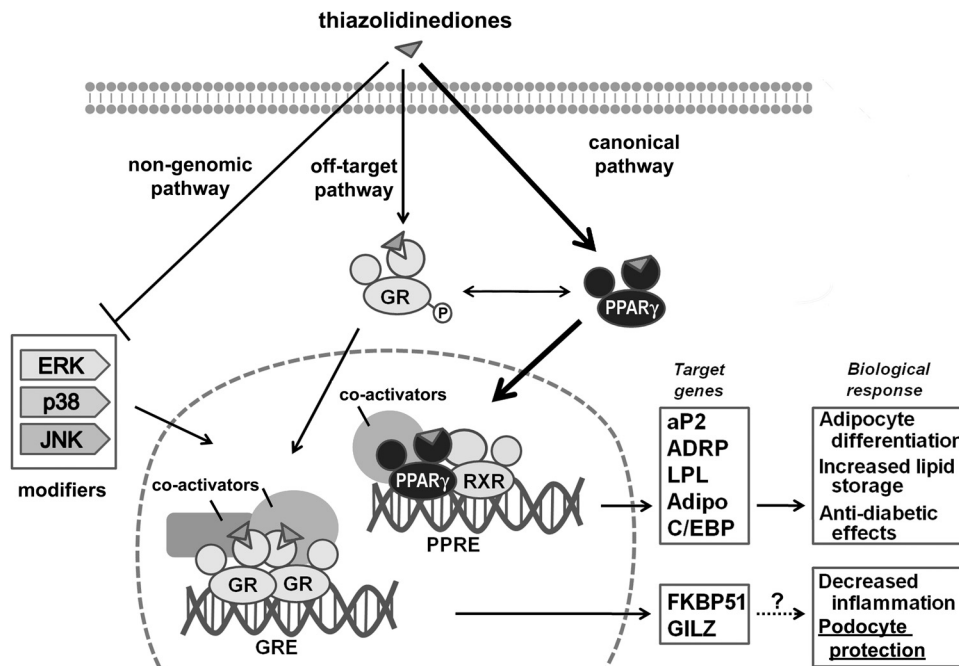
Fig. 9. Schematic of canonical and alternate modes of action of TZDs. Traditionally, TZDs bind to their receptor, PPARγ, which dimerizes with the nuclear receptor RXR and acts on peroxisome proliferator response elements together with coactivators to promote the transcription of genes involved (e.g., in antidiabetic effects). Our data and the literature suggest that the TZDs can act on the GR directly (off-target pathway), as well as via the canonical PPARγ pathway with considerable cross-talk between the two pathways. In the "off target" pathway, TZDs may imperfectly bind to and stimulate the GR via phosphorylation and nuclear translocation, possibly resulting in the expression of GC responsive genes. TZDs may also modulate the GR pathway indirectly via binding to PPARγ, resulting in subsequent interaction between the two receptors directly or through the involvement of common cofactors. TZDs may also deactivate MAPKs via a "nongenomic" pathway, which subsequently modifies other cellular systems including the phosphorylation of the GR and PPARγ. This schematic was modified from the Nuclear Receptor Resource (nrresource.org) with the permission of Dr. J. Vanden Heuvel, University Park, PA.

have also been reported in the glomeruli of patients with diabetic nephropathy (Sakai et al., 2005).

GCs are the prevailing therapy for NS and are also known to act directly on podocytes, although not much is known about their mechanism of protective action (Ransom et al., 2005; Guess et al., 2010). We studied the effects of TZDs on the GR pathway in podocytes and found that Rosi and Pio phosphorylate the GR at the site associated with its activation, although to a lesser extent than Dex. In support of this finding, other recent reports have also linked the action of TZDs to the GR pathway (Ialenti et al., 2005; Matthews et al., 2009). In these studies, the anti-inflammatory effects of Rosi and ciglitazone were shown to involve, to some extent, GR-dependent signaling. In addition, TZDs were shown to activate nuclear translocation of the GR in vitro, with no effect on its down-regulation and independent of the PPARγ. Likewise, in the present study, Rosi and Pio did not lead to down-regulation of the GR, which is a known negative feedback mechanism associated with GC treatment. In theory, such an effect could be clinically beneficial if TZDs were used in patients with steroid-resistant NS (or other steroid resistant diseases), because it might overcome this known negative feedback mechanism. Furthermore, we found that the phosphorylation of GR by Rosi and possibly Pio is PPARγ-dependent because it is abolished in the presence of GW9662, a selective PPARγ antagonist. This suggests that the PPARγ may interact with the GR signaling pathway by physical or cofactor dependent interaction upon activation as has also been shown recently in other systems (Nie et al., 2005; Lahiri et al., 2009).

Given the complex nature of both GR- and PPARγ agonist-mediated signaling, the interplay of transcription cofactors (activators or repressors) can be assumed to be altered by PPARγ agonists. Such cross-talk among signaling cascades may be the basis for the synergistic effects on GR signaling exerted by Rosi and Dex in a mouse model of inflammation or for their ability to modulate Dex- and Rosi-induced gene transcription and differentiation in osteoblastic cells (Johnson et al., 1999; Ialenti et al., 2005). In the latter report, Rosi did not exhibit any effect alone in activating the native mouse mammary tumor virus promoter, whereas it enhanced the effects of Dex on the same promoter. In a different report, Rosi and Pio minimally activated a GRE TAT3 promoter but repressed its activity when used in combination with Dex (Matthews et al., 2009). In the current study using a minimal GRE promoter with four GRE sites, combination treatments using Rosi or Pio with Dex did not significantly alter the GRE promoter activity. Despite this, Rosi and Pio both induced the expression of two endogenous GC-responsive genes, GILZ and FKBP51, and also enhanced Dex-induced expression of these genes. These findings, together with the above reports, suggest that the effects of TZDs on Dex-regulated gene expression can be very disparate, depending on the nature of the studied promoters and genes. Moreover, our findings that GR phosphorylation by Rosi and possibly Pio is PPARγ-mediated and that both TZDs modulate GR-responsive genes in podocytes in a PPARγ- and GR-dependent manner suggest that these drugs are indeed able to modulate GR signaling in podocytes and that their protective effects could possibly be mediated in part via these pathways.

Finally, because CaN inhibitors are often used as alternative treatments for NS, we investigated the ability of TZDs to alter CaN activity in podocytes. We also compared their modulation of CaN activity with that of Dex, because GCs are known to provide direct protection to podocytes against injury. Of particular note, we found that all three drugs that protected podocytes against PAN-induced injury (Pio, Rosi, and Dex) also increased the phosphatase activity of CaN in these cells. This finding is consistent with a previous report of transcription-independent activation of CaN by GCs in renal proximal tubular cells (Tumlin et al., 1997). However, our findings of increased podocyte CaN activity in response to Rosi, Pio, and Dex were somewhat surprising, because CaN inhibitors (cyclosporine and tacrolimus) are widely used clinically to treat steroid-dependent and steroid-resistant NS. Moreover, CaN inhibition has been suggested to be involved in cyclosporine's direct effects on podocytes via stabilization of the actin cytoskeleton (Faul et al., 2008). Despite this, the current findings suggest the possibility that inhibition of podocyte CaN activity may not be critical for the clinical efficacy of cyclosporine and tacrolimus in NS. Thus, although the role of podocyte CaN activity in response to injury remains unclear at this time, our results suggest yet another similarity between the molecular actions of GCs and TZDs in podocytes.

In summary, we have found that TZDs are able to modulate the GC pathway and exert direct protective effects on podocytes, similar to GCs, possibly via previously unrecognized effects on GC and MAPK signaling. These findings suggest that these FDA-approved drugs may have potential clinical utility as either primary or adjunctive therapy for NS or other diseases treated with GCs. This study may also offer mechanistic insight into the well established but poorly understood renal protective effects of Rosi and Pio in patients with diabetic nephropathy.

Authorship Contributions

Participated in research design: Agrawal, Benndorf, and Smoyer.
Conducted experiments: Agrawal and Guess.
Performed data analysis: Agrawal, Guess, Benndorf, and Smoyer.
Wrote or contributed to the writing of the manuscript: Agrawal, Benndorf, and Smoyer.

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