

Research Article

Troglitazone Induces Extracellular Matrix and Cytoskeleton Remodeling in Mouse Collecting Duct Cells

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Peroxisome proliferator-activated receptor (PPAR γ) has been shown to have a protective role in the nephron through its ability to inhibit a transforming growth factor- (TGF- β) mediated fibrotic response. In contrast, PPAR γ was also shown to induce a mesenchymal transformation in epithelial intestinal cells. A fibrotic response in the collecting duct has only recently been established; however, the entire collecting duct has not been fully examined. Inner medullary collecting duct cells (IMCD-K2) and mouse cortical collecting duct cells (M1), representing the cortical and medullary collecting duct, were exposed to 5–10 μ M troglitazone for 24 hours. Troglitazone resulted in an elongated morphology, 60% decreases in E-cadherin and β -catenin, a 35% decrease in α -catenin, and a 1.5-fold increase in fibronectin. These effects were not reversed with PPAR γ antagonists or affected with PPAR γ overexpression. Our results indicate that troglitazone induced a mesenchymal-like transformation in M1 and IMCD-K2 epithelial cells independently of PPAR γ .

1. Introduction

PPARs are ligand-activated transcription factors that heterodimerize with an RXR receptor. There are currently three cloned PPARs (α , γ , and β/δ), all of which are expressed in different tissues at various levels and are involved in numerous cellular events including metabolism, differentiation, growth, apoptosis, and tumorigenesis [1–4]. The glitazone receptor, PPAR γ , is abundant in brown adipose tissue, intestine, spleen, and kidney. Certain nonsteroidal anti-inflammatory drugs (NSAIDs) and thiazolidinediones (TZDs), of which troglitazone is a member, are synthetic PPAR γ ligands [5]. Natural ligands include 15-deoxy-prostaglandin J₂ (PGJ₂), certain polyunsaturated fatty acids (PUFAs) such as linoleic acid, and endogenous low-density lipoprotein (LDL) particles.

In the kidney, PPAR γ ligands have been shown to have a protective effect in various segments of the nephron including the glomerulus (podocytes and mesangial cells),

proximal tubule, and distal tubule. Although PPAR γ expression is among the highest in the collecting duct (CD), its role in the CD remains unclear. PPAR γ activation by TZDs has been shown to result in oedema through enhanced epithelial sodium cotransporter (ENaC) activity in the CD [6]. These effects were blocked by amiloride, a CD-specific diuretic. The deletion of PPAR γ from the collecting duct, using PPAR $\gamma^{\text{lox/lox}}$ mice, was also successful in reversing the fluid retention. The full impact of PPAR γ ligands in the collecting duct requires further characterization as the potential renal protective and antidiabetic effects are promising. Whether PPAR γ is an antifibrotic transcription factor is unclear as there have been studies indicating an induction of fibrotic responses by PPAR γ ligands. An example of this was found in the intestine where activation of PPAR γ was shown to induce a mesenchymal transition in epithelial intestinal cell line [7].

Epithelial cells form organized cell-cell adhesion junctions preventing movement of individual cells and producing a tight epithelial surface. Mesenchymal cells, however, tend

to be highly motile as they lack this level of organization. The transformation of EMT is described as the process in which epithelial cells that function as ion and fluid transporters lose their epithelial polarity, cellular adhesion molecules, and reorganize their actin cytoskeleton from a cortical bundle formation that supported adhesion molecules into stress fibers containing *de novo* expressed α -smooth muscle actin (α -SMA) that supports migration.

EMT has been described in the collecting duct where TGF- β induced activation of Smad3 and ERK1/2 leading to a mesenchymal morphology, increased vimentin and α -SMA, and a decrease in E-cadherin and β -catenin [8]. Expression of E-cadherin has been shown to vary inversely with fibronectin in several diseased states including a unilateral ureteral obstruction (UTO) model, a model which invokes scarring in the collecting duct [9]. This study, however, does not account for a full representation of the collecting duct as it focused on the terminal section.

E-cadherin is an integral, Ca^{2+} -dependent transmembrane adhesion protein that is generally localized at the adheren junctions of epithelial cells and plays an important role in the development and maintenance of renal epithelial polarity. The intracellular domain of E-cadherin is bound to β -catenin that is bound to α -catenin, which in turn links the entire complex to the actin cytoskeleton. Unbound β -catenin can also function as a component of the Wnt nuclear signaling pathway acting as a coactivator of T-cell factor (TCF)/lymphocyte enhancer factor (LEF) transcription factors [9, 10].

PPAR γ has been shown to interact with members of the cadherin-catenin cytoskeletal complex through the functional peroxisome proliferator response element (PPRE) described in E-cadherin [11], as well as its catenin-binding domain (CDB) which interacts directly with the TEC/LEF domain in β -catenin [10]. Studies have shown that PPAR γ activation induces proteasomal degradation of β -catenin as well as a reduction in E-cadherin in several cell types, including hepatocytes [12]. In normal cells, PPAR γ can function to suppress Wnt signaling by targeting phosphorylated β -catenin to the proteasome through a process involving the above-mentioned catenin-binding domain [1].

The overall purpose of this study is to clarify the mechanisms by which TRO/PPAR γ alters collecting duct structure and function. We report that TGF- β was unable to initiate an epithelial to mesenchymal transformation in the IMCD-K2 and M1 collecting duct cell lines. In contrast, Troglitazone caused morphological changes, decreased E-cadherin, α -catenin and β -catenin, and increased fibronectin. These effects were not reversed with PPAR γ antagonists or altered by PPAR γ overexpression.

2. Materials and Methods

2.1. Cell Culture. The IMCD-K2 cell line is derived from the initial section of the IMCD of an SV40-transgenic mouse. These were a generous gift from Dr. Bruce Stanton (Dartmouth Medical School). IMCD-K2 cells were grown in DMEM : F-12 (1 : 1) media supplemented with 10% FBS, 1% ITS, 1% Penicillin-Streptomycin, 1% L-glutamine, and 5 μM

dexamethasone and were maintained at 5% CO_2 and 37°C during culture and treatment. The M-1 cells are a mouse cortical collecting duct cell line (ATCC no. CRL-2038). M1 cells were grown at 37°C and 5% CO_2 in DMEM : F-12 media (1 : 1), pH 7.4, containing, 5% FBS, and 1% Pen-Strep.

2.2. Chemicals and Reagents. Troglitazone (Sigma) is a synthetic PPAR γ agonist and was used at 5 μM and 10 μM . Troglitazone is a well-studied synthetic PPAR γ agonist. It has high specificity for PPAR γ as it does not activate the PPAR δ or PPAR α isoforms at a concentration of 25 mM and higher. Troglitazone is a member of a class of anti-diabetic drugs that are currently used to improve insulin sensitivity in type 2 diabetics. GW9662 (Cayman Chemicals) is a PPAR γ antagonist that covalently modifies Cys₂₈₅ in PPAR γ 's LBD. PPAR γ antagonist (T0070907) (Cayman Chemical) covalently modifies Cys₃₁₃ thereby modulating PPAR γ cofactor recruitment. Both antagonists were used alongside Troglitazone at 10 μM . PGJ₂ (Cayman Chemical) is an endogenous PPAR γ agonist. All reagents were dissolved in DMSO to their suggested stock concentrations. Antibodies for α -SMA, α -catenin, β -actin, β -catenin, fibronectin, and E-cadherin were obtained from Sigma-Aldrich (ON, Canada). Anti-PPAR γ was obtained from Santa Cruz Biotech (CA, USA). AlexaFluor Phalloidin and DAPI were gifts from Dr. Chris Kennedy (University of Ottawa).

2.3. Western Blot. Following stimulations, protein isolations were performed using RIPA lysis buffer consisting of: RIPA buffer, 10 mM Na fluoride, 1 mM Na pyrophosphate, 100 μM Na orthovanadate, 1 : 100 protease inhibitor cocktail (Sigma), and 500 μM PMSF. The solution was centrifuged at 10,000 $\times g$ for 10 minutes, and protein was quantified by spectrophotometry using Bradford reagent and denatured by boiling the samples at 95°C for 5–10 minutes. Equal amounts of lysates (50–100 μg) were electrophoresed and transferred onto a nitrocellulose membrane (Amersham). The nitrocellulose membrane was blocked in 10% milk in TBS/T for 1 to 3 hours, and a primary antibody was applied directly to the milk and left overnight. Following three 20-minute washes, the primary antibody solution was removed, and the appropriate secondary antibody was applied in 10% milk-TBS/T for 90 minutes, after which the membranes were washed with TBS/T. The HRP-conjugated secondary antibodies were developed with Pierce SuperSignal chemiluminescent reagent for 5 minutes and exposed to Kodak X-Omat Blue XB-1 film as well as digitally exposed using the Alpha Innotech Fluorchem HD2 imaging system.

2.4. Immunofluorescence. Cells were plated on coverslips and allowed to adhere overnight. The culture media was replaced for 24 hours with serum-free DMEM : F-12 and stimulated with TRO and GW9662 for an additional 24 hrs. The coverslips were washed with PBS to remove traces of media and fixed for 30 minutes at room temperature in 4% paraformaldehyde or at -20°C in a (1 : 1) methanol-acetone solution. Excess fixative was washed with PBS/ Mg^{2+} / Ca^{2+} for 2 \times 5 minutes. Triton-X at 0.1% in PBS was used for permeabilization for 30 minutes at room temperature

followed by 3×5 -minute washes in PBS/Mg²⁺/Ca²⁺. The coverslips were blocked in 5% BSA in 0.1% Triton-X in PBS/Mg²⁺/Ca²⁺ for 1 hour prior to adding the antibody of choice. All antibodies were diluted in 5% BSA in 0.1% Triton-X in PBS/Mg²⁺/Ca²⁺. Cells were incubated with Phalloidin-FITC for 1 hour at RT in the dark. Next, 3×5 -minute PBS/Mg²⁺/Ca²⁺ washes were performed prior to the addition of the secondary, α -mouse FITC (Sigma) 1 : 300 for 90 minutes or DAPI (1 : 1000) for 15 minutes. The coverslips were then mounted in Fluoromount G-mounting media (Southern Biotech). Images were captured using the Zeiss AxioCam of an Axioskop2 MOT Fluorescent microscope.

2.5. PPAR γ Overexpression. pcDNA and pcDNA-PPAR γ vectors were obtained from Addgene as bacterial stabs. The sizes of each insert were verified with XhoI/HindIII digests. M1 cells were grown to 25% confluence prior to transfection. 4 μ g of plasmid DNA was diluted to a volume of 150 μ L in DMEM and 15 μ L of Polyfect Transfection Reagent (Qiagen) was added to the solution. The suspension was then incubated at room temperature for 10 minutes, mixed with 1 mL of culture media containing 0.1% ampicillin, and added to the culture plate. This was repeated 48 hours later for the final 24 hours of the 72 hours transfection period.

2.6. Statistics. GraphPad Prism v4.03 was used to plot and analyse the data collected. Values are expressed as means \pm standard error of the mean (S.E.M.). An unpaired *t*-test was used to assess statistical significance between selected experimental groups. A one-sample *t*-test was performed against a hypothetical value of 1.0 for all groups as the values represented fold controls where the control group all had values of 1.0. Statistical analysis for multiple comparisons was done using one-way ANOVA corrected with Bonferroni's posttest. A *P* value \leq 0.05 was regarded as statistically significant.

3. Results

3.1. TRO Induces Morphological Changes in M1 Cells. The presence of PPAR γ in both the M1 and IMCD-K2 cell lines was verified through Western blot and RT-PCR (Figure 1) with PPAR γ being successfully overexpressed in M1 cells as seen in Figure 1(a). The general effects of TZDs in the kidney have been protective; however, the treatment of M1 cells with TRO resulted in a decrease in cell-to-cell contact, disorganization of the F-actin cytoskeleton filament network, and a less rounded, elongated spindle-like shape at 5 μ M and 10 μ M, visualized through FITC-conjugated Phalloidin (Figure 2). These morphological changes were not attenuated by the PPAR γ antagonist GW9662 at 10 μ M with the same order of disorganization and cell elongation being observed after cotreatment in Figures 2(d) and 2(f).

3.2. Troglitazone Induces Cytoskeleton Reorganization in the IMCD-K2 and M1 Collecting Duct Cell Lines. Along with the aforementioned morphological changes, treatment of IMCD-K2 and M1 cells with TRO resulted in a decrease

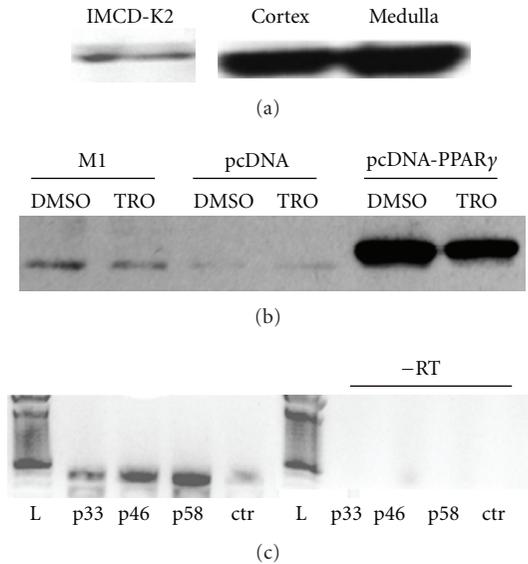


FIGURE 1: PPAR γ is present in the IMCD-K2 and M1 collecting duct cell lines. (a) Western blot analysis of PPAR γ detected a band at 67 kDa in IMCDK2 cells, also shown in mouse cortex and medulla lysates used as a positive control. (b) In M1 cells, endogenous PPAR γ levels as well as those of the pcDNA plasmid control and pcDNA-PPAR γ overexpression samples are shown, included for each are samples treated for 24 hours with DMSO (vehicle) or 10 μ M troglitazone (TRO). (c) In different passages of M1 cells (ctr, P33, 46, and 48), RT-PCR analysis produced a 400 bp product corresponding to PPAR γ detected on 1% agarose gel. The (RT-) samples were used as a negative control. Lanes identified as L contain DNA ladder.

in several epithelial adhesion markers. Figure 3 shows the analysis of α -smooth muscle actin (α -SMA) in M1 cells and vascular smooth muscle cells (VSMC, positive control). As seen, α -SMA was not detectable in M1 cells even upon TRO stimulation, whereas a strong signal was obtained by Western blotting and immunofluorescence in VSMC. By immunofluorescence, very weak staining for α -SMA was observed in image B only upon a prolonged exposure up to 500 ms, confirming the minimal TRO effect. This response was not strong enough for detection by Western blot, even in 100 μ g of lysate. In comparison, Figures 4(a) and 7(a) show a clear decrease in E-cadherin and increase in the intensity of fibronectin staining through immunofluorescence in response to TRO. Figures 4(b) and 4(c) show a 60% drop in E-cadherin with TRO in M1 and IMCD-K2 cells. Treatment with a lower dose of TRO resulted in a 30% drop in E-cadherin. The same trend is shown in Figure 5 with a 60% reduction in β -catenin levels after 24-hour TRO treatment, and a 30% reduction in α -catenin (Figure 6). Conversely, an upregulation of fibronectin was obtained in response to 24-hour TRO (Figures 7(b) and 7(c)), near 2-fold in M1 cells, and 1.5-fold in IMCD-K2 cells. In comparison, Figure 8 illustrates the response of M1 cells to PGJ₂. As shown, 24-hour treatment with 0.5 μ M PGJ₂ increased E-cadherin and fibronectin levels to 1.5–2-fold of control. A numerical increase in β -catenin was also observed.

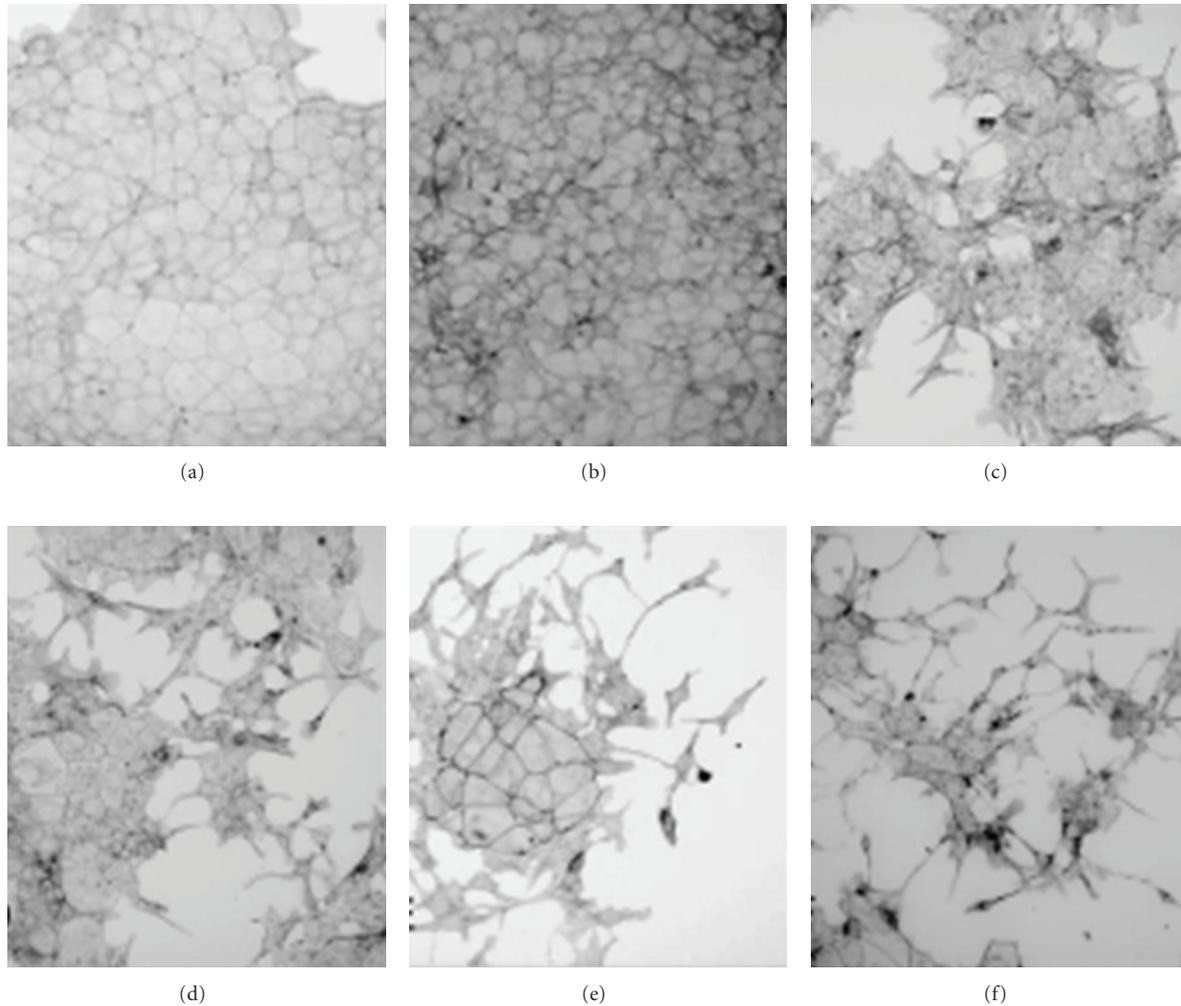


FIGURE 2: Troglitazone induces morphological changes in M1 cells independent of PPAR γ . Images of F-actin filaments stained with FITC-conjugated phalloidin are shown, in M1 cells stimulated for 24 hours with (a) DMSO (vehicle), (b) 10 μ M GW9662 (PPAR γ antagonist), (c) 5 μ M troglitazone (TRO), (d) GW9662 and 5 μ M TRO, (e) 10 μ M TRO, and (f) GW9662 and 10 μ M TRO. Magnification 100x.

3.3. TRO-Mediated Effects in the M1 Collecting Duct Cell Line Are PPAR γ Independent. TZDs are known to induce several PPAR γ -independent effects. In our study PPAR γ antagonists GW9662 and T0070907 had no effect on E-cadherin (Figure 4(d)), β -catenin (Figure 5(c)), or fibronectin (data not shown). However, as shown in Figure 6, T0070907 resulted in a further 2-fold reduction in α -catenin levels, but this was not statistically significant. The overexpression of PPAR γ in M1 cells was performed to see whether an enhancement of the abovementioned effects could be observed. However, PPAR γ overexpression did not significantly change the 50% decrease in E-cadherin in nontransfected M1 cells compared to cells transfected with the pcDNA plasmid control and the pcDNA-PPAR γ vector insert (Figure 4(e)). As seen in Figure 7(d) the 1.5-fold increase in fibronectin in nontransfected and plasmid control transfected cells was not increased with PPAR γ overexpression in pcDNA-PPAR γ transfected cells, instead yielding a modest 1.3-fold increase in fibronectin, lower than in nontransfected cells. Taken

together these results portray a system in which PPAR γ does not mediate TRO responses in collecting duct cells.

It should also be noted that the transformation of the epithelial CD cell lines via a cytotoxic response is unlikely. An inhibition of growth along with p38 activation was described in a previous study; however, no oxidative stress (ROS generation) or cell death (cleaved caspase-3 expression) were observed [13]. The reduction in cell number seen with TRO compared to control can be primarily attributed to the aforementioned decrease in proliferation. TRO is also known to drop pH levels [14]; however, we did not observe any significant levels of acidosis in our cell culture media in a 48 hr period.

4. Discussion

4.1. PPAR γ in the Collecting Duct. PPAR γ has previously been detected *in vivo* in the medullary and cortical collecting duct [13, 15] and was present in both IMCD-K2 and

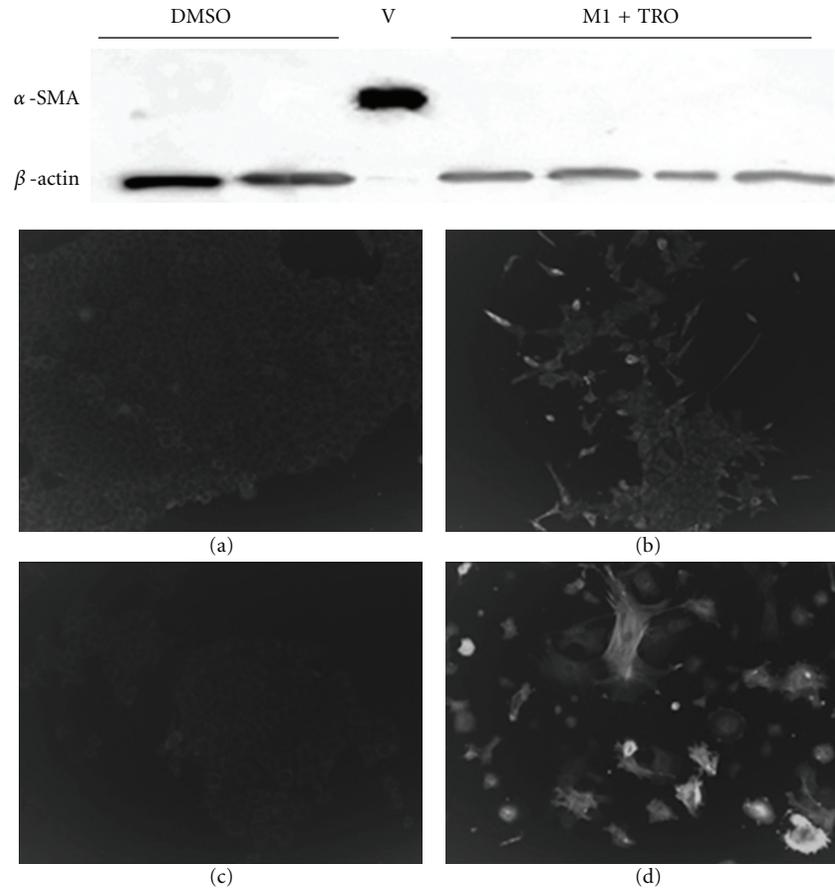


FIGURE 3: Troglitazone does not induce α -SMA expression in M1 cells. In the upper panel, α -smooth muscle actin (α -SMA) was assessed by Western blotting in 100 μ g lysates from M1 cells treated with DMSO, confirming the absence of α -SMA, vascular smooth muscle cells (V) were used as a positive control, and 4 lanes of M1 cells treated with 10 μ M troglitazone (TRO) for 24 hours. In the lower panel, α -SMA was assessed by immunofluorescence. Images are shown of M1 cells stimulated with (a) DMSO and (b) 10 μ M troglitazone (TRO) for 24 hours, (c) antimouse FITC conjugated secondary as a negative control, and (d) vascular smooth muscle cells were used as a positive control. Magnification 100x.

M1 cells in our study. To our knowledge the only other studies on PPAR γ and the collecting duct indicate that it may be involved in sodium and fluid balance, as seen with the PPAR γ knockout model reversing the oedemic effects resulting from TZD treatment [6, 16]. The use of PPAR γ agonists, specifically TZDs, on the other hand, has raised interest in several fields of research due to their potential antifibrotic and antitumorigenic effects. Troglitazone has been shown to suppress TGF- β -induced SMAD2/3 phosphorylation, thereby inhibiting EMT in human retinal pigment epithelial cells independent of PPAR γ [17]. It has also been suggested that the antiproliferative effects of TZDs in cancer cells are also independent of PPAR γ activation as TZD analogs, unable to activate PPAR γ , retain the ability to induce apoptosis at an equal potency in several cancer cell lines. Nonetheless, there is an emerging view that some antitumorigenic effects are partially PPAR γ dependent, whereas most are independent of the receptor [1, 18].

In our studies PPAR γ was successfully overexpressed in M1 cells. The increased PPAR γ levels did not appear to alter cell morphology alone; however, stimulation with

TRO in over-expressed cells resulted in a similar phenotype as in nontransfected M1 cells. Generally, PPAR γ overexpression alone is sufficient to induce a cellular response without the use of an agonist as seen in pancreatic islets where overexpression of PPAR γ protein suppressed insulin secretion induced by stimulatory concentrations of glucose [19, 20]. This was also seen in rat livers where PPAR γ was over-expressed using an additional adenoviral vector (AdPPAR γ), resulting in significantly lower levels of fibrosis compared to controls [21]. Troglitazone has been shown to act through both PPAR-dependent and -independent mechanisms in epithelial cells. For instance, in porcine renal epithelial tubules, TZDs can modulate the pathways influencing glucose and glutamine metabolism, as well as cellular acidosis favoring reduced plasma glucose *in vivo* [14, 22]. This alteration in cellular acidosis and glutamine metabolism was verified in pH-sensitive lung carcinoma cells where TZD-dependent PPAR γ activation was verified; however, the primary effects of the study were still independent of this activation, instead involving PKC/ERK. The task remains to decipher which effects obtained with TZDs are PPAR γ

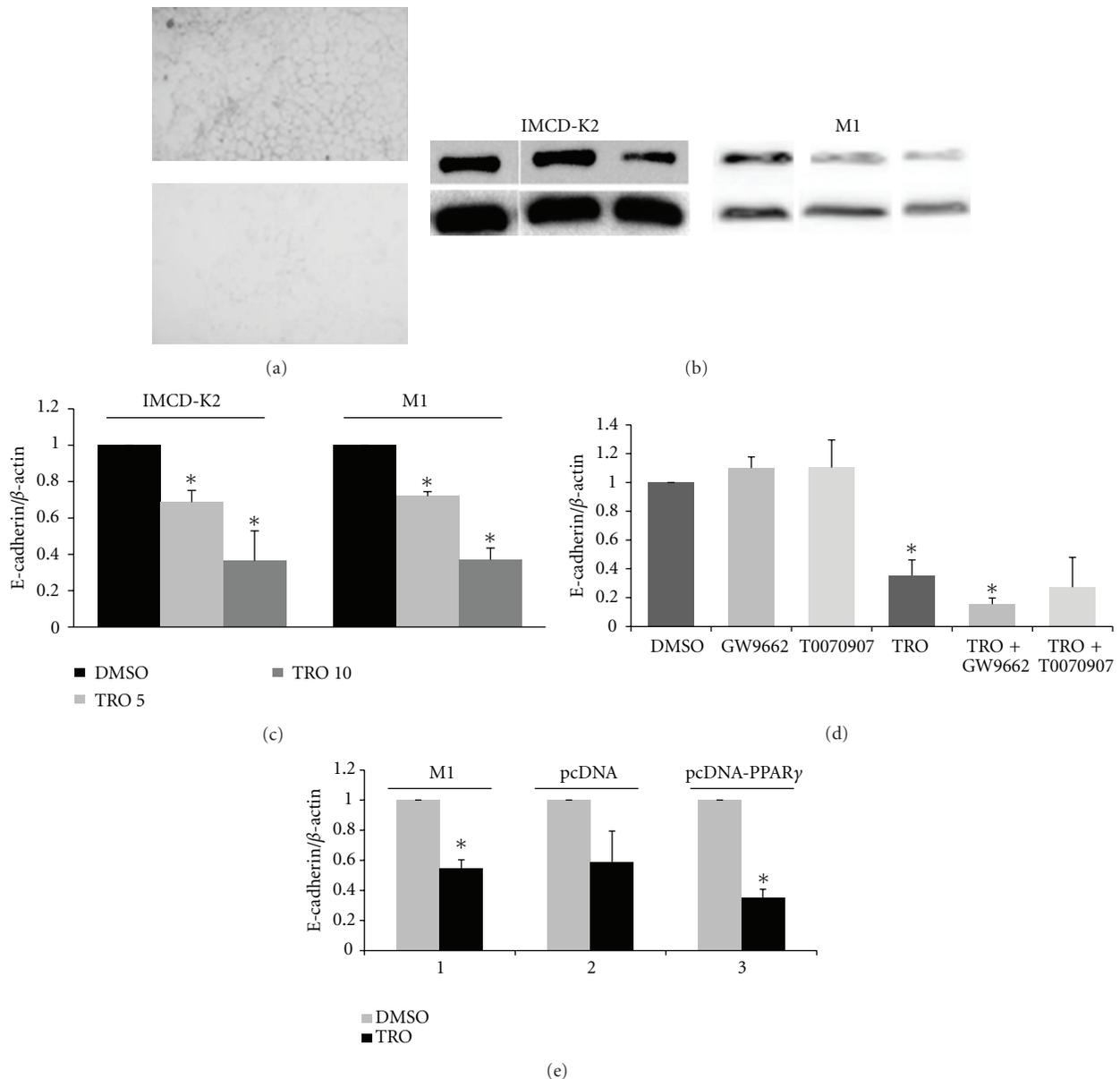


FIGURE 4: Troglitazone attenuates E-cadherin expression in IMCD-K2 and M1 cells. In (a) E-cadherin is detected by immunofluorescence in M1 cells stimulated with DMSO (vehicle, upper) and 10 μ M troglitazone (lower) for 24 hours (magnification 100x). In (b) IMCD-K2 and M1 cells were treated with DMSO (vehicle) or troglitazone (TRO), and a representative Western blot is shown for E-cadherin (upper bands) and corresponding β -actin (lower bands). In (c) the corresponding densitometric analysis is shown with data presented as mean \pm S.E.M. $P \leq 0.05$ (*). In (d) a densitometric analysis of E-cadherin is shown for M1 cells treated with 10 μ M TRO in the presence or absence of the PPAR γ antagonists GW9662 and T0070907. In (e) the densitometric analysis is shown for E-cadherin in M1 cells treated with DMSO or 10 μ M TRO with endogenous PPAR γ as well as those expressing the control pcDNA plasmid and pcDNA-PPAR γ overexpression. Data presented as mean \pm S.E.M. $P \leq 0.05$ (*).

mediated and how they are interconnected with the PPAR γ -independent pathways to fully understand how these drugs operate.

4.2. TGF- β and EMT in IMCD-K2 and M1 Cells. TGF- β was unable to induce EMT in either cell line. This was surprising as the IMCD-3 cell line, representing the terminal section of the inner medulla has recently been shown to undergo EMT

upon stimulation with TGF- β . Although our stimulation was extended to 96 hrs, the high levels of E-cadherin and β -catenin may have protected the tight junctions, requiring a longer time point to achieve the initial disruptions in cell contact required to initiate the expression of mesenchymal markers. The enhanced growth rate of the immortalized cell lines may have also contributed to the lack of a response. Both TGF- β receptors as well as endogenous cytokine have

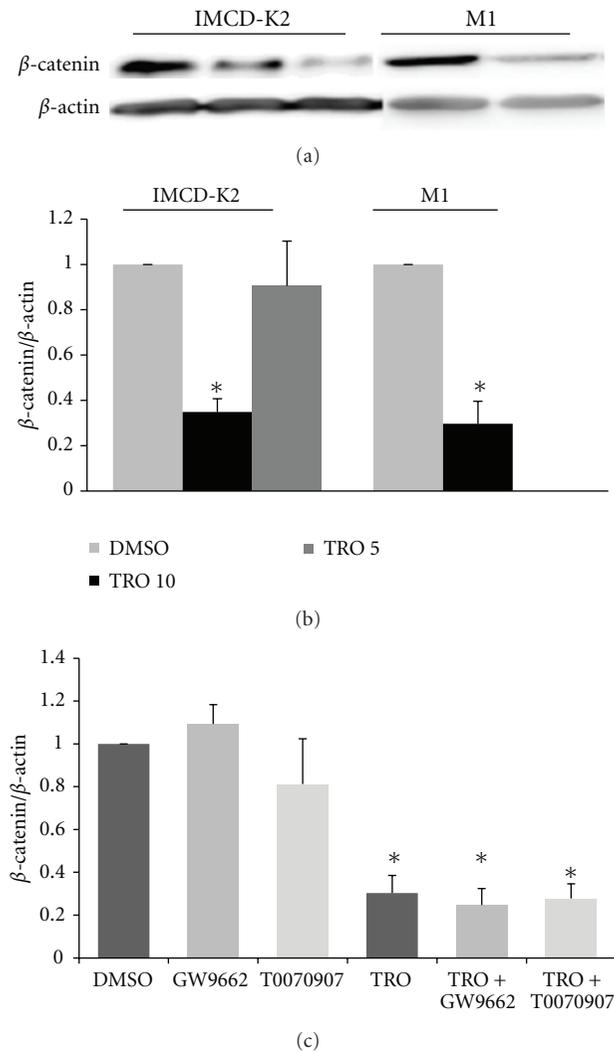


FIGURE 5: Troglitazone attenuates β -catenin expression in IMCD-K2 and M1 cells. IMCD-K2, and M1 cells were treated with DMSO (vehicle) or troglitazone (TRO), and in (a) a representative Western blot is shown for β -catenin and corresponding β -actin. In (b) the corresponding densitometric analysis is shown with data presented as mean \pm S.E.M. $P \leq 0.05$ (*). In (c) a densitometric analysis of β -catenin is shown for M1 cells treated with 10 μ M TRO in the presence or absence of the PPAR γ antagonists GW9662 and T0070907.

been previously characterized in both cell lines [23, 24]. The inability to induce EMT prevented a full assessment of whether PPAR γ activation could have reversed these effects, which would have confirmed a protective role for the transcription factor in collecting duct cells.

4.3. Troglitazone and Cell Morphology. TRO caused a loss in cell-to-cell contact and an elongated spindle-like phenotype in a percentage of the treated cell population. The changes in cell shape appear to be PPAR γ independent as GW9662 did not restore normal morphology or any cell-to-cell contact. Additionally the agonist may have resulted in a further disruption of cell integrity with TRO cotreatment. TRO may

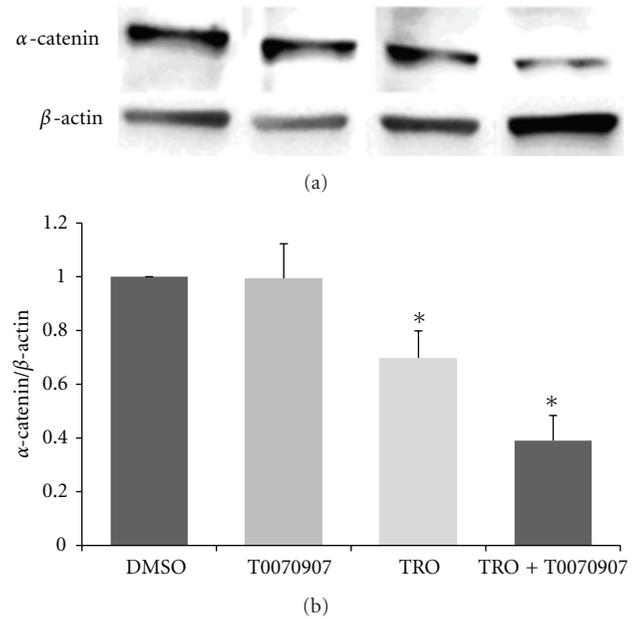


FIGURE 6: Troglitazone attenuates α -catenin in M1 cells. M1 cells were treated with 10 μ M troglitazone (Tro) in the presence or absence of the PPAR γ antagonist T0070907. Western blot was performed for α -catenin, and the results were normalized to β -actin. (a) A representative blot is shown, and (b) the corresponding densitometric analysis. Data presented as mean \pm S.E.M. $P \leq 0.05$ (*).

be acting through both PPAR γ -dependent and -independent pathways in inducing the aforementioned changes in morphology; however, with no partial reversal upon antagonism, it is more likely that a PPAR γ -independent pathway is able to fulfill the necessary steps to obtain the phenotype observed. PKC/Akt and MAP kinase activation may be involved as TZDs have been shown to enhance PKC/Akt phosphorylation in both diabetic and nondiabetic rats and MAP kinase activation is a well-characterized PPAR γ -independent response to TZDs [10]. This elongated phenotype was absent in populations that were able to reach confluence. This generally occurred if cells were stimulated with TRO at a confluence greater than 75%. Even with an inhibition in growth, highly populated plates were able to proliferate in close proximity. TRO does not appear to be able to alter morphology and initiate cell-to-cell contact disruption in confluent layers of IMCD-K2 and M1 cells. Similarly, intact tubular epithelial layers do not undergo EMT upon TGF- β stimulation either; however, loss of epithelial integrity (subconfluence, wounding, and contact disassembly by Ca²⁺ removal) restores the ability of the monolayer to undergo a mesenchymal transformation [25, 26]. This suggests that there may be a similar phenomenon observed in our study, with Troglitazone requiring a disruption in the cell monolayer in order to induce the morphological changes characterized.

4.4. Troglitazone and the ECM/Cytoskeleton. TRO induced decreases in epithelial adhesion markers such as E-cadherin,

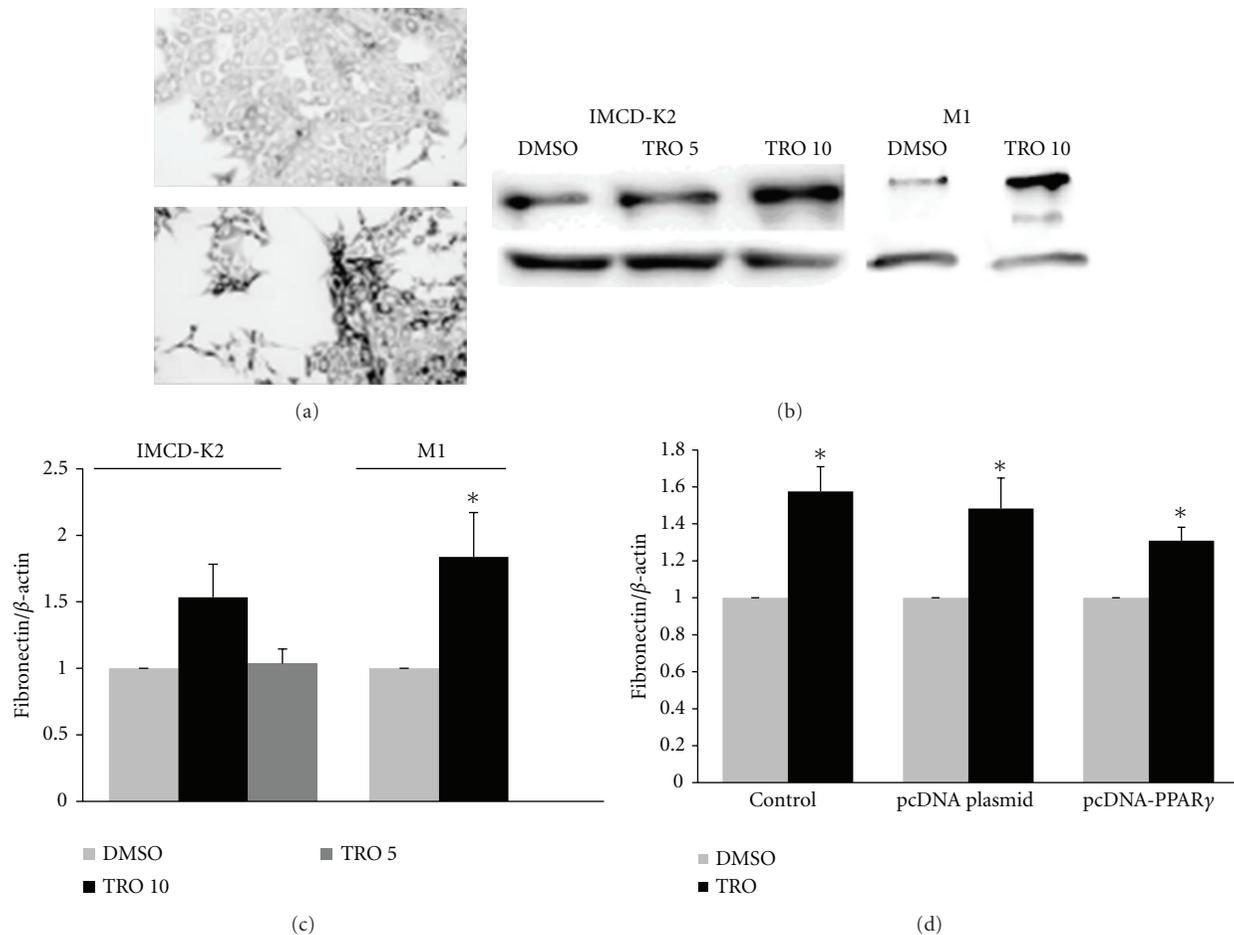


FIGURE 7: Troglitazone increases fibronectin expression in IMCD-K2 and M1 cells. In (a) fibronectin is detected by immunofluorescence in M1 cells stimulated with DMSO (vehicle, upper) and 10 μ M troglitazone (lower) for 24 hours (magnification 100x). In (b) IMCD-K2 and M1 cells were treated with DMSO (vehicle) or troglitazone (TRO), and a representative Western blot is shown for fibronectin (upper bands) and corresponding β -actin (lower bands). In (c) the corresponding densitometric analysis is shown with data presented as mean \pm S.E.M. $P \leq 0.05$ (*). In (d) the densitometric analysis is shown for fibronectin in M1 cells treated with DMSO or 10 μ M TRO with endogenous PPAR γ as well as those expressing the control pcDNA plasmid and pcDNA-PPAR γ overexpression. Data presented as mean \pm S.E.M. $P \leq 0.05$ (*).

α -catenin, and β -catenin. TRO also caused an increase in fibronectin protein but did not trigger *de novo* α -SMA expression, the hallmark of myofibroblast generation. PGJ₂ on the other hand increased E-cadherin, β -catenin, and fibronectin levels. This suggests that PPAR γ activation *in vivo*, as PGJ₂ is a supposed endogenous ligand of PPAR γ , may counteract the decreases in epithelial markers observed *in vitro* with a synthetic ligand. These inconsistencies among PPAR γ ligands are mirrored in MCF-7 breast cancer cells where conjugated linoleic acid, which acts as a PPAR γ agonist, also caused an upregulation and redistribution of β -catenin and E-cadherin [27]. E-cadherin has been shown to have a functional PPRE [11] suggesting that PPAR γ stimulation may result in an increase in E-cadherin levels. Although the PGJ₂ and TRO results contradict one another, TRO stimulation may still involve a PPAR response element since a PPAR γ co-repressor complex could have bound to E-cadherin's PPRE, preventing further transcription and thereby translation of the E-cadherin protein. A core function of p120-catenin is to regulate cadherin stability and

turnover by controlling cadherin entry into the degradative endocytic pathways [1, 28]. The decrease in protein levels could have been due to the prevention of E-cadherin turnover and enhanced degradation. A closer look at p120-catenin would clarify whether TRO's effects on E-cadherin were secondary, preventing the regeneration of E-cadherin protein, as opposed to causing the direct degradation of the adheren marker.

Nonetheless, the decrease in E-cadherin in our study occurs independently of PPAR γ . The PPAR γ antagonists T0070907 and GW9662 did not reverse the decrease observed. Overexpression of PPAR γ did not alter the drop in E-cadherin levels upon TRO stimulation either. The opposing effects of the two agonists, TRO and PGJ₂, create another argument that the effects of at least one of the ligands are PPAR γ independent. Another explanation is that one ligand activates PPAR γ at a higher capacity than the other as this paradox has been shown to induce opposing effects in breast cancer tissue, depending on the magnitude of PPAR γ activation in terms of cellular proliferation,

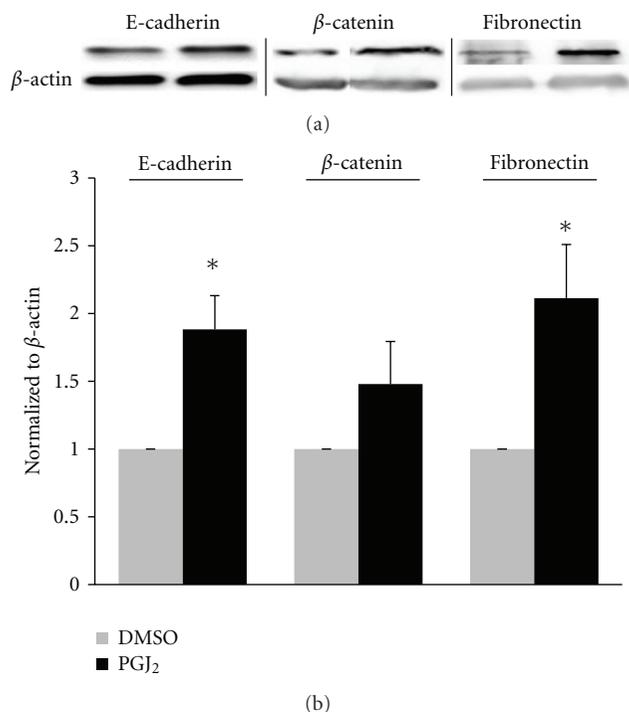


FIGURE 8: PGJ₂ increases ECM-cytoskeleton proteins in M1 cells. M1 cells were treated with 0.5 μ M PGJ₂ for 24 hours. Western blot was performed for E-cadherin, β -catenin, and Fibronectin. (a) The results were normalized to β -actin ($n = 4$) and (b) a densitometric analysis is shown. Data presented as mean \pm S.E.M. $P \leq 0.05$ (*).

differentiation, and apoptosis of cancer cells and tumors [1]. For example, lower concentrations increased cellular proliferation, whereas higher concentrations blocked cell growth.

It should be noted that the 50% reduction in β -catenin may have not only led to the destabilization of adherens junctions and loss of cell-to-cell contact but also to decreases in β -catenin activity as an LEF/TCF transcription factor, possibly accounting for some of the other effects observed upon TRO stimulation. Whether any one effect can be directly linked to catenin signaling would require a full restoration of β -catenin levels. TRO also caused a decrease in α -catenin in M1 cells. This decrease was not reversed by T0070907 suggesting the degradation of α -catenin to be independent of PPAR γ activation as well. Generally α -catenin is thought of as a static link between the cadherin-catenin chain and actin cytoskeleton; however, new roles for α -catenin are being described as α -catenin has been shown to exist as a monomer or a dimer with monomeric α -catenin binding more strongly to E-cadherin- β -catenin and the dimer preferentially binding to actin filaments [29]. Determining the proportion of monomeric to dimerized α -catenin affected by TRO stimulation may shed light on the local regulation of actin assembly and organization at sites of cadherin-mediated cell-cell adhesion.

Fibronectin was upregulated by TRO and PGJ₂. In a previous study we showed that lower concentrations of TRO had no effect on fibronectin in collecting duct cells [13].

This provides a further argument for the concentration dependence of TRO actions though the effect of fibronectin seems to be mainly PPAR γ independent (no effect of PPAR γ antagonism or overexpression). Generally TZDs have been shown to reverse fibronectin expression in diseased states, as in human lung carcinoma cells [3], in human cortical fibroblasts [30], in TGF- β -treated glomerular mesangial cells [31], and in diabetic mice [32]. Nonetheless, the complex nature and inconsistencies of PPAR γ activation and function in terms of its ligands are seen throughout the literature. An inverse relation between the adhesion molecule E-cadherin and the matrix protein fibronectin has been established in many diseased states, a trend observed in this study suggesting that the collecting duct may be altered upon stimulation with Troglitazone.

5. Conclusion

PPAR γ is present in the collecting duct; however, the use of TRO, a potent synthetic ligand, resulted in structural changes independent of its target receptor. These effects were not replicated with the use of PGJ₂, a proposed endogenous PPAR γ ligand. PPAR γ overexpression did not accentuate any of the effects obtained from stimulation with TRO. Taken together the data suggest that TRO can alter ECM and cytoskeletal marker expression in the CD leading to a partial epithelial to mesenchymal-like transformation, independent of PPAR γ . The beneficial effects of PPAR γ ligands in other diseased systems highlight the relevance of this study and underline the importance of fully understanding the effects of these drugs to improve their use and specificity.

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