Interaction of PPARalpha with the Wnt pathway, a mechanism for the therapeutic effect of fenofibrate on diabetic nephropathy

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ABSTRACT
Aberrant activation of canonical Wnt signaling plays a key role in renal fibrosis. Peroxisome proliferator-activated receptor-α (PPARα) has shown renoprotective effects with an unclear mechanism. Renal levels of PPARα were down-regulated in both type 1 and type 2 diabetic models. The PPARα agonist fenofibrate and over-expression of PPARα both attenuated expression of fibrotic factors and suppressed Wnt signaling in renal cells and in the kidney of diabetic rats. PPARα ablation enhanced activation of Wnt signaling in the kidneys of mice with diabetes or with obstructive nephropathy and in PPARα−/− tubular cells. PPARα did not block Wnt reporter expression induced by a constitutively active mutant of LRP6 or β-catenin. LRP6 stability was decreased by over-expression of PPARα and increased by PPARα ablation, suggesting that PPARα regulates Wnt signaling at the Wnt co-receptor level. LRP6 stability was enhanced by ROS and PPARα ablation while being suppressed by over-expression of PPARα. Diabetic PPARα−/− mice showed more prominent NOX4 over-expression, compared with diabetic wild-type mice, suggesting that PPARα regulates Wnt signaling likely through its anti-oxidant activity. These observations identified a novel interaction between PPARα and the Wnt pathway, which is responsible, at least partially, for the therapeutic effects of fenofibrate on diabetic nephropathy.
Diabetes nephropathy (DN) occurs in 30-40% of diabetic patients (1,2). Without therapeutic intervention, 80% of type 1 diabetic individuals with sustained microalbuminuria will develop overt nephropathy in 10–15 years (2). DN accounts for 40% of newly diagnosed end-stage renal disease (ESRD) in United States (3). A better understanding of the pathogenesis of DN could facilitate the development of novel and effective therapeutic strategies.

Peroxisome proliferator-activated receptor alpha (PPARα) is a ligand-dependent nuclear receptor, regulating lipid metabolism (4). PPARα is mainly expressed in tissues with high mitochondrial and β-oxidation activities, including the liver, renal cortex, intestinal mucosa and heart. The known functions of PPARα are primarily regulation of fatty acid oxidation. Two independent, perspective clinical studies, Fenofibrate Intervention and Event Lowering in Diabetes (FIELD) study and The Action to Control Cardiovascular Risk in Diabetes (ACCORD) study, reported that fenofibrate, a PPARα agonist, has robust therapeutic effects on DN in type 2 diabetic human patients, suggesting that PPARα is a potential target for the treatment of DN (5-7). Activation of PPARα was found to ameliorate DN in diabetic animal models through its anti-fibrosis and anti-inflammatory effects (8). PPARα deficiency has been shown to aggravate DN by increasing extracellular matrix formation and inflammation in the kidney (9). However, the molecular mechanism underlying its anti-fibrogenic and anti-inflammatory effects remains elusive.

The canonical Wnt pathway regulates multiple physiological and pathological processes including angiogenesis, inflammation and fibrosis. The activation of Wnt signaling is triggered by binding of Wnt ligands to a co-receptor complex consisting of a frizzled (Fz) receptor and low-density lipoprotein receptor-related protein 5/6 (LRP5/6), leading to activation of β-catenin, a transcription factor, which subsequently activates transcription of Wnt target genes including
multiple factors associated with angiogenesis, fibrosis and inflammation (10). Other groups and we have independently shown that the Wnt pathway is aberrantly activated in the kidney of diabetic patients and diabetic animal models (11-13). We have demonstrated that Wnt ligands and receptors are significantly up-regulated in diabetic kidneys, and hyperglycemia-induced oxidative stress contributes to the over-activation of Wnt signaling in diabetic complications (12). Inhibition of the Wnt pathway using a monoclonal antibody blocking LRP6 ameliorated proteinuria and renal fibrosis in a type 1 diabetic model, suggesting that Wnt signaling plays a role in DN-associated renal inflammation and fibrosis (12).

Several studies demonstrated the crosstalk between PPARγ and the Wnt signaling pathway in adipogenesis, osteoblastogenesis, kidney diseases and cancer. However, the regulatory effect PPARα on the canonical Wnt signaling pathway has not been well investigated. In this study, we explored a novel interaction between PPARα and the Wnt pathway in renal cells and the kidneys, which is responsible, at least in part, for the anti-inflammatory and anti-fibrogenic effects of PPARα and its agonists in DN.
RESEARCH DESIGN AND METHODS

Streptozotocin (STZ)-induced diabetic animal model

Diabetes was induced as described in a previous report (14). Briefly, 8-week-old Brown Norway rats (Charles River Laboratories, Wilmington, MA) were fasted overnight and then received a single intraperitoneal injection of STZ (Sigma-Aldrich, St. Louis, MO) at the dose of 55 mg/kg. Eight-week old PPARα−/− in C57/BL6J background and wild-type (Wt) C57/BL6J mice (Jackson Laboratories, Bar Harbor, MA) received 5 consecutive daily intraperitoneal injections of STZ at the dose of 55 mg/kg. Blood glucose levels were measured 72 hr after STZ injection for the rat or 1 week for the mouse after the last STZ injection. Animals with blood glucose levels higher than 350 mg/dl were defined as diabetic animals. All of the experiments were approved by the Institutional Animal Care and Use Committee of The University of Oklahoma.

At 6 weeks of diabetes, diabetic rats were randomly assigned into two groups, and fed regular chow or special chow containing 0.1% fenofibrate (LabDiet®/TestDiet®. Ft. Worth, TX) for another 6 weeks. Age-matched non-diabetic animals were used as the normal control.

Unilateral ureter obstruction (UUO) model

PPARα−/− mice were crossed with the BAT-gal Wnt reporter mice (Jackson Laboratories) to generate PPARα−/− or Wt mice carried the BAT-gal transgene. Mice received the UUO operation in the left kidney at the age of 8 weeks as described (15). Mice were euthanatized at the 7th day after the operation, and the kidney was dissected and sectioned for X-gal staining.

Measurement of urine albumin and creatinine

Metabolic cages were used for collection of 24-h urine. Levels of urine albumin and creatinine were measured and calculated as described previously (12).
X-Gal staining

X-Gal staining was performed in kidney sections as previously described (16). Briefly, mice were perfused with 4% paraformaldehyde (PFA) in PBS containing 2 mM MgCl$_2$ (pH 7.4). The post-obstructive kidney was dissected and post-fixed in 4% PFA for additional 2 h, followed by dehydration in 30% sucrose overnight. Tissues were embedded in OCT, and 30-µm cryosections were cut. Sections were washed in PBS with 0.01% sodium deoxycholate and 0.02% NP-40 for 2 hr and stained in X-gal solution (1 mg/ml X-Gal, 5 mM potassium ferricyanide, and 5 mM potassium ferrocyanide in PBS, Sigma-Aldrich, St. Louis, MO) for 6 hr at 37°C. Images were visualized and captured using an Olympus microscope.

Immunohistochemistry

Five-micrometer sections were prepared from paraffin-embedded kidney for immunohistochemistry as described (12). Briefly, kidney sections were blocked with normal goat serum and the Avidin/Biotin blocking kit (Vector Laboratories, Burlingame, CA) according to the manufacturer’s protocol. To quantify expression levels of renal Collagen IV, fibronectin and LRP6, Sections were incubated separately with anti-Collagen IV (Millipore, Bellerica, MA), anti-fibronectin (Santa Cruz Biotechnology, Dallas, Texas) and anti-LRP6 (in-house antibody) antibodies. ABC reagent (Vector Laboratories, Burlingame, CA) was used and the color was developed by DAB substrate (BD Biosciences, San Jose, California) according to the manufacturer’s instruction.

Cell culture and treatment.

HRPTC were purchased from ATCC (Manassas, VA) and cultured according to the recommendation of ATCC. HKC-8 cells, a cell line derived from human renal proximal tubular epithelial cells, were from Dr. L. Racusen (The Johns Hopkins University, Baltimore, MD) and
cultured in Dulbecco’s modified Eagle’s medium/F12 medium (DMEM/F12) with 5% fetal bovine serum (FBS, Life Technologies, Grand Island, NY), as described (18). L-cells and L-cells expressing Wnt3a were obtained from ATCC (Manassas) and maintained in DMEM supplemented with 10% FBS cultured following recommendations from ATCC. Wnt3a conditioned medium (WCM) and the control medium from L-cells were collected according to the manufacturer’s protocol. Tubular cells were starved in 0.2% FBS (HRPTC) or serum-free medium (HKC-8) overnight before treatment. Cells were treated with high glucose medium containing with 30 mM D-glucose (Sigma-Aldrich) or control low glucose medium containing 25 mM L-glucose plus 5 mM D-glucose (Sigma-Aldrich) for indicated time. Recombinant human TGF-β1 was from R&D Systems (Minneapolis, MN). Adenovirus expressing PPARα (Ad-PPARα) was generated as described previously (19). Cells were infected with Ad-PPARα or control adenovirus as described previously (12,20).

**Culture of primary mouse renal proximal tubular cells**

Mouse primary proximal tubular cells were cultured from 21 day-old PPARα<sup>−/−</sup> mice or their genetic background matched Wt littermates according to a documented protocol (21). The cells from these mice were isolated side by side, and used at the same passage. Proximal tubular fragments were maintained in renal proximal tubular cells special medium (ATCC) supplement with 5% FBS, 10 ng/ml EGF (R&D Systems), 1% antibiotic-antimycotic and ITS (Life Technologies), and seeded in collagen-coated dishes in 37°C humidified incubator with 5% CO<sub>2</sub>. Culture medium was changed 48 hr after seeding, and then changed every 2 days. Cells at passages 1 to 4 were used in this study. Mouse primary proximal tubular cells were identified at passage 1 by immunostaining with the antibody against SGLT1 (Millipore), Commercial HRPTC were used as the positive control for the immunostaining.
Luciferase assay

The transcriptional activity of β-catenin was measured by luciferase assay as described previously (22).

Measurement of the reactive oxygen species

Cells in 24-well plates were treated with different concentrations of 4-hydroxynonenal (HNE) for 2 hr. Intracellular ROS was quantified by CM-H₂DCF-DA staining (Molecular Probe, Invitrogen, Carlsbad, CA) according to the manufacturer’s instruction.

Extraction of nuclear protein

Nuclear fractions from renal cortical tissues were extracted using the Fraction PREP™ Cell Fractionation Kit (Biovision, Mountain View, CA) following manufacturer’s instruction.

Western blot analysis

Cells were lysed using 1×SDS lysis buffer (62.5 mM Tris-HCl [pH 6.8], 2% SDS, 10% glycerol, 0.1% bromophenol blue, 1×protein inhibitor cocktails [Thermo Fisher Scientific, Rockford, IL], 10 mM NaF [Sigma-Aldrich] and 2 mM sodium orthovanadate [Sigma-Aldrich]). Renal cortical lysates were prepared by adding 100 µl tissue lysis buffer (50 mM Tris-HCl, pH 7.8, 5 mM EDTA, 0.1% SDS, 1% NP-40, 2.5% glycerol, 100 mM NaCl, and 1 mM fresh PMSF, 1×protein inhibitor cocktails, 10 mM NaF and 2 mM sodium orthovanadate) per 10 mg tissue. After sonication, the supernatant of the tissue homogenate was separated by centrifugation at 1, 4000 ×g at 4°C for 15 min. Protein concentration was determined using BCA assay (Thermo Fisher). Fifty micrograms of proteins were subjected to Western blot analysis as published previously (23). The primary antibodies were used as following: anti-p-LRP6 (Ser1490, 1:1000, Cell Signaling Technology, Danvers, MA), anti-LRP6 (1:2000, in-house generated), anti-N-p-β-catenin (1:1000, Cell Signaling Technology), anti-β-catenin (1:3000, Santa-Cruz Biotechnology,
Santa Cruz, CA), anti-CTGF (1:500, Santa-Cruz Biotechnology), anti-fibronectin (1:100, Santa-
Cruz Biotechnology), anti-PPARα (1:500, Santa-Cruz Biotechnology), anti-NOX4 (1:3000
Abcam, Cambridge, MA), anti-β-actin (1:50,000, Sigma-Aldrich).

**Statistical analysis**

Data are expressed as mean ± SD. Statistical analysis was performed using Student’s \( t \) test
for the comparison between two groups or by ANOVA for the analysis among more than two
groups. A value of \( p<0.05 \) was considered statistical significance.
RESULTS

Activation of PPARα ameliorates renal fibrosis in diabetic kidney

PPARα expression was evaluated in the kidneys of STZ-induced diabetic rats (type 1 DM) at 12 weeks after the diabetic onset and db/db mice (type 2 DM) at 6 months of age. Expression of PPARα was significantly decreased in the renal cortex of diabetic animals at both the protein and mRNA levels, compared to respective non-diabetic controls (Fig. 1A - F).

To evaluate the protective effect of PPARα on renal fibrosis, STZ-induced diabetic rats at 6 weeks after diabetic onset were fed with special chow containing 0.1% fenofibrate for another 6 weeks. Fenofibrate treatment significantly reduced albuminuria in diabetic rats without changing body weight or blood glucose concentration, compared with those of diabetic rats with regular chow (Fig. 1G, Suppl. Fig. 1A and B). Fenofibrate significantly decreased collagen IV and fibronectin accumulation in diabetic rat kidneys (Fig. 1H). The over-expression of TGF-β1, fibronectin and CTGF was attenuated by fenofibrate in the diabetic kidneys (Fig. 1I and J). These results suggested that activation of PPARα has an anti-fibrotic activity in DN models.

Activation of PPARα attenuates renal fibrosis through blocking the canonical Wnt signaling pathway.

Proximal tubule is an important player in renal hypertrophy, inflammation and fibrosis in DN by generating and secreting fibrosis-related proteins in diabetes, such as fibronectin, CTGF, TGF-β and collagen IV (24). Expression of CTGF and fibronectin in primary human renal proximal tubular cells (HRPTC) was up-regulated by high glucose, compared with those in the control cells treated with low glucose. Fenofibrate significantly suppressed high glucose-induced CTGF and fibronectin expression in a concentration-dependent manner (Fig. 2A - C). Over-expression of PPARα by an adenovirus over-expressing PPARα (Ad-PPARα) significantly
attenuated high glucose-induced expression of CTGF and fibronectin (Fig. 2D to F). These results demonstrate an anti-fibrogenic activity of PPARα in renal cells under diabetes condition.

We have previously reported a pathogenic role of over-activation of canonical Wnt signaling in renal inflammation and fibrosis in DN (12). Wnt3a condition medium (WCM) induced fibronectin and CTGF expression was significantly inhibited by fenofibrate and Ad-PPARα (Fig. 2G - L). Fenofibrate or Ad-PPARα significantly attenuated high glucose- or WCM-induced up-regulation of phosphorylated LRP6 (p-LRP6), a major co-receptor in Wnt signaling, and non-phosphorylated β-catenin (N-p-β-catenin), an effector of canonical Wnt signaling, in renal tubular cells (Fig. 3). Consistently, a luciferase-based promoter assay demonstrated that both fenofibrate and Ad-PPARα dramatically inhibited the WCM-induced transcriptional activity of β-catenin (Fig. 4). The inhibitory effect of PPARα on the canonical Wnt pathway was further confirmed in the kidney of diabetic rats. As shown by elevated levels of LRP6 and nuclear levels of β-catenin, the canonical Wnt pathway was significantly activated in the kidney of STZ-induced diabetic rats. Fenofibrate chow attenuated the activation of the Wnt pathway in the STZ-induced diabetic rat kidneys (Fig. 5A and B). The effect of fenofibrate on LRP6 levels was also demonstrated by immunostaining of kidney sections (Fig. 5C). These results suggested that PPARα negatively regulated the canonical Wnt pathway in the kidney.

**PPARα deficiency results in more prominent activation of the canonical Wnt pathway and more severe DN in diabetic animals.**

To evaluate the inhibitory effects of PPARα on Wnt signaling activation in vivo, diabetes was induced by STZ in PPARα<sup>−/−</sup> mice and age- and genetic background-matched Wt mice. PPARα<sup>−/−</sup> mice with 24 weeks of STZ-induced diabetes showed more severe proteinuria, and dramatically enhanced renal expression of fibronectin, compared to the diabetic Wt mice (Fig.
5D and E), consistent with a previous report (9). However, there was no difference in body weights and blood glucose concentrations between diabetic PPARα+/− mice and Wt mice (Suppl. Fig. 2C and D). Interestingly, diabetic PPARα+/− mice showed more prominent increases of p-LRP6 and N-p-β-catenin levels in the kidney, suggesting higher Wnt signaling activities, compared to diabetic Wt mice (Fig. 5E and F). There were no significant differences in these Wnt signaling components or proteinuria between non-diabetic Wt mice and PPARα+/− mice. These results indicated that PPARα deficiency enhanced the activation of the canonical Wnt pathway induced by diabetic conditions.

The relationship between PPARα and the canonical Wnt pathway was further studied using the transgenic BAT-gal mouse, a Wnt signaling reporter mouse line that expresses the β-galactosidase reporter gene under the control of a promoter containing β-catenin/TCF/LEF-binding sites (25). PPARα+/− mice were crossed with BAT-gal mice to generate PPARα+/− mice carrying the BAT-gal transgene. To activate Wnt signaling in the kidney, these mice were subjected to UUO, a commonly used model for renal fibrosis study. It was previously reported that the canonical Wnt pathway was over-activated in the UUO kidneys (26,27). X-gal staining performed at 7 days after UUO operation showed significantly higher β-catenin transcriptional activities in the obstructive kidney of PPARα+/−/BAT-gal mice, compared to those of their Wt/BAT-gal littermates with UUO (Fig. 5G). These results indicated that activation of PPARα has an inhibitory effect on the canonical Wnt pathway in the kidney.

Primary murine proximal tubular cells were isolated and verified with immunostaining using an antibody against SGLT-1, a specific proximal tubular cell marker (Suppl. Fig. 2). PPARα+/− tubular cells demonstrated more prominent up-regulation of p-LRP6, N-p-β-catenin and fibronectin levels induced by high glucose, compared to Wt cells (Fig. 6A and B).
also induced more prominent increases of p-LRP6 levels and transcriptional activity of β-catenin in PPARα<sup>−/−</sup> cells, compared to Wt cells (Fig. 6C - E). There were no differences in fibronectin expression and p-LRP6 levels between PPARα<sup>−/−</sup> cells and Wt cells with L-glucose or control L cell medium treatment.

**PPARα does not inhibit the transcriptional activity of β-catenin induced by a constitutively active mutant of LRP6 or β-catenin.**

To delineate the mechanism by which PPARα suppresses the canonical Wnt pathway, a constitutively active LRP6 mutant lacking the extracellular domain (LRP6∆N) (28,29) and a constitutively active β-catenin mutant with Serine37 substituted by Alanine (S37A) (12) were used. Both LRP6∆N and S37A induced significant increases of the transcriptional activity of β-catenin in the absence of Wnt ligands (Fig. 7A and B). Although Ad-PPARα blocked the β-catenin transcriptional activity induced by Wnt ligand, Ad-PPARα had no inhibitory effect on LRP6∆N- or S37A-induced transcriptional activity of β-catenin (Fig. 7A and B). These results suggested that PPARα inhibits the canonical Wnt pathway at the Wnt receptor level.

**PPARα decreased LRP6 stability through anti-oxidant effects.**

LRP6 is a crucial co-receptor in the canonical Wnt pathway. To determine if the regulation of PPARα on Wnt signaling is through modulation of LRP6, we measured the protein stability of LRP6 in renal proximal tubular cells. Over-expression of PPARα significantly accelerated degradation of LRP6 protein, as shown in protein stability assay (Fig. 7C). In contrast, primary proximal tubular cells from PPARα<sup>−/−</sup> mice showed a delayed degradation and prolonged half-life of LRP6, compared to Wt cells (Fig. 7D). These data suggested that PPARα inhibits the canonical Wnt pathway through regulating LRP6 protein stability.
We have previously demonstrated that oxidative stress contributes to the activation of the canonical Wnt pathway through stabilizing LRP6 (30). To investigate whether PPARα decreases the stability of LRP6 through reducing oxidative stress, we measured ROS levels in cultured proximal tubular cells. Over-expression of PPARα significantly inhibited ROS generation induced by HNE, a major lipid peroxidation product (Fig. 8A). On the other hand, deficiency of PPARα resulted in more prominent increases of 4-HNE-induced intracellular ROS levels (Fig. 8B). These results suggested that PPARα inhibits the canonical Wnt pathway through promoting LRP6 protein degradation, which is mediated by its anti-oxidant activities.

To delineate the underlying mechanism for PPARα’s anti-oxidant activities, we examined expression levels of NOX4, a key enzyme responsible for ROS generation in DN. Hyperglycemia increased NOX4 expression levels in HKC-8 cells (Fig. 8C and D). Diabetic PPARα−/− mice showed more prominent NOX4 over-expression in the kidney, compared with diabetic Wt mice (Fig. 8E and F). To evaluate the effect of NOX4 on the activation of the Wnt pathway, the transcriptional activity of β-catenin was measured. Over-expression of NOX4 using an adenoviral vector significantly enhanced Wnt3a-induced Wnt signaling (Fig. 8G). These data suggested that PPARα regulates the canonical Wnt pathway, at least in part, through inhibiting the NOX4/ROS pathway in renal cells.
PPARα agonists have displayed therapeutic effects on diabetic nephropathy (8). The aberrant activation of the Wnt signaling pathway has been identified to play a crucial role in renal fibrosis (11-13,31). Tissue- and organ-specific interactions between the Wnt signaling pathway and PPARγ have been reported (32,33). However, the role of PPARα in the regulation of the Wnt signaling pathway has not been previously documented. The present study demonstrated that fenofibrate, a PPARα agonist, ameliorates proteinuria and renal fibrosis, and inhibits aberrant activation of the canonical Wnt pathway in the kidney of diabetic rats. Activation and over-expression of PPARα significantly inhibit Wnt signaling induced by diabetic conditions and by a Wnt ligand in cultured renal cells. In contrast, ablation of PPARα results in a more prominent activation of the canonical Wnt pathway in the kidneys of both DN and obstructive nephropathy models. As expression of PPARα was down-regulated in the kidney of both type 1 and type 2 diabetic animal models, the reduced renal PPARα levels may be responsible, at least in part, for the over-activation of Wnt signaling in the kidney of diabetic models, contributing to renal inflammation and fibrosis in DN. Toward the molecular basis by which PPARα regulates Wnt signaling, the present study demonstrated that PPARα destabilizes the crucial Wnt co-receptor LRP6 through inhibiting the renal NOX4/ROS production. These observations provide the first evidence that PPARα functions as a negative regulator of Wnt signaling in the kidney and reveal a new mechanism responsible for the anti-inflammatory and anti-fibrogenic activities of PPARα.

Members in the PPAR family control distinct signaling pathways by regulating specific gene cassettes. In the PPAR family, PPARγ is highly produced in adipose tissue and regulates lipogenesis, and PPARα is mainly expressed in tissues with high mitochondrial and β-oxidation
activities (34). It has been reported that PPARγ inhibited Wnt signaling in adipogenesis and kidney diseases, partly through down-regulating levels of β-catenin (35,36). Recently, the PPARα antagonist MK886 was reported to increase the expression level of β-catenin in co-cultured spheroid and endometrial cells (37), and activation of Wnt/β-catenin pathway inhibits PPARα-mediated induction of cytochromes p450 genes (38). In the present study, we demonstrate an inhibitory effect of PPARα on Wnt signaling using gain-of-function and loss-of-function approaches. Using a constitutively active LRP6 mutant and β-catenin mutant, we confirmed that the inhibition of the Wnt pathway by PPARα does not occur intracellularly. Further studies showed that PPARα decreases protein stability of LRP6. LRP6 plays a crucial role in Wnt signaling over-activation in diabetes, as blockade of LRP6 alone is sufficient to attenuate Wnt signaling over-activation and ameliorate diabetic retinopathy and DN (12,17). Thus, destabilization of LRP6 is likely responsible for the inhibitory effect of PPARα on Wnt signaling, suggesting that PPARα interacts with the Wnt pathway at a different target than that of PPARγ.

Fenofibrate has been reported to have therapeutic effects on DN in both diabetic human patients and in diabetic animal models (8,39). However, a unifying mechanism responsible for the anti-inflammatory and anti-fibrotic effects of fenofibrate remains unclear. The Wnt pathway has been shown to mediate inflammation through upregulation of inflammatory factors such as TNF-α and ICAM-1, and to enhance fibrosis through up-regulation of CTGF and fibronectin (40,41). Previous studies have shown that over-activation of Wnt signaling as found in both type 1 and type 2 diabetic models, play a crucial role in renal dysfunction of DN (11,12,31,42). Therefore, suppression of Wnt signaling over-activation by PPARα may represent a mechanism for the anti-inflammatory and anti-fibrotic effects of fenofibrate.
Hyperglycemia-induced oxidative stress is considered the primary cause for tissue damage in DN (43). The anti-oxidant effects of PPARα and its agonist have been reported in the retina and kidney of diabetic models by our group and others (8,19,44). We have reported that enhanced oxidative stress contributed to the activation of the Wnt pathway in the retina of diabetic rats via stabilizing LRP6 (30). In our study, 4-HNE-induced ROS production was significantly inhibited by over-expression of PPARα. PPARα deficiency dramatically enhanced 4-HNE-induced ROS generation. These observations suggest that the anti-oxidant effects of PPARα in the kidney results in the destabilization of LRP6, which leads to inhibition of Wnt signaling.

The NOX family is believed to be one of the major oxidases regulating ROS production in DN (45,46). NOX4 is a crucial isoform contributing to DN, as deficiency of NOX1 and NOX2 has no effect on the albuminuria and renal fibrosis in diabetic animals (47,48). In contrast, mice with NOX4 knockout or treated with NOX4 inhibitors showed ameliorated urine albumin excretion and down-regulation of renal fibrosis-related proteins in a long duration of diabetes (48-50). In the present study, diabetic PPARα−/− mice showed significantly higher renal levels of NOX4, compared with diabetic Wt mice, demonstrating that PPARα negatively regulates NOX4 expression. We have previously identified that fenofibrate suppressed expression of NOX4 in retinal pericytes (19). In this study, we have demonstrated that over-expression of NOX4 significantly enhanced Wnt3a-induced transcriptional activity of β-catenin, suggesting that the negative regulation of NOX4 by PPARα may contribute to the inhibition of Wnt signaling. However, the detailed molecular mechanism responsible for the regulation of NOX4 by PPARα remains to be further studied.
Our results showed that non-diabetic PPARα<sup>−/−</sup> mice lack significant changes in renal levels of p-LRP6 and N-p-β-catenin, suggesting that PPARα deficiency alone is not sufficient to activate Wnt signaling under non-diabetic conditions. This notion is supported by observation from primary renal cells, which showed that PPARα deficiency alone does not activate the Wnt pathway under normal glucose medium or in the absence of Wnt ligands. ROS generation in the PPARα<sup>−/−</sup> cells were increased only in the presence of 4-HNE and not in vehicle control. These results suggest that PPARα only inhibits the activation of Wnt signaling under stress conditions such as oxidative stress, and the mechanism remains unclear.

In summary, we identified that PPARα confers a renal protective effect through blocking activation of the canonical Wnt pathway in DN. This inhibitory effect of PPARα on Wnt signaling is through destabilizing LRP6, which is mediated by the anti-oxidant activity of PPARα. This finding suggests that the interaction between PPARα and the canonical Wnt pathway renders a new therapeutic target for renal fibrosis.
Acknowledgments

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Duality of Interest

No potential conflicts of interest relevant to this article were reported.

Author Contributions

R.C. contributed to designing and performing most of the experiments, data analysis and manuscript writing. L.D. assisted in animal studies. X.H. purified adenoviruses and assisted in animal studies. Y.T. made and provided adenoviruses and plasmids in this study. J.M designed and directed the study, and contributed to writing and editing the manuscript.

J.M. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.
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Figure Legends

Figure 1. Activation of PPARα ameliorates renal fibrosis in diabetic kidneys. Representative Western blots (A), semi-quantitative densitometry analyses (B) and real-time RT-PCR results (C) show PPARα protein and mRNA levels in the renal cortex of non-diabetic rats (Non-DM) and STZ-induced diabetic rats (STZ-DM) at 12 weeks following the onset of diabetes. n=4 to 7. Representative Western blots (D), semi-quantitative analyses (E) and real-time RT-PCR results (F) show PPARα protein and mRNA levels in the renal cortex of db/db mice and age-matched wild-type (Wt) mice at the age of 6 months. n=5. Albumin in 24-hr urine from Non-DM and STZ-induced diabetic rats with fenofibrate chow (DM-Feno) or control chow (STZ-DM) was measured by ELISA (G). Representative images of immunohistochemistry show the expression of collagen IV and fibronectin in Non-DM, STZ-DM and DM-Feno (×200) (H). Levels of fibronectin, CTGF, and TGF-β1 in renal cortex lysates were determined by Western blot analysis (I) and the semi-quantitative analysis (J) in Non-DM, STZ-DM and DM-Feno rats. Values are mean±SD, n=4 in Non-DM and STZ-DM; n=6 in DM-Feno. *P<0.05 and **P<0.01.

Figure 2. PPARα attenuates renal fibrosis in proximal tubular cells. Representative Western blots (A) and densitometry analyses of fibronectin (B) and CTGF (C) in primary HRPTC treated with high glucose (HG, 30 mM D-glucose) and indicated concentrations of fenofibrate (Feno) for 72 hr. Low glucose (LG, 25 mM L-glucose + 5 mM D-glucose) was used as negative control. Primary HRPTC were infected with adenovirus expressing PPARα (Ad-PPARα) for 24 hr, with adenovirus expressing GFP (Ad-GFP) as control, and then treated with HG for another 72 hr. Levels of fibronectin and CTGF were determined by Western blot analysis (D) and semi-
quantified by densitometry (E and F). Levels of fibronectin and CTGF were measured with Western blot analyses (G) and quantified (H and I) in primary HRPTC treated with Wnt3a conditioned medium (WCM) and indicated concentrations of fenofibrate for 24 hr. L-cell medium (LCM) was used as negative control. Primary HRPTC were infected with Ad-PPARα, and then treated with WCM for 24 hr. Representative Western blots (J) and densitometry analyses (K and L) show the expression of fibronectin and CTGF in HRPTC. Values are mean±SD, n=3. **P<0.01.

Figure 3. Inhibitory effects of PPARα on high glucose- and Wnt3a-induced activation of Wnt signaling. Representative Western blots (A) and densitometry analyses of phosphorylated LRP6 (p-LRP6) (B), non-phosphorylated β-catenin (N-p-βctnn) (C) in HRPTC exposed to 30 mM D-glucose (HG), with 5 mM-D-glucose + 25 mM L-glucose (LG) as control, and treated with indicated concentrations of fenofibrate (Feno) for 24 hr. Levels of p-LRP6 and N-p-β-ctnn were determined in HRPTC treated with HG and adenovirus expressing PPARα (Ad-PPARα) for 24 hr by Western blot (D) and densitometry analyses (E and F). Levels of p-LRP6 and N-p-β-ctnn were measured by Western blot analysis (G) and densitometry analysis (H and I) in HRPTC treated with Wnt3a conditioned medium (WCM) and indicated concentrations of fenofibrate for 12 hr. L-cell medium (LCM) was used as negative control. Representative Western blots (J) and densitometry analyses (K and L) show changed levels of p-LRP6, N-p-β-ctnn in HRPTC infected with Ad-PPARα treated with WCM for 12 hr. Values are mean±SD, n=3. **P<0.01.

Figure 4. Inhibitory effects of PPARα on Wnt3a-induced transcriptional activity of β-catenin. Luciferase assay was used to quantify the effects of fenofibrate (Feno) (A) and over-expression of PPARα (Ad-PPARα) (B) on Wnt3a conditioned medium (WCM)-induced transcriptional activity of β-catenin in tubular cells. L-cell conditioned medium (LCM) and
adenovirus expressing β-galactosidase (Ad-βgal) were used as controls. Values are mean±SD, 

\( n=4 \). **\( P<0.01 \).

**Figure 5. Inhibitory effects of PPARα activation on Wnt signaling in renal fibrosis models.**

Representative Western blots (A) and densitometry analyses (B) show levels of total LRP6 and nuclear β-catenin in the renal cortex in non-diabetic rats (Non-DM) and STZ-diabetic rats with fenofibrate chow (DM-Feno) or control chow (STZ-DM). \( n=4 \) in Non-DM and STZ-DM; \( n=6 \) in DM-Feno. Representative images of immunohistochemical staining (brown) show changes of renal LRP6 levels in the diabetic rats treated with fenofibrate (C) (×200). ELISA results show albumin levels in 24-hr urine in diabetic PPARα\(^{-/-}\) mice (PKO) and age-matched wild-type (Wt) mice (D). \( n=6 \). Representative Western blots (E) and semi-quantitative analyses (F) compare levels of renal phosphorylated LRP6 (p-LRP6), non-phosphorylated β-catenin (N-p-βctnn) and fibronectin in diabetic and non-diabetic PKO mice and age-matched Wt mice. \( n=6 \). PPARα\(^{-/-}\) /BAT-gal mice (PKO/BAT-gal) and their wild-type/BAT-gal littermates (Wt/BAT-gal) received unilateral ureteral obstruction (UOO) (G). The kidney with UOO was subjected to X-gal staining (blue) at post-operation day 7 to detect the transcriptional activity of β-catenin in the kidney (Top panels, ×40; Bottom panels, higher magnification of the boxed areas). \( n=3 \). Values are mean±SD. *\( P<0.05 \) and **\( P <0.01 \).

**Figure 6. Deficiency of PPARα promoted Wnt pathway activation in proximal tubular cells.** Primary mouse renal proximal tubular cells (MRPTC) were isolated and cultured from 21-day-old PPARα\(^{-/-}\) mice (PKO) and wild-type (Wt) littermates. Representative Western blots (A) and semi-quantitative analyses (B) of phosphorylated LRP6 (p-LRP6), non-phosphorylated β-catenin (N-p-βctnn) and fibronectin in primary MRPTC treated with 30 mM D-glucose (HG) for 72 hr, with the 5 mM D-glucose + 25 mM L-glucose (LG) as control. Wnt3a conditioned
medium (WCM)-induced phosphorylation of LRP6 was measured by Western blot analysis (C) and semi-quantitative analysis (D) in primary MRPTC. Luciferase assay was used to measure transcriptional activity of β-catenin in Wt and PPARα−/− MRPTC (E). Values are mean±SD. n=3 to 4. *P<0.05, **P<0.01.

Figure 7. PPARα inhibits the canonic Wnt pathway through destabilizing LRP6. HKC-8 TOPFLASH cells were transfected with plasmids expressing LRP6ΔN (A) or a constitutively active mutant of β-catenin (S37A) (B), then infected with Ad-PPARα for another 24 hr. Transcriptional activity of β-catenin was measured using luciferase assay. Twenty-four hours after transfection with a plasmid expressing PPARα, the cells were treated with Wnt3a conditioned medium for 24 hr, then incubated with cycloheximide (CHX, 50 µM) for indicated times. Levels of LRP6 in cell lysates were measured using Western blot analysis (C). Primary mouse renal proximal tubular cells (MRPTC) were cultured from PPARα−/− mice (PKO) and age-matched wild-type mice (Wt). Cells were incubated with Wnt3a conditioned medium for 24 hr, and then treated with CHX for the indicated times. LRP6 stability was measured by Western blot analysis (D). Values are mean±SD, n=3. **P<0.01. n.s., no significance.

Figure 8. Anti-oxidant effects of PPARα. Levels of intracellular ROS in HKC-8 cells infected with Ad-PPARα (A) or in primary mouse renal proximal tubular cells (MRPTC) from PPARα−/− (PKO) and wild-type (Wt) mice (B) were determined using CM-H2DCFDA staining (n=4 to 5). Ad-β-galactosidase (Ad-βgal) was used as control virus. Representative Western blots (C) and semi-quantitative analyses (D) show NOX4 levels in HRPTC treated with 30 mM D-glucose (HG) or 5 mM D-glucose + 25 mM L-glucose (LG) for 72 hr (n=3). Representative Western blots of NOX4 levels in the renal cortex from STZ-diabetic Wt (Wt-DM) and diabetic PPARα−/− (PKO-DM) mice (E) and densitometry analysis (F) (n=5). Luciferase assay shows the effect of
over-expression of NOX4 using Ad-NOX4 infection on transcriptional activity of β-catenin in the presence of Wnt3a conditioned medium (WCM) (G). n=4. Values are mean±SD, **P<0.01.

Supplementary Figure Legends

Supplementary Figure 1. Blood glucose levels and body weights in diabetic animals. (A and B) Body weights (g) (A) and blood glucose levels (mg/dl) (B) of STZ-induced diabetic rats fed with fenofibrate chow (DM-Feno) or control chow (STZ-DM) at 12 weeks after diabetes onset. n=4 in age-matched non-diabetic rats (Non-DM) and STZ-DM, n=6 in DM-Feno. Body weights (g) (C) and blood glucose levels (mg/dl) (D) of STZ-induced diabetic (DM) and non-diabetic (N-DM) PPARα⁻/⁻ mice (PKO) and age-matched wild-type mice (Wt) at 24 weeks after diabetes onset. n=6. Values are mean±SD, n=3. **P<0.01. n.s., no significance.

Supplementary Figure 2. Characterization of cultured murine renal proximal tubular cells. Primary murine renal proximal tubular cells (MRPTC) at passage 1 were immunostained with an antibody against SGLT-1. Commercial human renal proximal tubular cells (HRPTC) were used as positive control cells. MRPTC incubated without the primary antibody were used as negative control. (×100)
Cheng et al., Figure 1
Cheng et al., Figure 2
Cheng et al., Figure 3

A) HG+Feno (µmol/L)  LG  0  6  12  24  48
p-LRP6  N-p-βcatnn  β-actin

B) Ad-GFP Ad-PPARα

C) Ad-GFP Ad-PPARα

D) Ad-GFP Ad-PPARα

E) Ad-GFP Ad-PPARα

F) Ad-GFP Ad-PPARα

G) WCM+Feno (µmol/L)  LCM  0  6  12  24  48
p-LRP6  N-p-βcatnn  β-actin

H) Ad-GFP Ad-PPARα

I) Ad-GFP Ad-PPARα

J) Ad-GFP Ad-PPARα

K) Ad-GFP Ad-PPARα

L) Ad-GFP Ad-PPARα
Cheng et al., Figure 5
Cheng et al., Figure 7
A

4-HNE (µmol/L)

ROS intensity

0  2000  4000  6000  8000  10000  12000  14000  16000

Wt MRPTC  PKO MRPTC

B

4-HNE (µmol/L)

ROS intensity

0  2000  4000  6000  8000  10000  12000  14000  16000

Wt MRPTC  PKO MRPTC

C

LG  HG

NOX4  β-actin

D

NOX4 level

LG  HG

E

Wt-DM  PKO-DM

NOX4  β-actin

F

NOX4 level

Wt-DM  PKO-DM

G

β-catenin activity (% of control)

LCM  WCM  WCM  Ad-β-gal  Ad-NOX4

Cheng et al., Figure 8
**Supplementary Figure 1. Blood glucose levels and body weights in diabetic animals.** (A and B) Body weights (g) (A) and blood glucose levels (mg/dl) (B) of STZ-induced diabetic rats fed with fenofibrate chow (DM-Feno) or control chow (STZ-DM) at 12 weeks after diabetes onset. n=4 in age-matched non-diabetic rats (Non-DM) and STZ-DM, n=6 in DM-Feno. (C and D) Body weights (g) (C) and blood glucose levels (mg/dl) (D) of STZ-induced diabetic (DM) and non-diabetic (N-DM) PPARα−/− mice (PKO) and age-matched wild-type mice (Wt) at 24 weeks after diabetes onset. n=6. Values are mean±SD, n=3. **P<0.01. n.s., no significance.
Supplementary Figure 2. Characterization of cultured murine renal proximal tubular cells. Primary murine renal proximal tubular cells (MRPTC) at passage 1 were immunostained with an antibody against SGLT-1. Commercial human renal proximal tubular cells (HRPTC) were used as positive control cells. MRPTC incubated without the primary antibody were used as negative control. (×100)