Pirfenidone Is Renoprotective in Diabetic Kidney Disease

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ABSTRACT

Although several interventions slow the progression of diabetic nephropathy, current therapies do not halt progression completely. Recent preclinical studies suggested that pirfenidone (PFD) prevents fibrosis in various diseases, but the mechanisms underlying its antifibrotic action are incompletely understood. Here, we evaluated the role of PFD in regulation of the extracellular matrix. In mouse mesangial cells, PFD decreased TGF-β promoter activity, reduced TGF-β protein secretion, and inhibited TGF-β–induced Smad2-phosphorylation, 3TP-lux promoter activity, and generation of reactive oxygen species. To explore the therapeutic potential of PFD, we administered PFD to 17-wk-old *db/db* mice for 4 wk. PFD treatment significantly reduced mesangial matrix expansion and expression of renal matrix genes but did not affect albuminuria. Using liquid chromatography with subsequent electrospray ionization tandem mass spectrometry, we identified 21 proteins unique to PFD-treated diabetic kidneys. Analysis of gene ontology and protein–protein interactions of these proteins suggested that PFD may regulate RNA processing. Immunoblotting demonstrated that PFD promotes dosage-dependent dephosphorylation of eukaryotic initiation factor, potentially inhibiting translation of mRNA. In conclusion, PFD is renoprotective in diabetic kidney disease and may exert its antifibrotic effects, in part, *via* inhibiting RNA processing.

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Diabetic nephropathy (DN) is the single major cause of the emerging epidemic of ESRD in the United States,[1] accounting for nearly 50% of all new cases.[2] Characteristic morphologic lesions of DN include glomerular hypertrophy, thickening of the basement membrane, and mesangial expansion.[3] Several interventions, such as tight glycemic control and antihypertensive therapy, especially angiotensin-converting enzyme inhibitors (ACEIs) and angiotensin II receptor blockers,[4–9] have been shown to slow the progression of established disease. Nevertheless, DN remains a major long-term complication of both types 1 and 2 diabetes,[10,11] because treatment commenced after the manifestation of overt clinical nephropathy often does not arrest progression to ESRD.[11] The annual medical cost for treatment of patients with diabetes ESRD is expected to be $18 to 30 billion (US) during the next decade.[12–15] It is therefore imperative to identify

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novel drug-therapeutic regimens that can ideally arrest further progression of the disease after manifestation of nephropathy.

Pirfenidone (PFD; 5-methyl-1-phenyl-2-(1H)-pyridone) is a low molecular weight synthetic molecule that exerts dramatic antifibrotic properties in cell culture and various animal models of fibrosis.[16,17] PFD has emerged as a promising oral treatment with few adverse effects in open-label clinical studies. A study of hemodialysis patients with a history of sclerosing peritonitis demonstrated that it may not be necessary to adjust dosages of PFD for renal impairment and that the drug is well tolerated even in ESRD.[18] In an open-label study wherein PFD was administered to patients with advanced refractory focal sclerosis, there was a good safety profile in patients with impaired renal function and heavy proteinuria, and PFD slowed the rate of decline of renal function by 25%.[19] In a Phase III trial for patients with idiopathic pulmonary fibrosis in Japan, PFD was reported to promote stabilization and improvement of lung function.[20] Of note, there have been no reports that PFD may worsen renal blood flow, lower BP, affect glycemic parameters, or cause hyperkalemia, thereby making this treatment approach truly unique as compared with presently available renin-angiotensin-aldosterone antagonists. Thus, the combined experience with PFD in patients and in animal models of progressive kidney disease suggests that the compound is safe and may provide stabilization of renal function.

To determine whether PFD is potentially beneficial in diabetic kidney disease, we studied the effects of PFD in cell culture experiments and in the *db/db* mouse model of diabetic kidney disease. In cell culture studies, PFD inhibited TGF-β production and TGF-β signaling and reduced TGF-β–induced reactive oxygen species (ROS) production. In the *db/db* mouse, PFD promoted resolution of mesangial matrix when administered after the onset of nephropathy. For identification of novel pathways of PFD relevant to DN, proteomic studies of the whole kidneys followed by bioinformatic analyses revealed RNA processing as a novel mechanism of PFD action. In support of a role of PFD to affect mRNA translation, PFD was found to regulate the activity of eukaryotic initiation factor (eIF4E), a key mRNA cap-structure binding protein, in mesangial cells in culture.

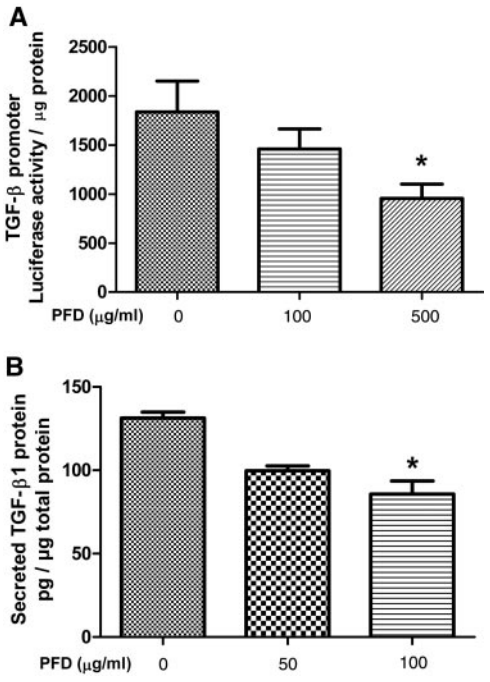

Figure 1. (A) PFD effects on glucose-induced TGF-β1 promoter A 835-luciferase activity. PFD decreases the glucose-induced activity of TGF-β1 promoter A835-luciferase in MMCs at 100 μg/ml, that becomes significant at 500 μg/ml. *$P < 0.05$ PFD 500 μg/ml *versus* control ($n = 4$). (B) PFD reduces TGF-β1 protein levels. To determine whether PFD reduces matrix by lowering TGF-β production, we measured TGF-β1 levels by ELISA in the conditioned media. As compared with control, PFD decreased secreted TGF-β1 significantly at all dosages tested.*$P < 0.05$, PFD treatment *versus* control ($n = 3$).

RESULTS

Pirfenidone Reduces TGF-β Production and Inhibits TGF-β Signaling

To determine whether PFD regulates TGF-β production in murine mesangial cell (MMC) line, we performed studies to evaluate the effect of PFD on the glucose-responsive TGF-β1 promoter (pA835)[21] and on TGF-β1 protein secreted in the conditioned media (Figure 1). PFD was able to reduce both the TGF-β1 promoter activity and TGF-β1 protein secretion but at different dosages. The promoter activity was significantly inhibited at 500-μg/ml concentrations, whereas PFD inhibited secreted TGF-β protein at 100 μg/ml (Figure 1). Thus, in relation to the TGF-β system, PFD seems to be more sensitive to inhibit protein production as compared with gene transcription.

To evaluate effects of PFD on TGF-β signaling, we assessed Smad2 phosphorylation and 3TP-Lux activity. PFD inhibited TGF-β–induced Smad2 phosphorylation (Figure 2A) at all dosages of 100 to 1000 μg/ml. Surprisingly, there was also an effect of PFD to reduce total Smad2/3 protein. PFD also inhibited TGF-β1–induced 3TP-Lux activity as shown in Figure 2B, but the inhibition reached significance only at a concentration of 1000 μg/ml.

Pirfenidone Reduces TGF-β–Induced Mesangial Cell ROS Generation

We recently demonstrated that TGF-β stimulates ROS production *via* an NADPH oxidase pathway and with a time course that is likely independent of Smads.[22] Because PFD has been postulated to inhibit NADPH oxidase in other cell types,[23] we determined whether there was a similar effect of PFD to block TGF-β–induced ROS in mesangial cells. As shown in Figure 3, PFD blocked the TGF-β–induced increase in the ROS production in mesangial cells (100 to 1000 μg/ml, in a dosage-dependent manner).

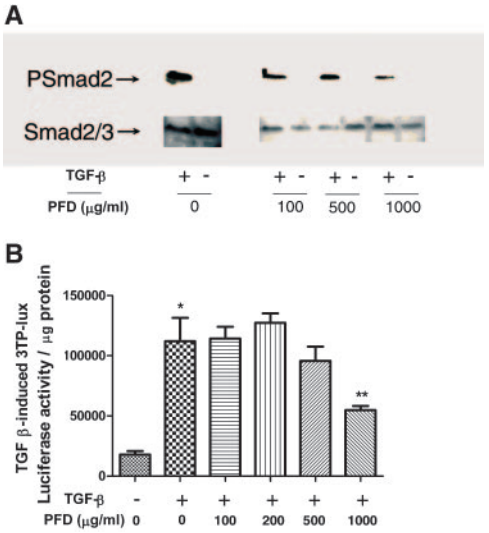

Figure 2. (A) Effect of PFD on phospho-Smad2 protein upregulation by TGF-β. As shown in this Western blot, low-dosage PFD had no effect on Smad2 phosphorylation, but, at higher dosages, it partially reduced Smad2 phosphorylation. (B) PFD effects on 3TP-lux activity and TGF-β1 promoter activity. TGF-β1 increased 3TP-LUX promoter activity by six-fold in HEK293 cells. PFD reduced TGF-β1 promoter activity only at dosages 600 μg/ml and significantly blocked 50% of the increase at 1000 μg/ml dosage. *$P < 0.05$, TGF-β treatment *versus* control ($n = 5$); **$P < 0.05$, PFD + TGF-β treatment *versus* TGF-β treatment alone ($n = 5$).

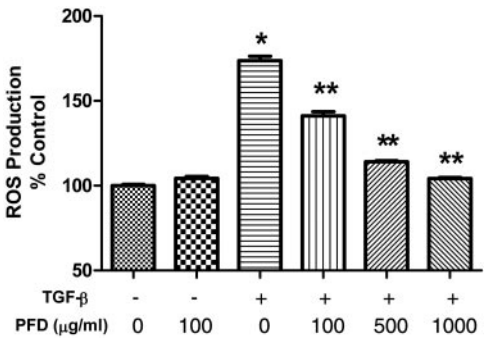

Figure 3. ROS quantification in MMCs treated with TGF-β and PFD. MMCs grown in 96-well plates were serum-deprived and treated with TGF-β in the presence of varying amounts of PFD as described in the Concise Methods section. ROS generated in MMCs subsequent to treatment with these reagents was quantified using Amplex Red Assay kit. *$P < 0.05$, TGF-β treatment *versus* control ($n = 6$); **$P < 0.05$, PFD+TGF-β treatment *versus* TGF-β treatment alone ($n = 6$).

PFD Inhibits TGF-β–Induced Matrix Gene Expression in Mesangial Cells

Mesangial cells exposed to TGF-β1 (10 ng/ml) for 24 h exhibit a stimulation of α1(I) collagen and α1(IV) collagen mRNA levels (Figure 4). Pretreatment with PFD (added 30 min before TGF-β1 addition) reduced TGF-β–stimulated type I collagen (Figure 4A) and type IV collagen (Figure 4B).

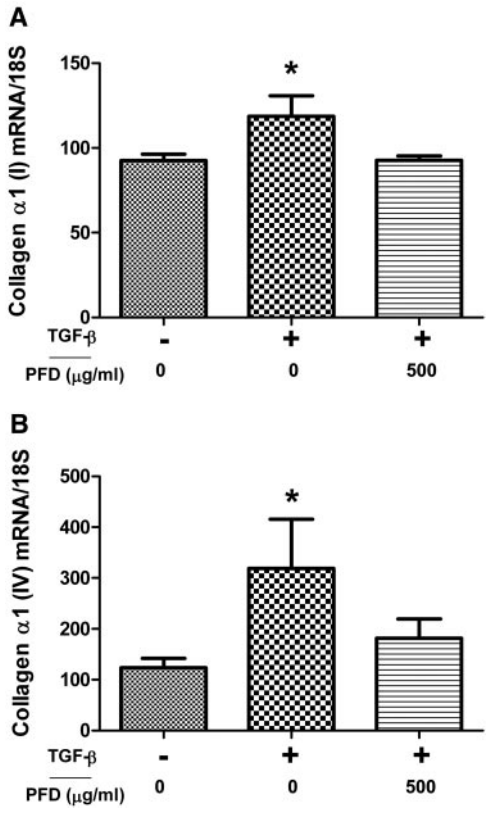

Figure 4. MMCs were treated with TGF-β alone, PFD alone, or PFD and TGF-β (30 min before TGF-β treatment) and left in culture for 24 h before harvesting. Total RNA was isolated from the MMCs using the RNeasy Mini Kit (Qiagen, Valencia, CA) according the manufacturer's instructions. (A and B) Real-time PCR was performed using the primers for α1-type I collagen (A) and α1-type IV collagen (B) and normalized against 18S as listed in supplemental section. *$P < 0.05$, TGF-β treatment *versus* control.

PFD Inhibits Mesangial Matrix Expansion in *db/db* Mice but Does not Affect Albuminuria

The *db/db* mice in a C57Bl6KS background demonstrate albuminuria and mesangial matrix expansion by 12 to 16 wk of age. To determine whether PFD would have benefit after the onset of diabetic kidney disease, we treated *db/db* mice with oral PFD from week 17 to week 21. There was no effect of PFD on blood glucose levels or albuminuria (Table 1). At 21 wk of age, there was a marked increase in grade 3 and grade 4 glomerulosclerosis in *db/db* mice as compared with *db/m* mice. This was accompanied by arteriolar hyalinosis. Four weeks of PFD treatment led to a significant reduction in the degree of mesangial matrix expansion as the percentage of glomeruli with grade 4 lesions decreased by >50% and there were more glomeruli with the mild grade 2 lesions (Figure 5A, Table 2). The glomerular volume was increased in the *db/db* mice, and PFD treatment did not significantly affect glomerular size (Table 2); however, PFD reduced the diabetic stimulation of renal type I collagen, type IV collagen, and fibronectin gene expression to control levels (Figure 5B).

Table 1. Clinical parameters of mice at the beginning and the end of study[a]

Mice	Body Weight (g)	Urinary Albumin:Creatinine (μg/mg)	Blood Glucose (mg/dl)
Db/m			
baseline	26.65 ± 2.20	36.42 ± 45.00	216.90 ± 43.00
end of study	29.90 ± 2.40	35.00 ± 17.00	147.00 ± 41.00
Db/db			
baseline	41.90 ± 2.50[b]	670.90 ± 334.50[b]	560.60 ± 62.00[b]
end of study	46.00 ± 5.20[c]	427.00 ± 249.00[c]	537.00 ± 72.00[b]
Db/db + PFD			
baseline	41.40 ± 2.40[b]	443.00 ± 391.00[b]	462.90 ± 76.00[b]
end of study	42.00 ± 2.95[c]	779.00 ± 561.00[c]	540.00 ± 77.00[b]

[a]Data are means ± SEM.
[b]$P < 0.05$ *versus db/m* at baseline.
[c]$P < 0.05$ *versus db/m* at end of study.

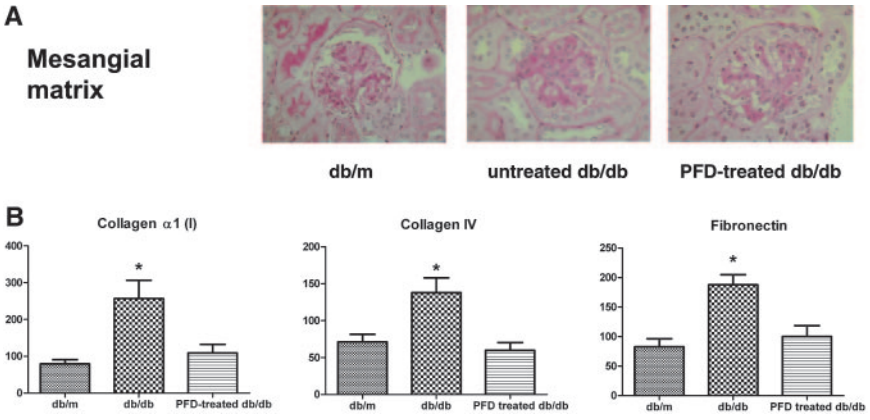

Figure 5. (A) PFD reduces the mesangial matrix expansion in *db/db* mice. Representative micrographs taken from kidney in *db/m*, untreated *db/db* and PFD-treated *db/db* mice. See Table 2 for semi-quantitative scoring of the glomerular matrix. (B) PFD inhibits renal collagen and fibronectin expression in *db/db* mice. Quantitative real time PCR was performed with kidney cortex from all 3 groups (type I collagen, type IV collagen, and fibronectin), each normalized against 18S. *$P < 0.05$ *db/db versus db/m*.

PFD Treatment Alters Protein Expression in the Kidney

To identify potential new pathways by which PFD may be beneficial in diabetic kidney disease, we carried out proteomics of kidneys from the nondiabetic, diabetic, and diabetic mice treated with PFD. Proteomics were performed on kidney lysate by liquid chromatography with subsequent tandem mass spectrometry (LC-MS/MS), and the results are summarized in the Venn diagram shown in Figure 6. Twenty-one proteins were uniquely found in PFD-treated diabetic kidneys (Table 3). Several of these proteins were involved in calcium signaling, RNA translation, nucleotide-binding proteins, and nuclear translocation.

Network-Based Analysis Reveals a Possible New Mechanism of PFD Action in Diabetic Kidney Disease

To gather more insight into the PFD-activated pathways in the diabetic kidney, we explored the 21 newly identified PFD-unique proteins (Table 3) with respect to their physical interactions in a protein–protein interaction network (PPI). Due to the paucity of murine PPI data, we assembled a corresponding human PPI network, comprising 57,235 interactions among 11,203 proteins, integrated from yeast two-hybrid experiments,[24,25] predicted interactions *via* orthology,[26] and curation of the literature.[27–30] Of the 21 PFD-unique mouse proteins, 14 have clear human orthologs (Table 4) and 11 were able to be mapped onto the PPI network (Figure 7). Aiming to construct a PFD-centered network, we then pulled out the first interacting neighbors of the 11 PFD-unique proteins, resulting in a network comprising 518 proteins and 655 interactions. Interestingly, there was only one interaction between two PFD-unique proteins: The splicing factor arginine/serine-rich involved in premRNA splicing (SFRS4) and the heterogeneous nuclear ribonucleoprotein heterogeneous nuclear ribonucleoprotein H (hnRNPH2; Figure 7). Gene ontology and pathways enrichment analysis revealed that the majority of the significantly enriched biologic functions are related to posttranscriptional or posttranslational regulation pathways such as ubiquitin cycle, control of protein translation, and mRNA processing (Supplemental Figure 1, Supplemental Table 1). Of particular interest is the interplay between hnRNPH2 and SFRS4. Both of these proteins not only physically interact with each other but also share 90 interacting proteins with members of the heterogeneous nuclear ribonucleoproteins, splicing factors, and mRNA polymerase protein families. These proteins are central to pathways involved with pre-mRNA processing, splicing, and localization (Figure 7).

PFD Treatment Regulates eIF4E Phosphorylation

Several studies have shown that increased phosphorylation of eIF4E stimulates initiation of mRNA translation.[31–34] eIF4E phosphorylation increases binding to capped mRNA *in vitro*,[35] thereby modulating mRNA translation initiation in cells.[34]

Table 2. PFD reduces glomerular mesangial matrix expansion

Parameter	% of Glomeruli Counted (Mean Grid No.)				Glomerular Area	
	Grade 1	Grade 2	Grade 3	Grade 4	Mean Grid No.	% of Control
Db/m	78	22	0	0	18.9200 ± 0.6610	100.00
Db/db	0	0	83[a]	17	23.3500 ± 0.3459[a]	123.40
Db/db + PFD	0	7	86[a]	7[b]	22.6000 ± 1.1000[a]	119.45

[a] $P < 0.05$ *versus db/m.*
[b] $P < 0.05$ *versus db/db.*

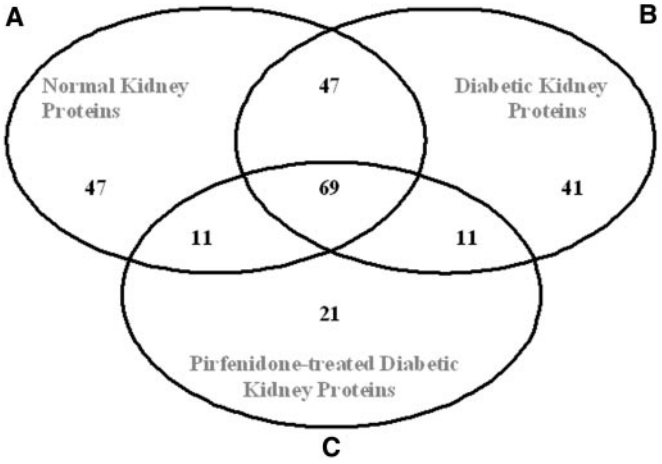

Figure 6. (A) Venn diagram depicting the distribution of proteins in normal *db/m* mouse kidney. (B) PFD-treated *db/db* diabetic mouse kidney. (C) *db/db* diabetic mouse kidney ($n = 4$).

PFD induced dephosphorylation of eIF4E in a dosage-dependent manner (Figure 8).

DISCUSSION

Novel treatments for DN are urgently required. Antifibrotic agents that are orally available and work *via* a nonhypoglycemic and nonhypertensive pathway would be especially attractive as disease-modifying therapies. In this study, we demonstrate the effects of PFD to inhibit numerous pathways in cell culture and in a mouse model of diabetic kidney disease. Although the exact mechanism of action remains unclear, we demonstrate that PFD can block TGF-β production at the transcriptional and protein levels, inhibit TGF-β–induced Smad phosphorylation and TGF-β–induced gene transcription, and inhibit TGF-β–induced matrix stimulation in mesangial cells. In the *db/db* mouse, PFD provides histologic protection after the onset of diabetic kidney disease. Using a proteomic approach, we identified novel pathways that may be relevant to the *in vivo* effects of PFD in diabetic kidney disease.

The inhibition of the glucose-responsive promoter of TGF-β suggests that PFD would have effects on the regulation of signaling pathways and transcription factors involved in high-glucose stimulation of the TGF-β1 gene. The signaling pathways stimulated by high glucose to regulate TGF-β include protein kinase C, extracellular signal–regulated kinase, p38, and ROS production. Which of these pathways may be involved to inhibit high-glucose stimulation is unclear.

PFD seems to have several effects in the Smad pathway. Our data demonstrate that PFD inhibits Smad2 phosphorylation. PFD is able to inhibit the stimulation of both type I collagen

Table 3. Proteins unique to *db/db* mouse kidneys treated with PFD

Name of Protein Identified	Accession No.
Calbindin 2 [Mus musculus]	34098931
Calbindin-28K [Mus musculus]	6753242
Clathrin, light polypeptide (Lcb) [Mus musculus]	30794164
Eukaryotic translation elongation factor 1 α 1 [Mus musculus]	51873060
Eukaryotic translation elongation factor 1 α 2 [Mus musculus]	6681273
Glutamyl-prolyl-tRNA synthetase [Mus musculus]	82617575
Glycine amidinotransferase (L-arginine:glycine amidinotransferase)	13385454
Heterogeneous nuclear ribonucleoprotein H2 [Mus musculus]	9845253
Histidine triad nucleotide binding protein 2 [Mus musculus]	110625719
NADH dehydrogenase (ubiquinone) Fe-S protein 6 [Mus musculus]	56711244
PREDICTED: similar to bifunctional aminoacyl-tRNA synthetase	94364712
PREDICTED: similar to heat-shock protein 1 (chaperonin) [Mus musculus]	94373522
PREDICTED: similar to NADH dehydrogenase (ubiquinone) Fe-S protein 6	82906344
PREDICTED: similar to NADH dehydrogenase (ubiquinone) Fe-S protein 6	82913708
PREDICTED: similar to SMT3 suppressor of mif two 3 homolog 2	94366596
PREDICTED: similar to SMT3 suppressor of mif two 3 homolog 2	94406927
RNA binding motif protein 3 [Mus musculus]	37497112
SMT3 suppressor of mif two 3 homolog 2 [Mus musculus]	19111164
Spectrin β 2 isoform 1 [Mus musculus]	117938332
Splicing factor, arginine/serine-rich 4 (SRp75) [Mus musculus]	10181126
sulfotransferase family, cytosolic, 1C, member 2 [Mus musculus]	34328501

Table 4. Human orthologs of PFD-unique mouse proteins

Mouse Entrez Gene ID	GI Accession	RefSeq Protein	Symbol	Gene Name	Human Entrez Gene ID
12307	6753242	NP_033918	CALB1	Calbindin-28K	793
12308	34098931	NP_031612	CALB2	Calbindin 2	794
74325	30794164	NP_083146	CLTB	Clathrin, light polypeptide (Lcb)	1212
13627	51873060	NP_034236	EEF1A1	Eukaryotic translation elongation factor 1 α 1	1915
13628	6681273	NP_031932	EEF1A2	Eukaryotic translation elongation factor 1 α 2	1917
107508	82617575	NP_084011	EPRS	Glutamyl-prolyl-tRNA synthetase	2058
67092	13385454	NP_080237	GATM	Glycine amidinotransferase (L-arginine:glycine amidinotransferase)	2628
56258	9845253	NP_063921	HNRPH2	Heterogeneous nuclear ribonucleoprotein H2	3188
407785	56711244	NP_035018	NDUFS6	NADH dehydrogenase (ubiquinone) Fe-S protein 6	4726
19652	37497112	NP_058089	RBM3	RNA binding motif protein 3	5935
57317	10181126	NP_065612	SFRS4	Splicing factor, arginine/serine-rich 4 (SRp75)	6429
170930	19111164	NP_579932	SUMO2	SMT3 suppressor of mif two 3 homolog 2	6613
20742	117938332	NP_787030	SPNB2	Spectrin β 2 isoform 1	8904
69083	34328501	NP_081211	SULT1C2	Sulfotransferase family, cytosolic, 1C, member 2	27233

and α1(IV) collagen by TGF-β in mesangial cells. Our cell culture data demonstrate that PFD is able to inhibit both the stimulation of TGF-β and its downstream pathways to stimulate matrix gene expression.

Apart from effects of PFD on matrix regulation, there is a growing recognition that PFD has potent anti-inflammatory activities. Of interest is the potent effects of PFD to inhibit TGF-β–induced ROS production in mesangial cells (in this study), suggesting that PFD may be inhibiting NADPH oxidase. Indeed, PFD can inhibit NADPH oxidase, act as a scavenger of ROS and OH−, and inhibit lipid membrane peroxidation, suggesting that one effect of PFD may be *via* its antioxidant pathway.[23,36,37]

To determine whether PFD may be renoprotective after the onset of diabetic kidney disease, we chose the C57BLKS *db/db* mouse model. A major benefit of the model is the consistent temporal development of hyperglycemia (6 to 8 wk), albuminuria (10 to 12 wk), and mesangial matrix expansion (14 to 16 wk). Our treatment protocol therefore started with oral PFD at 17 wk, and the treatment was continued for 4 wk. Even with such a short treatment protocol, we found a significant reduction in the degree of diffuse glomerular mesangial matrix expansion and a reduction in renal gene expression of type IV collagen. The degree of resolution of both mesangial matrix expansion and renal type IV collagen gene expression was similar to previous studies with intraperitoneally administered anti–TGF-β antibodies.[38–40] The results of the preclinical studies in this accepted mouse model of diabetic kidney disease is strong evidence in support of potential renoprotective benefit of oral PFD in human DN.

Interestingly, we did not see a benefit of PFD to reduce albuminuria in the *db/db* mice. The results are again similar to the effect of anti–TGF-β antibodies in that there is a dissociation of renoprotective benefits and reduction of albuminuria. The data would support the concept that different pathways are involved in regulating albuminuria from mesangial matrix expansion and that renoprotection could occur in the face of persistent albuminuria. Previous reports of animal models of kidney disease demonstrated a consistent reduction of renal matrix accumulation with PFD but varying effects on proteinuria.[41–43] These data are important in interpreting clinical data from antifibrotic approaches. It is clearly conceptually possible to have effects to reduce mesangial matrix expansion and by inference protect the GFR, without necessarily reducing albuminuria. Another important factor that seems to be refractive to PFD treatment is the BP. A BP-lowering effect as a result of PFD treatment cannot be ruled out in our studies; however, PFD was not found to lower BP in previous animal or human studies when BP was measured.[44–46]

Angiotensin receptor antagonists have been used in the *db/db* mouse model,[47–53] and generally show DN to be delayed by the use of ACEIs.[47] Although we have not performed comparative studies with blockers of renin-angiotensin system (RAS), we speculate that treatment with PFD will be additive to that of ACEIs and angiotensin antagonists. As PFD has no reported effect on BP or blocking of the RAS, the mechanism of renoprotection by PFD is likely distinct to blockers of the RAS. Of note, one clinical study with PFD in patients with FSGS did not find any reduction of BP.[46]

Thus far, the only clinical study reported to date for PFD in kidney disease is an open-label study of patients with advanced FSGS.[19] This study demonstrated that patients with refractory FSGS, whose disease did not respond to steroids, may have a slower decline in the renal function with PFD. Interestingly, PFD was found to have a significant reduction in slowing the rate of progression without affecting albuminuria.[19] However, the lack of effect on proteinuria raises the question of how to monitor patients with antifibrotic therapies to determine

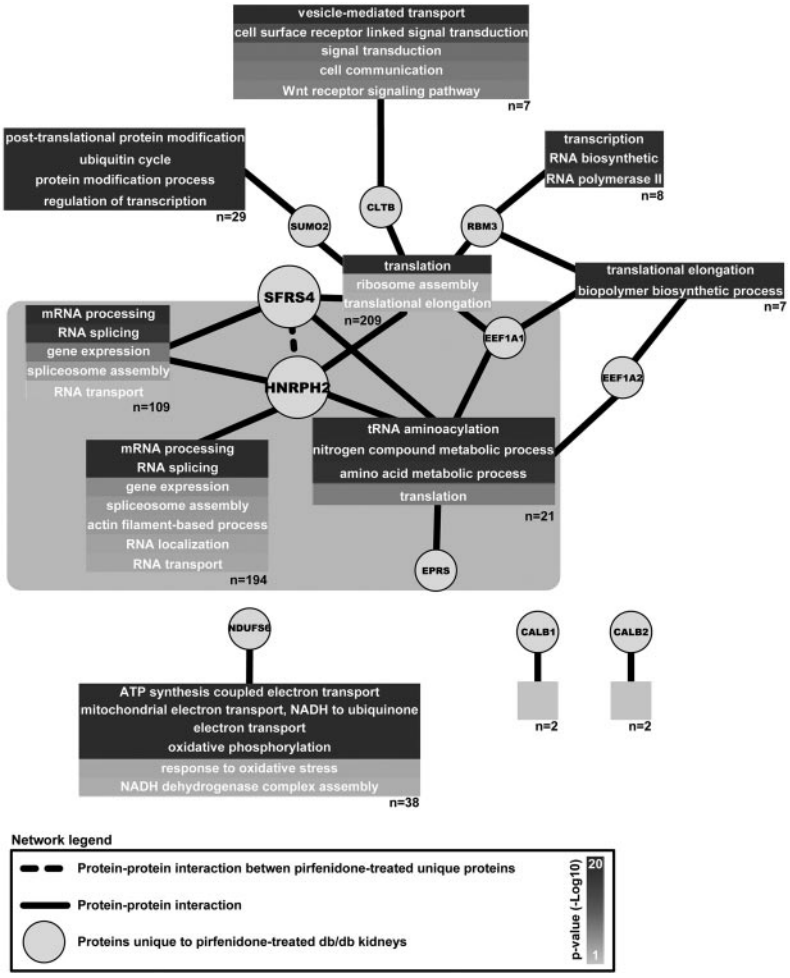

Figure 7. Protein–protein interaction network of PFD-unique *db/db* kidney proteome. The figure depicts the human protein–protein interaction network centered on 11 human proteins orthologous to the mouse proteins identified only in PFD-treated *db/db* kidneys. The network comprises 518 proteins and 655 interactions. The PFD-unique interacting proteins are grouped in the meta-nodes depicted by rectangular structures. The enriched gene ontology categories are reported for each meta-node with shades based on the enrichment *P* value. The total number of proteins in each meta-node is also featured.

whether such an approach provides benefit in our animal study. It is also possible that the lack of rapid weight gain could potentially play a role in reducing the degree of progressive kidney disease; however, it is unlikely that this would be the major reason for the PFD-induced improvement, because the mice did continue to gain weight while on the drug. Clearly, for advancing the progress of testing novel clinical therapies, new biomarkers related to ongoing renal fibrosis and inflammation are urgently needed.

As an initial attempt to identify novel biomarkers that would be relevant to PFD treatment of diabetic kidney disease and to understand the potential mechanism of renoprotection, we performed a proteomic screen of the kidneys in our study with *db/db* mice. Identification of proteins uniquely present in the PFD-treated kidneys demonstrated that several were involved in mRNA translation. Gene ontology and interaction analyses of the human orthologs of PFD-unique mouse proteins revealed a network comprising 518 proteins and 655 interactions and an interplay between hnRNPH and SFRS4. hnRNPH2 binds heterogeneous nuclear RNA and has been shown to have either exon-enhancing or -silencing activity, depending on the context of the binding site.[54,55] SRFS4 binds to pre-mRNA transcripts and components of the spliceosome and can either activate or repress splicing, depending on the location of the pre-mRNA binding site. Its ability to activate splicing is regulated by phosphorylation and interactions with other splicing factor–associated proteins (http://harvester.fzk.de/harvester/human).[56–58]

Several studies have previously linked DN to dysregulation of mRNA translational processing,[32,33,59,60] therefore we postulated that the effect of PFD on induction of mRNA processing pathways may underlie its beneficial effects in diabetic kidney disease. One of the key proteins involved in mRNA translation is eIF4E, which binds to the 5′ cap structure of mRNA. Phosphorylation increases the binding of eIF4E to capped mRNA *in vitro* and results in increased protein synthesis.[34] In mesangial cells, PFD inhibited the eIF4E phosphorylation in a dosage-dependent manner. These data strongly suggest that PFD modulates mRNA/protein synthesis. Interestingly, eIF4E phosphorylation is stimulated by high glucose.[33] The new data are the first evidence that PFD can potentially regulate mRNA translation of proteins, and the proteomic and network analysis suggests that this pathway may be of major significance in diabetic kidney disease.

In summary, PFD has beneficial effects in cell culture systems to reduce TGF-β production and activity. PFD also has potent anti-inflammatory effects as a result of its ability to block ROS production in mesangial cells. Administration of oral PFD led to a dramatic reduction of glomerular mesangial matrix with a short course of therapy after the onset of established disease in the *db/db* mouse model. As numerous candidate pathways are affected by PFD, further studies using unbiased approaches are necessary to establish the dominant pathways by which PFD may confer renoprotective benefits. These insights will be crucial to best determining the clinical monitoring of patients with progressive kidney disease and improve the likelihood of rapid

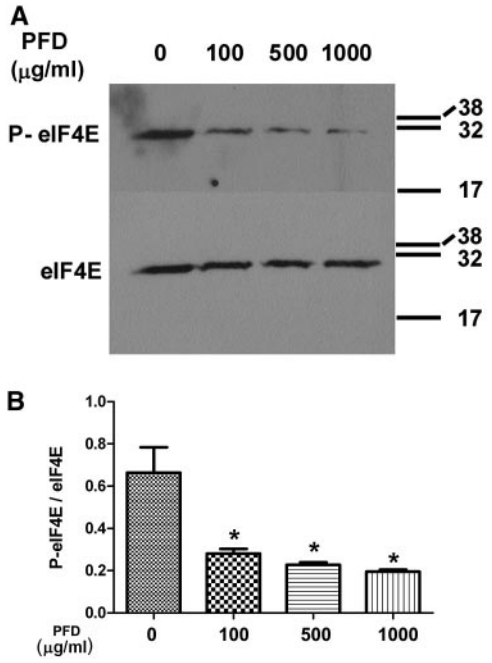

Figure 8. (A) Effect of PFD dosage on phosphorylation of eIF4E protein. As shown in this Western blot, PFD decreased eIF4E phosphorylation in a dosage-dependent manner. (B) Quantification of P-eIF4E/total eIF4E from immunoblots in A ($n = 4$). Data are means ± SEM. *$P < 0.05$ PFD treatment *versus* control.

advancement of novel clinical therapies focused on reducing inflammation and fibrosis.

CONCISE METHODS

Cell Culture and Transfection Studies

MMCs and HEK293 cells were used in cell culture studies. MMCs were originally isolated from kidneys of SJL/J(H-2) normal mice and transformed with non–capsid-forming SV-40 virus to establish a permanent cell line.[61] MMCs were maintained at 37°C in a humidified incubator with 5% CO_2/95% air and propagated in DMEM (Life Technologies BRL, Gaithersburg, MD) containing 10 mM D-glucose, 10% FCS, 100 U/ml penicillin, 100 mg/ml streptomycin, and 2 mM supplemental glutamine. HEK293 cells were obtained from American Type Culture Collection (ATCC) (Manassas, VA) and cultured in DMEMG450/10% FCS medium. For transient transfection studies, MMCs or HEK293 cells were transfected using Lipofectamine2000 (Invitrogen, Carlsbad, CA). Plasmids used included the glucose-responsive TGF-β1 promoter-reporter construct pA835-luciferase (pA835-luc),[21] the 3TP-lux TGF-β–sensitive plasmid, and a plasmid for β-galactosidase (pLENXZ) to standardize for transfection efficiency. After transfection, cells were treated with varying dosages of PFD (100 to 1000 μg/ml) for up to 24 h. Cells were then harvested, and luciferase and β-galactosidase activity was measured, as described previously.[21]

TGF-β1 Protein Level Measurement by ELISA

TGF-β1 protein level was measured by double-sandwich ELISA (Quantikine; R&D Systems, Minneapolis, MN) in the conditioned medium of MMCs after treatment with varying dosages of PFD (100 to 1000 μg/ml). TGF-β1 levels were factored for total cell protein as described previously.[21]

Western Blot Analysis

MMCs were grown in six-well plates with DMEM/10% FCS until 80% confluence. Cells were then serum-starved for 16 h and modulated with various dosages of PFD before TGF-β1 treatment. The total protein was isolated by cell lysis buffer (50 mM Tris [pH 7.2], 150 mM NaCl, 1% TritonX-100, 1 mM EDTA, and protease inhibitor cocktail). For phospho-Smad2 detection, 40 μg of total protein was electrophoresed on 10% SDS gel and transferred to the nitrocellulose membrane, blocked, and incubated with pSmad2 antibody at 1:500 dilution. Enhanced chemiluminescence by horseradish peroxidase–tagged secondary antibody enabled visualization of the protein band as described previously.[63] The membranes were subsequently stripped and immunoblotted with total Smad2/3 (Santa Cruz Biotechnology, Santa Cruz, CA). Immunoblotting for phospho-eIF4E was carried out as already described, followed by total eIF4E immunoblotting (Cell Signaling Technology, Danvers, MA).

ROS Quantification by Amplex Red Assay

As a quantitative index of ROS generation, the Amplex Red reagent (10-acetyl-3,7-dihydroxyphenoxazine; Amplex Red Hydrogen Peroxide/Peroxidase Assay Kit; Molecular Probes, Eugene, OR) was used, as described previously.[22] MMCs cultured on 96-well black plates were rested overnight and modulated with PFD and/or TGF-β. Fluorescence intensity was kinetically recorded with excitation at 544 nm and emission at 590 nm at 37°C over a 20-cycle period with 10 flashes per well *via* fluorescence plate reader (POLARstar OPTIMA; BMG Labtechnologies, Durham, NC). Measurements were made at 1-min intervals during a 20-min period. The data are reported as the mean value from each well during a 20-cycle period with $n = 6$ wells per experiment. Each experiment was repeated three times.

Interventional Animal Studies

Five- to 6-wk-old male, homozygous, obese KSJ *db/db* mice (*Lepr-db/db*; Jackson Laboratories, Bar Harbor, ME) and the corresponding heterozygote lean controls (*db/m*) were obtained. Mice were housed four to five per cage in micro-isolators with bedding changes two or three times per week. Animal studies were approved by the institutional animal care and use committee at Thomas Jefferson University. At week 17, the *db/db* mice were randomly assigned to two test groups: *db/db* untreated and *db/db* treated with PFD. The *db/db* group that was randomized to PFD treatment had 0.5% PFD added to their food at this time, and the mice were individually caged. PFD was provided by Dr. Solomon Margolin (Marnac, Dallas, TX). Mice that were fed PFD took in approximately 25 mg/d PFD and were treated for 4 wk total from week 17 to week 21. At the end of 21 wk, mice underwent 24-h urine collections in metabolic cages and were killed. Urine albumin:creatinine was performed with ELISA specific for albumin (Exocel, Philadelphia, PA) and standardized by urine creatinine (Nova

16CRT) as described previously. The left kidney was snap-frozen in liquid nitrogen for RNA analysis and proteomic analysis. The right kidney was fixed using phosphate-buffered formalin for further histopathology studies to determine degree of glomerular matrix accumulation.

RNA Isolation and Analysis

Total RNA was extracted from mouse kidneys using the RNeasy Mini Kit (Qiagen, Valencia, CA) according the manufacturer's instructions. Real-time PCR was performed as described previously[64,65] using the primers for α1-type I collagen, α1-type IV collagen, fibronectin, and 18S as listed in the Supplemental Methods.

Histopathology

The right kidney was sliced sagittally and placed in buffered formalin. After paraffin embedding, 4-μ sections were stained with PAS. Fifty glomeruli were evaluated for each kidney. The histologic specimens from both treated and nontreated *db/db* mice as well as controls from *db/m* mice were scored using a semiquantification scale from 1 to 4 as previously reported.[66] Morphometry of sections of kidneys from each of the *db/m*, *db/db*, and PFD-treated *db/db* mice was carried out as previously reported.[67] Twenty-five randomly selected glomeruli in the outer cortex of each kidney section were evaluated in a blinded manner.

Proteomic Analyses of the *db/m*, *db/db*, and PFD-Treated *db/db* Mouse Kidney Proteins

In solution, trypsinization of the mouse kidney proteins was carried out as follows: 10 μg of kidney proteins was taken in a final reaction volume of 100 μl, reduced by addition of dithiothreitol in 100 mM ammonium bicarbonate to a final concentration of 20 mM dithiothreitol, incubated at 50°C for 1 h, and then alkylated with 20 mM iodoacetamide at room temperature in the dark for 1 h. Assuming 10 μg of protein per sample and 1/50th of trypsin (wt/wt), 10 μl of trypsin solution (concentration 10 ng/μl in 100 mM ammonium bicarbonate solution) was added to each protein tube and digested for 24 h at 37°C, and subsequently the pH was adjusted to approximately 2 to 3 with 5% formic acid. The reaction mixture solvent resulting from in-solution trypsinization was solvent-evaporated to dryness. The dried down tryptic digests were resuspended in 15 μl of 1% formic acid. A total of 10 μl of this peptide preparation was loaded onto a Thermo Hypersil-Keystone BioBasic C18 column (0.18 $\times$ 100 mm) that served as the Surveyor HPLC front end of a ThermoElectron ProteomeX Workstation. The peptides were separated at a flow rate of 175 nl/min, using a gradient with Buffer A (0.1% formic acid in water), Buffer B (0.1% formic acid in acetonitrile), and a 2 to 50% gradient over 45 min. As peptides eluted from the column, they were subjected to ESI-Ion-trap MS in an LCQ DecaXP Plus electrospray-ion-trap MS workstation. Single full MS scan to determine the masses of analytes and MS/MS of the three biggest peaks from the preceding full scan were performed to yield the sequence information of the peptide. This entire process was repeated every 15 s during the gradient.

Bioinformatic Analysis of Proteomic Data

The peptide sequence data from MS/MS spectra were analyzed using the SEQUEST algorithm to determine the best protein matches. For obtaining reliable protein identification, Human Proteome Organization directives were used: (*1*) Only peptides with a ΔCn score of >0.1 were qualified; (*2*) the cross-correlation scores of single, double, and triple charged peptides had to be greater than 1.9, 2.2, and 3.75, respectively; and (*3*) rank of preliminary scoring value had to be ≤ 4. In addition, a peptide had to be a tryptic cleavage to be accepted, and, as modifications, the oxidation of methionine and carbamidomethylation of cysteine were permitted.

Statistical Analysis

For continuous variables, a two-tailed ANOVA was used to test means. Histology of mesangial matrix and arteriolar hyalinosis was evaluated using frequencies in a two-tailed Fisher exact test. For proteomics data analyses, total protein from three different mouse kidneys from each of the three populations (1, *db/m* control; 2, *db/db* without treatment; and 3, *db/db* treated with PFD) were separately analyzed by LC/MS/MS as described, and only the statistically significant identifications common to all mice ($P < 0.05$) from any single group were qualified for subsequent analysis.

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DISCLOSURES

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