Renoprotective mechanisms of pirfenidone in hypertension-induced renal injury: through anti-fibrotic and anti-oxidative stress pathways

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ABSTRACT

Pirfenidone (PFD) is a novel anti-fibrotic agent that targets TGFβ. However, the mechanisms underlying its renoprotective properties in hypertension-induced renal injury are poorly understood. We investigated the renoprotective properties of PFD and clarified its renoprotective mechanisms in a rat hypertension-induced renal injury model. Dahl salt-sensitive rats were fed a high-salt diet with or without 1% PFD for 6 weeks. During the administration period, we examined the effects of PFD on blood pressure and renal function. After the administration, the protein levels of renal TGFβ, Smad2/3, TNFα, MMP9, TIMP1, and catalase were examined. In addition, total serum antioxidant activity was measured. Compared to untreated rats, PFD treatment significantly attenuated blood pressure and proteinuria. Histological study showed that PFD treatment improved renal fibrosis. PFD may exert its anti-fibrotic effects via the downregulation of TGFβ-Smad2/3 signaling, improvement of MMP9/TIMP1 balance, and suppression of fibroblast proliferation. PFD treatment also increased catalase expression and total serum antioxidant activity. In contrast, PFD treatment did not affect the expression of TNFα protein, macrophage or T-cell infiltration, or plasma interleukin 1β levels. PFD prevents renal injury via its anti-fibrotic and anti-oxidative stress mechanisms. Clarifying the renoprotective mechanisms of PFD will help improve treatment for chronic renal diseases.
12, 13, 20). Oxidative stress is related to progressive renal injury (28). PFD is reported to exert anti-oxidative stress in mesangial cells and in a cirrhosis model (27, 30). Therefore, the anti-inflammatory and anti-oxidative stress effects of PFD are also expected to ameliorate renal injury. At present, although some renoprotective mechanisms of PFD have been reported, no systematic study has clarified the renoprotective mechanisms of PFD. Furthermore, the mechanisms underlying the renoprotective action of PFD in hypertension-induced renal injury are poorly understood. Therefore, the present systematic study investigated the anti-fibrotic, anti-inflammatory, and anti-oxidative stress effects of PFD. To elucidate the mechanisms underlying the renoprotective action of PFD, we administered PFD to Dahl salt-sensitive rats, and measured blood pressure and renal function. We also investigated the effects of PFD on TGFβ protein levels as well as its upstream and downstream signaling pathways. The PFD-induced protein expression changes of MMP9 and TIMP1 were examined. The effects of PFD on fibroblast proliferation were also investigated by immunohistochemistry. In addition, the effects of PFD on inflammatory factors such as macrophage and T-cell infiltration, TNFα protein expression, and plasma levels of IL1β were examined. Finally, we investigated catalase protein expression and total serum antioxidant activity.

MATERIALS AND METHODS

Experimental animals. Dahl salt-sensitive rats were obtained from SLC Japan (Shizuoka, Japan). The rats were housed in a temperature-controlled pathogen-free room with light from 0700 to 1900 h (daytime) and had free access to food and water. Experiments were performed in accordance with the guidelines of the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and the experimental protocols were approved by the National Cerebral and Cardiovascular Center for the Care and Use of Experimental Animals.

Renoprotective effects of PFD in Dahl salt-sensitive rats. Dahl salt-sensitive rats were fed a high-salt diet [8% NaCl (w/w); Oriental Yeast, Tokyo, Japan] from 4.5 weeks of age. PFD-treated Dahl salt-sensitive rats (PFD group, n = 10) were fed the high-salt diet with mixed with 1% PFD (approximately 700 mg kg⁻¹ day⁻¹). Control Dahl salt-sensitive rats (Control group, n = 10) were administered only the high-salt diet. Rats were treated with PFD for 6 weeks. PFD was provided by Shionogi (Osaka, Japan).

Blood pressure was measured once every 2 weeks using a tail-cuff method (BP-98A; Softron, Tokyo, Japan). Rats were housed in metabolic cages once every 2 weeks for 24-h urine collection. Urinary protein levels were measured using a BCA protein assay kit (Thermo Scientific, Rockford, IL, USA). Creatinine clearance (C_Cr) at week 6 of administration was calculated using the following formula: C_Cr = (U_Cr × V) / P_Cr; U_Cr is the concentration of urinary creatinine (mg • mL⁻¹), P_Cr is the concentration of plasma creatinine (mg • mL⁻¹), and V is the urine flow rate (mL • min⁻¹). Creatinine levels were measured using a QuantiChrom creatinine assay kit (DIUR-500; BioAssay Systems, Hayward, CA, USA).

Measurement of TGFβ mRNA in the Dahl salt-sensitive rat kidneys. After 6 weeks with or without PFD treatment, the rats were anesthetized with pentobarbital (25 mg • kg⁻¹). Venous blood was collected from the vena cava. Plasma (EDTA as an anticoagulant) and serum were isolated by centrifugation and stored at −80°C until measurement. The left kidneys of the rats were removed, weighed, and sectioned longitudinally. Half of each left kidney was frozen in liquid nitrogen and stored at −80°C until measurement, while the other half was used to isolate RNA with TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions. TGFβ mRNA expression levels were determined by real-time RT-PCR using a commercial kit (Rn01442102_m1; Applied Biosystems, Foster City, CA, USA) and normalized to the expression of β-actin mRNA; these measurements were expressed as log₁₀ (2^35-CT1 / 2^25-CT2), where 2^35-CT1 and 2^25-CT2 correspond to the expression levels of TGFβ and β-actin mRNA, respectively, as described previously (17).

Western blotting. Western blot analysis was performed using the extracts from rat kidneys frozen in liquid nitrogen and homogenized in 1% NP-40 lysis buffer as described previously (15). Equal amounts of protein (40 μg) were separated by SDS-PAGE (10% gels) and then transferred to a nitrocellulose membrane (Hybond ECL, 0.22 μm; GE Healthcare, Buckinghamshire, UK) or a PVDF membrane (Hybond-P; 0.45 μm; GE Healthcare). After blocking with 5% (w/v) skim milk powder, the membranes were incubated overnight at 4°C with primary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA) against TGFβ, Smad2/3, phosphorylated Smad2/3 (pSmad2/3), chromosome 3p kinase (3pK), homeodomain-interacting protein kinase 2 (HIPK2),...
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Statistical analysis. Values are expressed as mean ± standard error of the mean (SEM). Statistical analyses were performed using JMP (SAS Institute, Cary, NC, USA). Analysis of variance (ANOVA) was used to estimate differences among groups, and the significance of differences was tested using Student’s t-test.

RESULTS

Effects of PFD treatment on body weight, urinary protein, Ccr, heart rate, and blood pressure

Between the Control and PFD groups, there were no significant differences in body weight (Fig. 1A) and food intake (data not shown). Neither urine volume (Fig. 1B) nor water intake (data not shown) was significantly different between the Control and PFD groups. In contrast, urinary protein excretion was significantly attenuated at weeks 2 and 4 after the PFD treatment, indicating that PFD exerted a renoprotective effect (Fig. 1C). Although a trend demonstrating the improvement of Ccr was observed after 6 weeks of PFD treatment (Fig. 1D), there was no significant difference between the Control and PFD groups (1.58 ± 0.22 vs. 2.93 ± 0.66 mL • min⁻¹, respectively; *P* = 0.13).

PFD treatment did not affect heart rate as measured by the tail-cuff method (Fig. 1E). Six weeks of 8% NaCl diet feeding increased systolic blood pressure (SBP) in the Control group significantly. PFD treatment significantly attenuated this increase in SBP at weeks 2 and 4 after the PFD treatment, indicating that PFD exerted a renoprotective effect (Fig. 1C). Although a trend demonstrating the improvement of Ccr was observed after 6 weeks of PFD treatment (Fig. 1D), there was no significant difference between the Control and PFD groups (1.58 ± 0.22 vs. 2.93 ± 0.66 mL • min⁻¹, respectively; *P* = 0.13).

From the results described above, we conclude that PFD treatment ameliorates hypertension and renal injury.

Effects of PFD treatment on renal weight and fibrosis

There were no significant differences in renal weight between the Control and PFD groups at week 6 (Fig. 2A). Six weeks of 8% NaCl diet feeding caused tubular dilatation, interstitial fibrosis, and glomerular sclerosis (Fig. 2C); PFD treatment significantly attenuated these pathological changes (Fig. 2D). PFD treatment markedly reduced fibrotic areas (Fig. 2B).

Plasma IL1β measurement.

Plasma IL1β was measured in duplicate with an enzyme-linked immunosorbent assay (ELISA) kit (IBL, Gunma, Japan).

Measurement of total serum antioxidant activity.

To evaluate reactive oxygen species, we determined the total serum antioxidant activity by using the total antioxidant power colorimetric microplate assay kit (Oxford Biomedical Research, Oxford, MI, USA), which measures the reductive capacity by detecting the reduction of Cu²⁺ to Cu⁺.
PFD treatment also attenuated the ratio of pSmad2/3 and total Smad2/3 (Control vs. PFD treatment, 1.40 ± 0.16 vs. 0.83 ± 0.11, respectively; \( P = 0.002 \)). These results confirm that PFD treatment does not affect total Smad2/3 production but decreases Smad2/3 activity.

TGFβ also activates Smad-independent signaling pathways of renal TGFβ compared to the Control group. TGFβ activates a unique signal transduction pathway that acts via the Smad family of proteins (9, 27). In the present study, PFD treatment did not affect the protein expression of total Smad2/3 (Fig. 3C). However, PFD treatment significantly attenuated pSmad2/3 protein expression in the kidneys (Fig. 3D). PFD treatment also attenuated the ratio of pSmad2/3 and total Smad2/3 (Control vs. PFD treatment, 1.40 ± 0.16 vs. 0.83 ± 0.11, respectively; \( P = 0.002 \)). These results confirm that PFD treatment does not affect total Smad2/3 production but decreases Smad2/3 activity.

TGFβ also activates Smad-independent signaling

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Fig. 1 Effects of PFD treatment on body weight, urinary protein, Ccr, heart rate, and blood pressure. PFD-treated Dahl salt-sensitive rats (PFD group, \( n = 10 \)) were fed a high-salt diet mixed with 1% PFD for 6 weeks. Control Dahl salt-sensitive rats (Control group, \( n = 10 \)) were administered only the high-salt diet. Heart rate and SBP were measured by the tail-cuff method. There were no significant differences with respect to body weight (A), urine volume (B), Ccr (D), or heart rate (E) between the Control and PFD groups. Six weeks of the 8% NaCl diet significantly increased urinary protein excretion (C) and SBP (F) in the Control group; PFD treatment significantly attenuated these increases in urinary protein excretion (C) and SBP (F) at weeks 2 and 4. **\( P < 0.01 \), ***\( P < 0.001 \), significantly different from the Control group (unpaired Student’s \( t \)-test).
pathways, including MAPK-family proteins (9). Also known as MAPKAPK3, 3pK is activated by three members of the MAPK family: extracellular signal-regulated kinase (ERK), p38MAPK, and Jun N-terminal kinase (JNK) (22). In the present study, renal 3pK protein levels were not significantly different between the Control and PFD groups (Fig. 3E). HIPK2 regulates renal fibrosis by activating the TGFβ pathway (18). In the present study, there was no significant difference between the Control and PFD groups with respect to HIPK2 protein expression (Fig. 3F).

From the results described above, we conclude that PFD exerts its renoprotective effect via downregulation of the TGFβ and Smad2/3 signaling pathways.

Effects of PFD treatment on renal MMP/TIMP balance
MMP9 and its main inhibitor, TIMP1, play important roles in renal fibrosis (11, 21, 23). Nephritis-induced renal injury is more severe in MMP9-deficient mice than wild-type mice (21). Moreover, the significant rise of TIMP1 expression was attenuated by PFD in calcineurin inhibitor-induced nephrotoxicity (6). Therefore, the aim of the present study was to evaluate the effects of PFD treatment on renal MMP9/TIMP1 balance in hypertensive renal injury. Compared to the Control group, PFD treatment significantly increased renal MMP9 protein expression (Fig. 4A), whereas it significantly decreased that of renal TIMP1 (Fig. 4B).

Effects of PFD treatment on renal proliferation
FSP1, also called S100A4, is a specific fibroblast marker (37). In the present study, we did not observe significant suppression of fibroblast proliferation in renal interstitium after PFD treatment; we observed PFD treatment significantly attenuated the number of FSP1-positive cells in the fibrosis areas (Fig. 5).
sion (2, 12, 13, 20). However, we did not observe significant attenuation of renal TNFα protein expression (Fig. 6G) or plasma IL1β levels (Fig. 6H) after PFD treatment using Western blotting and ELISA, respectively.

**Effects of PFD treatment on antioxidant activity**

PFD was reported to have exhibited anti-oxidative stress effects in mesangial cells and in a cirrhosis model (27, 30). In the present study, a total antioxi-
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3pK protein levels were not significantly different between the Control and PFD groups. The uniqueness of 3pK is due to it being a novel convergence point of three MAPK pathways: ERK, p38MAPK, and JNK (22). From the results described above, we conclude that

1) PFD treatment decreases renal TGFβ expression, which may mediate the downregulation of Smad2/3 activity; and
2) the Smad-independent signaling pathways of ERK, p38MAPK, and JNK are not affected by PFD treatment upon renal injury and fibrosis. Recent reports also indicate that TGFβ and its downstream Smad pathway play important roles in renal diseases (4, 26, 31). In particular, PFD inhibits total Smad2/3 protein expression and TGFβ-induced Smad2 phosphorylation in murine mesangial cells (27). The balance between MMPs and TIMPs is also influenced by TGFβ in the kidneys (8, 10, 25). Furthermore, TGFβ stimulates fibroblast proliferation in the renal interstitium via a Smad-independent signaling pathway (39). From the results of the present and previous studies, we conclude that TGFβ is a potential central regulator of renal fibrosis. HIPK2 is reported to be a key regulator of renal fibrosis via the activation of the TGFβ-Smad3 pathway (18). However, PFD treatment did not affect HIPK2 protein expression in the kidneys in this study. This result suggests PFD treatment does not affect the TGFβ upstream signaling of HIPK2.

TIMP1 is the natural inhibitor of MMP9; both of these proteins play pivotal roles in kidney diseases. Therefore, disturbances in the balance of two systems lead to renal fibrosis (11, 21, 23). Nephritis-induced renal injury is more severe in MMP9-deficient mice than wild-type mice, which indicates that MMP9

**DISCUSSION**

PFD is a novel anti-fibrotic agent that inhibits the progression of renal fibrosis (1, 5–7, 24, 27, 34–36, 38). PFD has demonstrated anti-fibrotic, anti-inflammatory, and anti-oxidative stress effects in animal models and patients with renal diseases (6, 12–14, 27, 36). In the present study, we observed that PFD treatment decreased SBP, reduced proteinuria, and ameliorated renal fibrosis in a rat model of hypertensive renal injury.

TGFβ is a key protein in renal fibrosis (3, 4, 33), and PFD is an antagonist of TGFβ. PFD also decreases TGFβ expression in several animal nephropathy models (1, 5–7, 24, 27, 34–36, 38). In the present study, we also observed that PFD treatment decreased renal TGFβ mRNA and protein levels. TGFβ frequently modulates the transcription of key target genes via the Smad signaling pathway, which directly transduces TGFβ receptor activation to the nucleus (4, 9, 26, 31). In addition, Smad-independent signaling pathways via ERK, p38MAPK, and JNK provide alternative gene-activation mechanisms (9, 20).

In the present study, we observed that PFD treatment decreased Smad2/3 activity. In addition, renal 3pK protein levels were not significantly different between the Control and PFD groups. The uniqueness of 3pK is due to it being a novel convergence point of three MAPK pathways: ERK, p38MAPK, and JNK (22). From the results described above, we conclude that

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**Fig. 4** Effects of PFD treatment on renal MMP/TIMP balance. PFD treatment significantly increased renal MMP9 protein expression (A). Renal TIMP1 protein levels in the PFD group were significantly lower than that in the Control group (B). (n = 10 for Control and PFD groups)
Fig. 5 Effects of PFD treatment on renal fibroblast proliferation. Immunohistochemical examination revealed fibroblasts, which were identified by positive staining for anti-FSP1 (a specific marker of fibroblasts) antibody. FSP1-positive cells were counted and compared between the Control and PFD groups. There were significantly more fibroblasts in the kidneys of the Control group (A) than the PFD group (B). Average numbers of immune cells per square millimeter in the Control (n = 10) and PFD (n = 10) groups (C). Fibroblast proliferation was significantly attenuated in the PFD group compared to the Control group.

Fig. 6 Effects of PFD treatment on renal inflammatory reaction. Immunohistochemical examination revealed macrophages (A and B) and T cells (D and E), which were identified by positive staining for anti-CD68 and anti-CD3 antibodies, respectively. The numbers of cells positive for CD68 and CD3 were counted and compared between the Control and PFD groups. PFD treatment did not markedly attenuate macrophage (C) or T-cell (F) infiltration. Western blotting analysis also showed that PFD treatment did not significantly attenuate renal TNFα protein expression (G). ELISA revealed that PFD treatment did not affect the plasma level of IL1β (H). (n = 10 for Control and PFD treatment groups)
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Ever, if the injury continues toward end-stage renal disease, the anti-inflammatory effect of PFD is insufficient. Furthermore, the anti-fibrotic effect of PFD is the main mechanism that improves renal function.

Oxidative stress is also involved in progressive renal injury (28). Catalase deficiency promotes oxidant renal injury and fibrosis in mice (19). Moreover, PFD inhibited the production of reactive oxygen species in mesangial cells (27). We also observed that PFD treatment significantly increased renal catalase expression and total serum antioxidant activity. Therefore, we conclude that the anti-oxidative stress effect of PFD ameliorates renal injury and fibrosis.

Renal fibrosis can be induced via several mechanisms in different renal diseases but it ultimately produces identical fibrotic changes in the kidneys. In hypertension-induced renal injury, the therapeutic mechanisms of PFD are mediated by its anti-fibrotic and anti-oxidative stress pathways, not its anti-inflammatory pathway. PFD exerts its anti-fibrotic effect via three mechanisms:

1) the downregulation of TGFβ-Smad2/3 signaling,
2) improvement of MMP9/TIMP1 balance, and
3) suppression of fibroblast proliferation (Fig. 8).

The association between renal inflammation and the development of renal fibrosis has been demonstrated in chronic kidney disease models (15, 16, 29). Moreover, PFD reduces the production of inflammatory mediators such as TNFα and IL1β (2, 12, 13, 20). Therefore, we also investigated the effects of PFD on inflammation in hypertension-induced renal injury. Unexpectedly, there were no significant differences between the Control and PFD groups with respect to macrophage or T-cell infiltration, namely TNFα protein expression or plasma levels of IL1β. From these results, we conjecture that PFD ameliorates the initial inflammation; however, if the injury continues toward end-stage renal disease, the anti-inflammatory effect of PFD is insufficient. Furthermore, the anti-fibrotic effect of PFD is the main mechanism that improves renal function.

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Fig. 8 The renoprotective mechanisms of PFD in hypertensive renal injury: through anti-fibrotic and anti-oxidative stress pathways. The therapeutic mechanisms of PFD on renal injury are mediated by its anti-fibrotic and anti-oxidative stress activities, not its anti-inflammatory activity. PFD exerts its anti-fibrotic effect via three pathways: (1) downregulation of TGFβ-Smad2/3 signaling, (2) improvement of MMP9/TIMP1 balance, and (3) suppression of fibroblast proliferation. TGFβ is a central regulator of renal fibrosis and is inhibited by PFD.

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