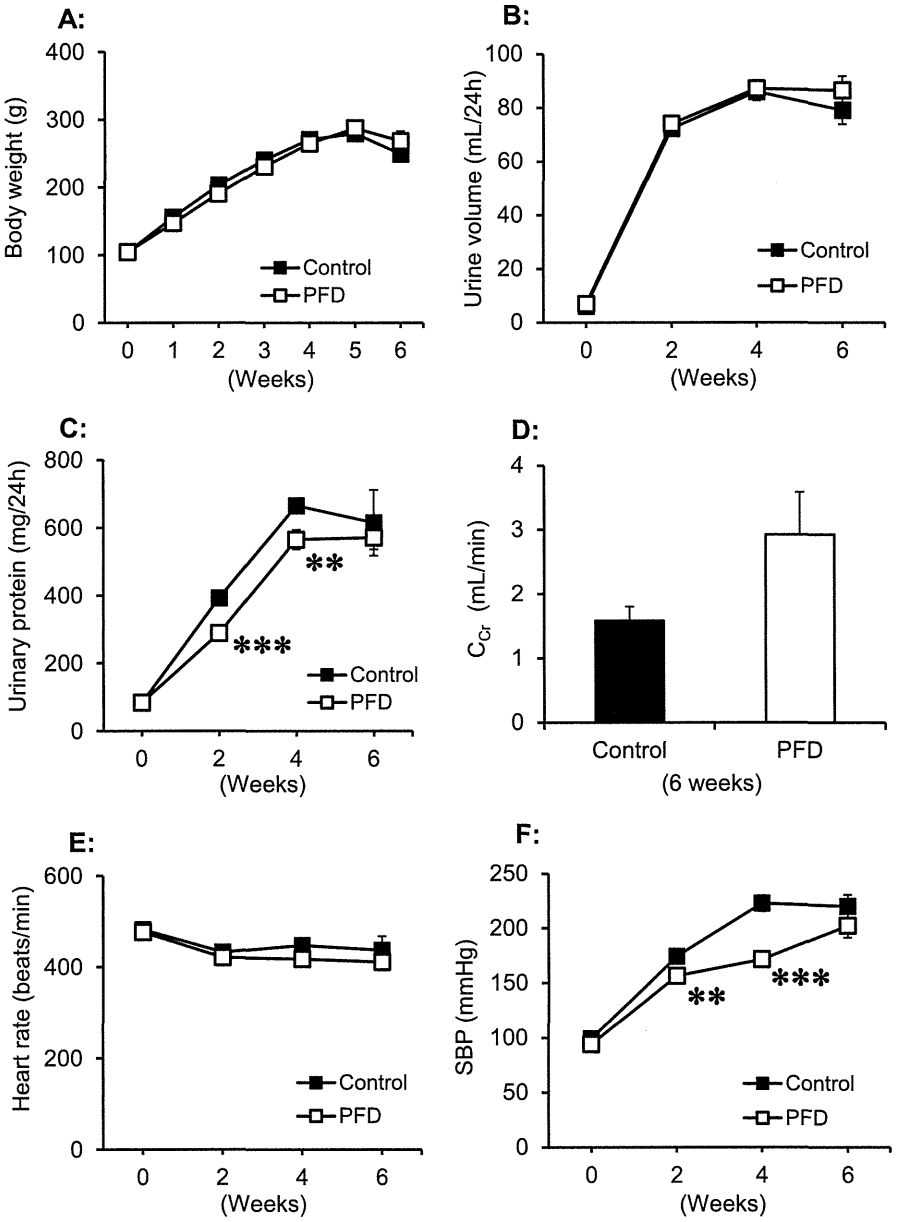

Fig. 1 Effects of PFD treatment on body weight, urinary protein, C_{Cr}, heart rate, and blood pressure. PFD-treated Dahl salt-sensitive rats (PFD group, n = 10) were fed a high-salt diet mixed with 1% PFD for 6 weeks. Control Dahl salt-sensitive rats (Control group, n = 10) were administered only the 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), C_{Cr} (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).

sions 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

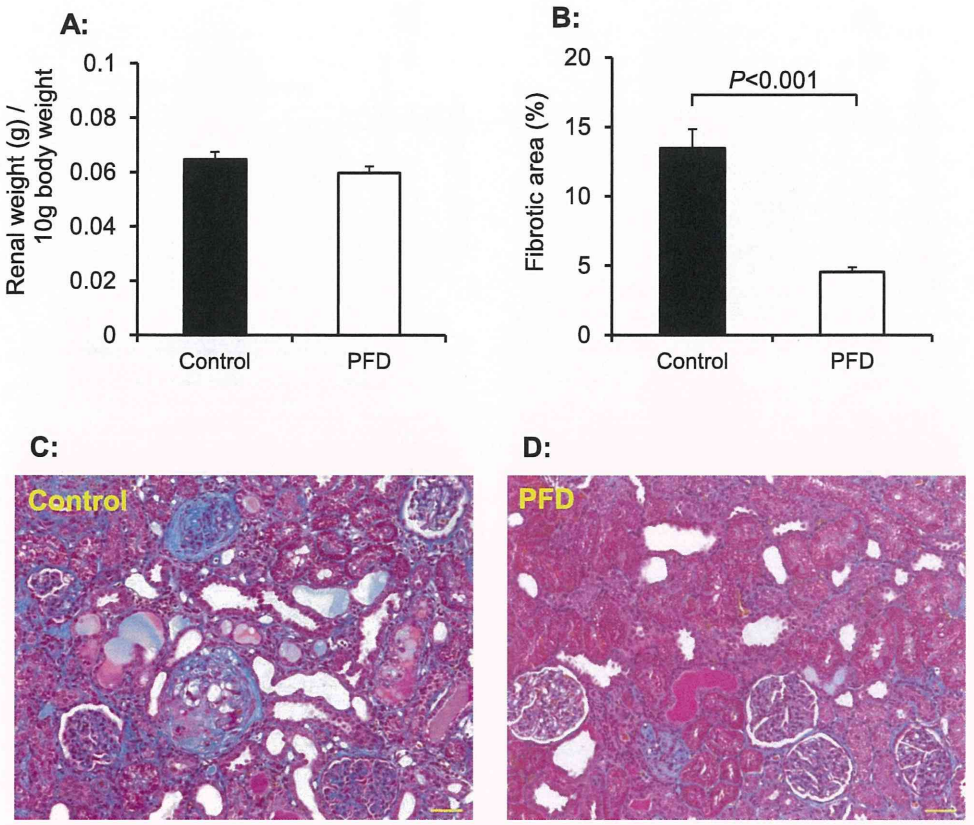

Fig. 2 Effects of PFD treatment on renal weight and fibrosis. (A) Relative renal weight in the Control (n = 10) and PFD (n = 10) groups at week 6; values represent renal organ weight per 10 g body weight. Renal weight was not significantly different between the Control and PFD groups. Representative images of the kidneys of the Control (C) and PFD (D) groups stained with Masson's trichrome exhibiting interstitial fibrosis and glomerular sclerosis (blue-colored area). PFD treatment markedly reduced the percentage of fibrotic areas (B).

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 down-regulation 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).

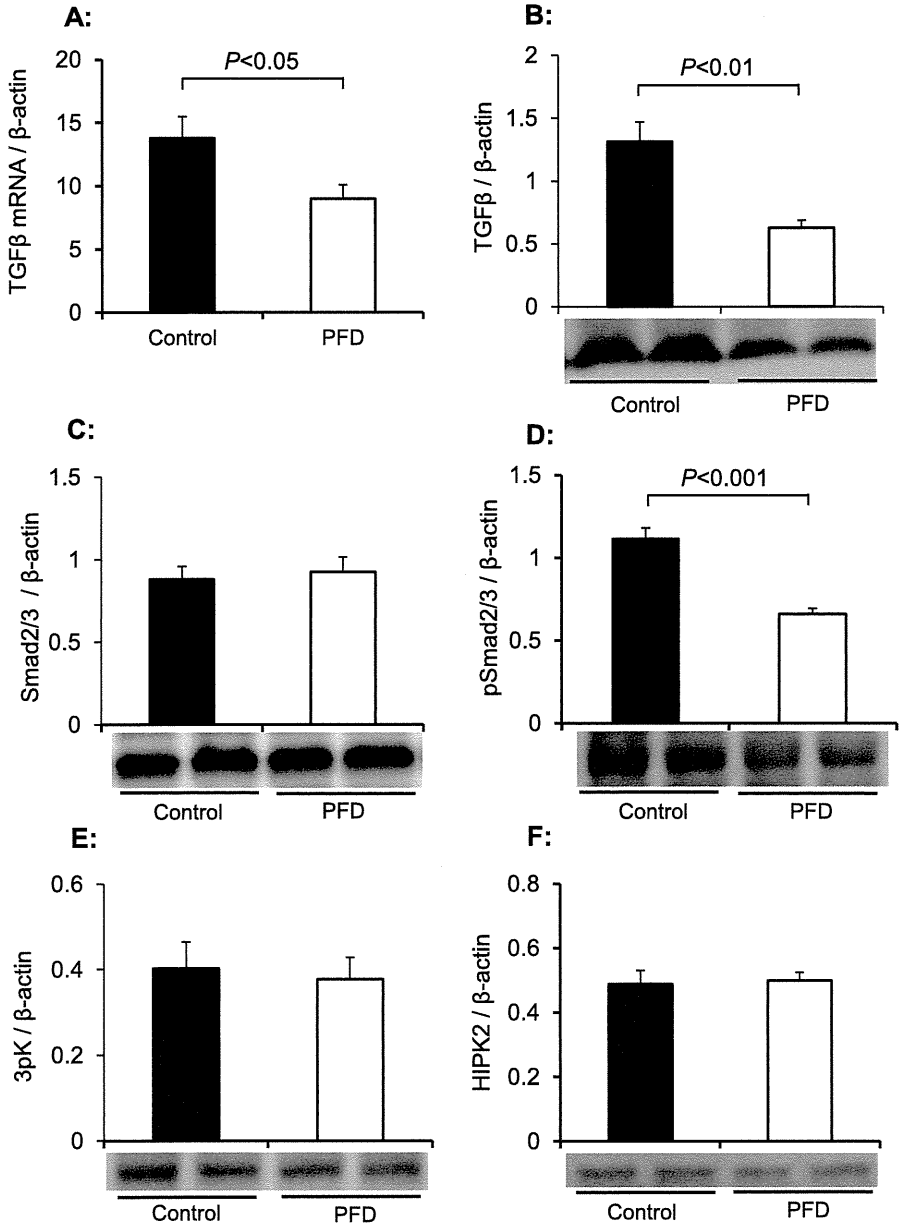

Fig. 3 Effects of PFD treatment on renal TGFβ, Smad2/3, pSmad2/3, 3pK, and HIPK2 expression. Expressions of TGFβ mRNA (A), TGFβ protein (B), Smad2/3 protein (C), pSmad2/3 protein (D), 3pK protein (E), and HIPK2 protein (F) in the kidneys of the Control and PFD groups. (n = 10 for Control and PFD groups)

Effects of PFD treatment on renal inflammatory reaction

To evaluate the renal inflammation caused by salt-induced hypertension, tissue sections of the kidneys were stained with CD68 (a macrophage marker) and CD3 (a T-cell marker). There were no significant differences between the Control and PFD groups with respect to macrophage (Fig. 6C) or T-cell infiltration (Fig. 6F).

PFD is reported to inhibit TNFα and IL1β expression (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-

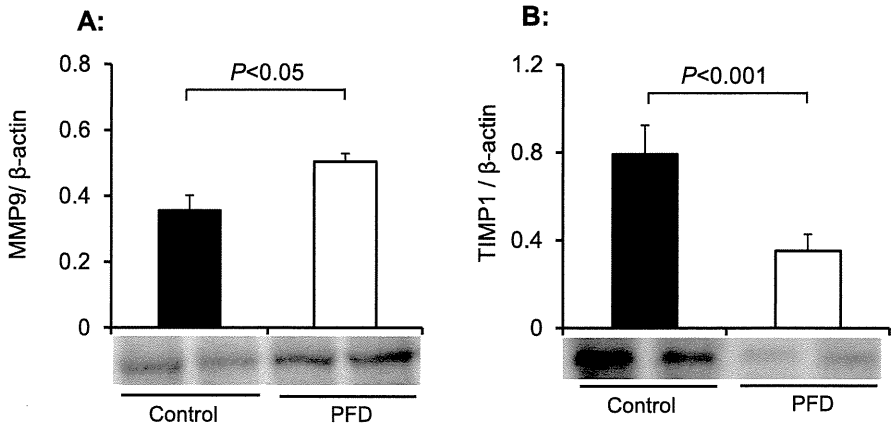

Fig. 4 Effects of PFD treatment on renal MMP/TIMP balance. PFD treatment significantly increased renal MMP9 protein expression (A). Renal TIMP1 protein levels in the PFD group were significantly lower than that in the Control group (B). (n = 10 for Control and PFD groups)

dant power kit was used to measure total serum antioxidant activity; we found that PFD treatment significantly increased the total antioxidant activity in serum (Fig. 7A; Control vs. PFD treatment, 10.17 ± 0.42 vs. 12.88 ± 0.76 nmol • mg protein^{-1}; $P < 0.05$). In addition, Western blotting revealed that PFD treatment significantly increased catalase protein expression in the kidneys (Fig. 7B).

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 *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

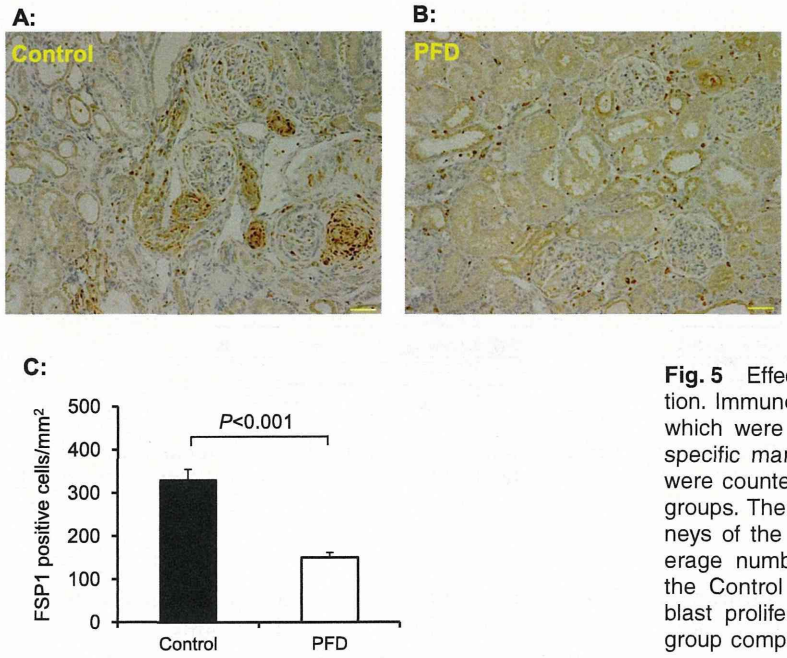

Fig. 5 Effects of PFD treatment on renal fibroblast proliferation. Immunohistochemical examination revealed fibroblasts, which were identified by positive staining for anti-FSP1 (a specific marker of fibroblasts) antibody. FSP1-positive cells were counted and compared between the Control and PFD groups. 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.

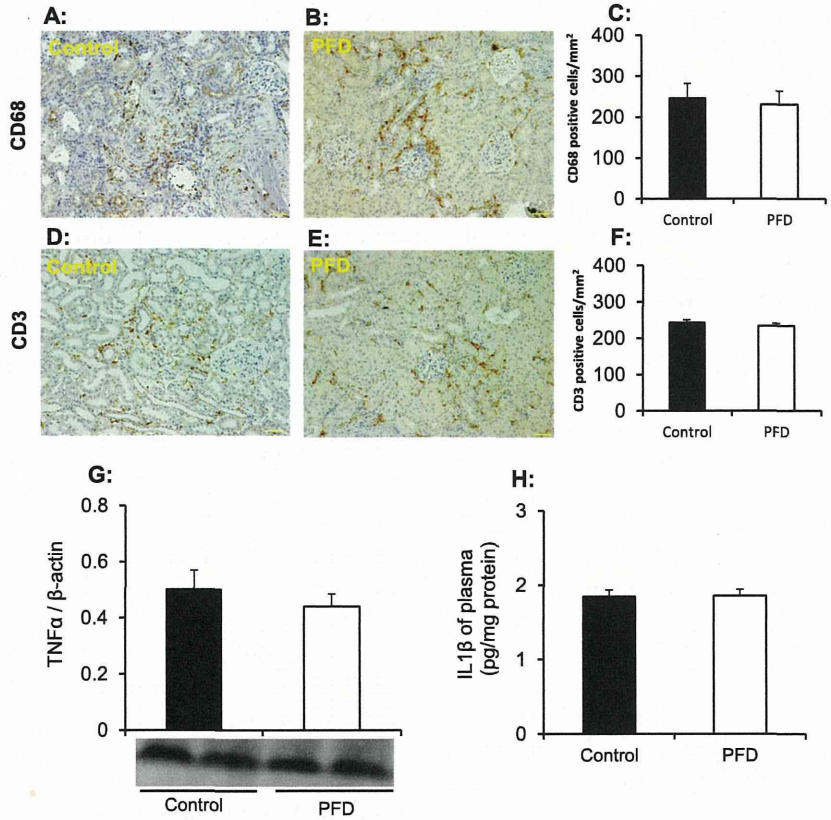

Fig. 6 Effects of PFD treatment on renal inflammatory reaction. Immunohistochemical examination revealed macrophages (A and B) and T cells (D and E), which were identified by positive staining for anti-CD68 and anti-CD3 antibodies, respectively. The numbers of cells positive for CD68 and CD3 were counted 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)

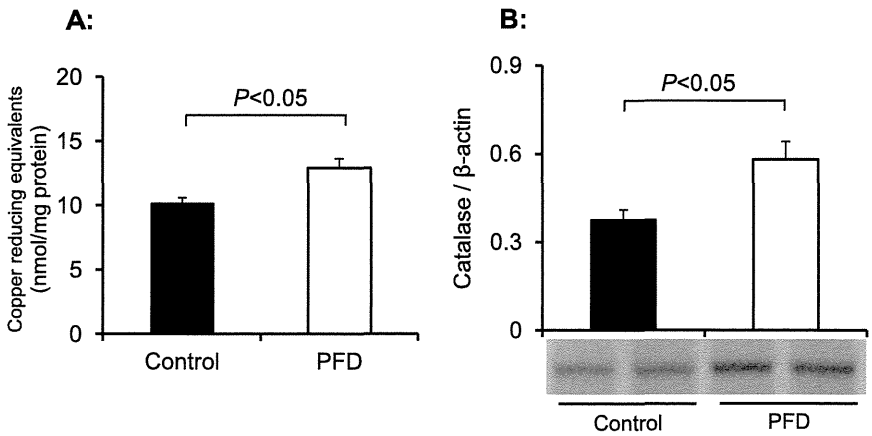

Fig. 7 Effects of PFD treatment on antioxidant activity. Total serum antioxidant activity was measured using the total antioxidant power kit. The activity level (expressed as copper-reducing equivalents) was significantly higher in the PFD group than the Control group after 6 weeks of high-salt diet intake (A). Western blotting showed that catalase expression was significantly higher in the PFD group than the Control group (B). (n = 10 for Control and PFD groups)

plays an important role in protecting renal function (21). Moreover, PFD affects MMP9/TIMP1 balance (6, 10, 20, 36). In the present study, PFD treatment increased MMP9 expression and decreased TIMP1 expression, suggesting that it may increase extracellular matrix degradation, leading to the attenuation of renal fibrosis.

PFD is also reported to attenuate renal fibroblast activation and proliferation *in vitro* (14). In the present study, PFD treatment significantly attenuated the number of FSP1-positive cells compared to the Control group. These results suggest that PFD treatment attenuates fibroblast proliferation; this may represent another possible mechanism of the anti-fibrotic effect of PFD.

From the results described above, we conclude that PFD exerts its anti-fibrotic effect via three mechanisms: *1)* 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.

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). TGFβ is a central regulator of renal fibrosis and is inhibited by PFD. Furthermore, PFD is a promising agent for the prevention of hypertensive renal injury. The clinical effectiveness of PFD has been evaluated in human diabetic nephropathy (32) and focal segmental glomerulosclerosis (7). Clarifying the renoprotective mechanisms of PFD will help us create new therapeutic strategies and could confer further benefits beyond the therapies currently used for the treat-

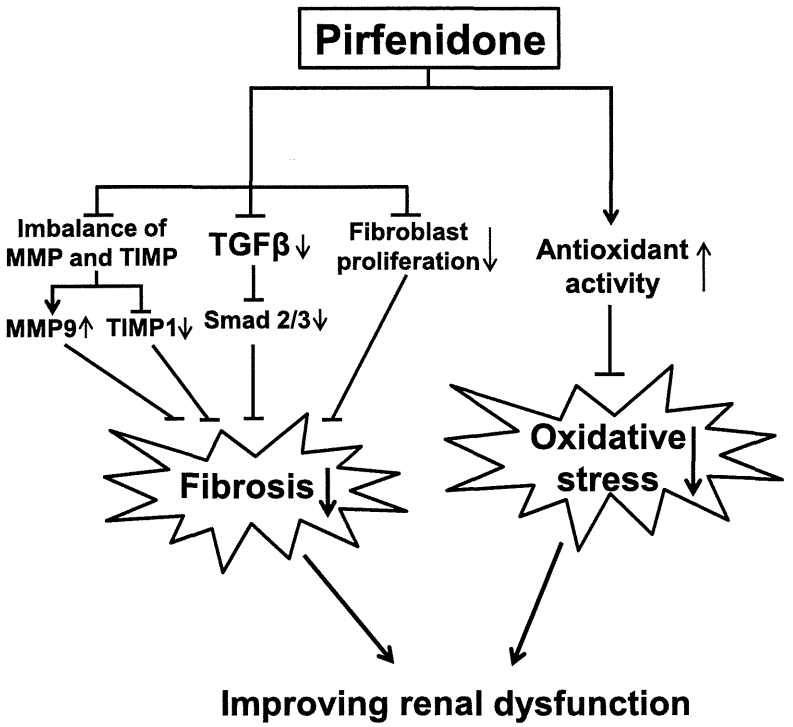

Fig. 8 The renoprotective mechanisms of PFD in hypertensive renal injury: through anti-fibrotic and anti-oxidative stress pathways. The therapeutic mechanisms of PFD on renal injury are mediated by its anti-fibrotic and anti-oxidative stress activities, not its anti-inflammatory activity. PFD exerts 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.

ment of chronic renal diseases.

Acknowledgements

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