Pirfenidine exhibits cardioprotective effects by regulating myocardial fibrosis and vascular permeability in pressure-overloaded hearts

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Yamagami K, Oka T, Wang Q, Ishizu T, Lee J-K, Miwa K, Akazawa H, Naito AT, Sakata Y, Komuro I. Pirfenidine exhibits cardioprotective effects by regulating myocardial fibrosis and vascular permeability in pressure-overloaded hearts. Am J Physiol Heart Circ Physiol 309: H512–H522, 2015. First published June 8, 2015; doi:10.1152/ajpheart.00137.2015.—Although cardiac fibrosis causes heart failure, its molecular mechanisms remain elusive. In this study, we investigated the mechanisms of cardiac fibrosis and examined the effects of the antifibrotic drug pirfenidine (PFD) on chronic heart failure. To understand the responsible mechanisms, we generated an in vivo pressure-overloaded heart failure model via transverse aortic constriction (TAC) and examined the effects of PFD on chronic-phase cardiac fibrosis and function. In the vehicle group, contractile dysfunction and left ventricle fibrosis progressed further from 4 to 8 wk after TAC but were prevented by PFD treatment beginning 4 wk after TAC. We isolated cardiac fibroblasts and vascular endothelial cells from the left ventricles of adult male mice and investigated the cell-type-specific effects of PFD. Transforming growth factor-β (TGF-β) induced upregulated collagen 1 expression via p38 phosphorylation and downregulated claudin 5 (Cldn5) expression in cardiac fibroblasts and endothelial cells, respectively; both processes were inhibited by PFD. Moreover, PFD inhibited changes in the collagen 1 and Cldn5 expression levels, resulting in reduced fibrosis and serum albumin leakage into the interstitial space during the chronic phase in TAC hearts. In conclusion, PFD inhibited cardiac fibrosis by suppressing both collagen expression and the increased vascular permeability induced by pressure overload.

NEW & NOTEWORTHY

Pirfenidine, which is used clinically in patients with idiopathic pulmonary fibrosis, exerted antifibrotic effects and improved left ventricular systolic dysfunction even at an advanced stage of pressure overload-induced heart failure and suppressed an increase in vascular permeability that was possibly associated with reduced Claudin 5 expression in vascular endothelial cells.

CARDIAC FIBROSIS IS A FEATURE of myocardial failure and is considered a cause of arrhythmia and cardiac dysfunction as well as an important therapeutic target in cases of heart failure. Although fibroblasts and the extracellular matrix are essential cardiac structural elements, the precise molecular and cellular characteristics of these components remain incompletely understood because of the lack of specific markers.

Recent clinical trials (14, 22) revealed that pirfenidone (PFD, 5-methyl-1-phenyl-2-[1H]-pyridone) prevented pulmonary fibrosis progression and ameliorated lung function, leading to a better prognosis in patients with idiopathic pulmonary fibrosis. Recently, several experimental studies reported that PFD has antifibrotic and anti-inflammatory effects in various disease models (26). Ouku et al. (24) demonstrated the antifibrotic effects of PFD on murine bleomycin-induced lung fibrosis. An in vitro study of lung fibroblasts also revealed that PFD prevented transforming growth factor-β (TGF-β)-induced migration and profibrotic effects (5). PFD also exerted antifibrotic effects on experimental animal models of liver (9, 13) and kidney fibrosis (18, 30). In models of myocardial infarction (20), pressure overload (33), or angiotensin II administration-induced heart failure (36), PFD treatment from the early stage of heart failure prevented cardiac dysfunction, left ventricular (LV) remodeling, cardiac fibrosis, and cardiac hypertrophy. However, it remains unclear whether PFD exhibits antifibrotic and cardioprotective effects during the chronic decompensated stage of heart failure. Furthermore, the precise regulatory mechanisms of PFD are not fully understood.

To elucidate these issues, we examined the effects of PFD on murine models of chronic pressure overload-induced heart failure and elucidated the underlying molecular mechanisms.

MATERIALS AND METHODS

Animals, TAC, drug administration, and measurement of blood pressure and heart rate. All mouse procedures and cellular experiments conducted in this study were approved by the Osaka University Ethics Committee for Animal Experiments. Seven-week-old male C57BL/6J mice were purchased from CLEA Japan (Tokyo, Japan) and housed in temperature-controlled rooms with a 12-h:12-h light/dark cycle. Transverse aortic constriction (TAC)-induced pressure overload was performed as described previously (23, 35). Briefly, 8-wk-old male C57BL/6J mice were anesthetized with isoflurane (2.0–2.5% in medical grade oxygen). The transverse aortic arch was visualized through a thoracotomy, and a 7-0 silk suture was tied around a 27-gauge needle at the aortic arch. Beginning at 4 wk after TAC or sham surgery, the mice were orally administered 400 mg/kg of PFD dissolved in 0.5% carboxymethylcellulose (CMC) or 100 μl of 0.5% CMC (vehicle) via gastric gavage twice daily for 4 wk. At 8 wk after TAC, the echocardiographic parameters, heart weight (HW), lung weight (LW), and tibial length (TL) were measured, and the mice were killed for histological and molecular analyses. Blood pressure and heart rate of the mice were measured by noninvasive recording.
using a BP-98A tail-cuff device (Softron, Tokyo, Japan) without using an anesthetic. PFD was kindly provided by Shionogi (Osaka, Japan).

Echocardiographic analysis. Transthoracic echocardiographic analyses were performed using a Vevo770 instrument (VisualSonics, Toronto, Ontario, Canada) as described previously (32). Two-dimensional guided M-mode tracings of an LV long-axis cross section at the tip of the papillary muscles were obtained to measure the interventricular septum thickness (IVSth, mm), posterior wall thickness (PWth, mm), LV end-diastolic diameter (LVEDd, mm), LV end-systolic diameter (LVEDs, mm), and heart rate (beats/min). Fractional shortening (FS) was calculated as \[
\frac{(LVEDd - LVEDs)}{LVEDd} \times 100\%
\].

Histological, immunohistochemical, and immunocytochemical analyses. Hearts were fixed in 10% Mildform (Wako, Osaka, Japan), embedded in paraffin, and cut into 5-μm sections for Masson’s trichrome staining. The LV %fibrosis area was calculated as the ratio of the total interstitial fibrosis area to the LV longitudinal sectional area of an LV section using ImageJ software (National Institutes of Health, Bethesda, MD). Optimal cutting temperature compound (Tissue-Tek; Sakura Fintek Torrance, CA)-embedded frozen heart sections and cardiac fibroblasts and endothelial cells were subjected to immunohistochemistry and immunocytochemistry, respectively, using the following antibodies: goat anti-troponin T-C (catalog no. 8121; Santa Cruz Biotechnology, Santa Cruz, CA), rabbit anti-vimentin (catalog no. 3932; Cell Signaling Technology, Beverly, MA), goat anti-VE-cadherin (catalog no. 6458, Santa Cruz Biotechnology), rat anti-periostin (catalog no. 3548; R&D Systems, Minneapolis, MN), rabbit anti-snail (catalog no. 3879, R&D Systems), rat anti-CD31 (catalog no. 553370; BD Biosciences, San Jose, CA), goat anti-discoidin domain receptor 2 (DDR2) (catalog no. 7555, Santa Cruz Biotechnology), rabbit anti-collagen type 1 (catalog no. 765; EMD Millipore, Billerica, MA), rabbit anti-claudin 5 (Clhn5) (catalog no.

**Fig. 1.** Assessment of cardiac hypertrophy and function. **A:** systolic blood pressure (sBP, mmHg) and heart rate (HR, beats/min, bpm) were measured by the tail-cuff method in 12-wk-old mice before and 1, 3, and 6 h after the administration of vehicle (Veh, 0.5% carboxymethylcellulose) or pirfenidone (PFD, 400 mg/kg) (n = 6 per group) and in transverse aortic constriction (TAC) mice at 8 wk after treatment with vehicle or PFD (n = 5 per group). The sBP and HR in the PFD group did not significantly change relative to the Veh group at any time point. **B:** schema of the in vivo study protocol. PFD administration was initiated at 4 wk after TAC. **C** and **D:** heart weight (HW, mg) to tibial length (TL, mm) ratio (C) and lung weight (LW, mg) to TL ratio (D) were measured in sham and TAC mice at 4 wk before treatment and sham and TAC mice at 8 wk after treatment with vehicle or PFD. **E:** assessment of fractional shortening (FS, %). Numbers of mice are indicated in the graph bars. *P < 0.05 vs. Sham, †P < 0.05 vs. TAC8w/Veh.
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Table 1. Echocardiographic assessment of cardiac structure and function of sham and TAC mice in the indicated groups

<table>
<thead>
<tr>
<th>Group</th>
<th>Sham4w</th>
<th>TAC4w</th>
<th>Sham8w/Veh</th>
<th>Sham8w/PFD</th>
<th>TAC8w/Veh</th>
<th>TAC8w/PFD</th>
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<tr>
<td>Number</td>
<td>n = 6</td>
<td>n = 10</td>
<td>n = 5</td>
<td>n = 5</td>
<td>n = 13</td>
<td>n = 16</td>
</tr>
<tr>
<td>IVSth, mm</td>
<td>0.83 ± 0.02</td>
<td>1.03 ± 0.02*</td>
<td>0.77 ± 0.01</td>
<td>0.80 ± 0.01</td>
<td>1.02 ± 0.02*</td>
<td>1.07 ± 0.01*</td>
</tr>
<tr>
<td>PWh, mm</td>
<td>0.84 ± 0.01</td>
<td>0.96 ± 0.01*</td>
<td>0.76 ± 0.01</td>
<td>0.76 ± 0.01</td>
<td>0.99 ± 0.02*</td>
<td>1.03 ± 0.02*</td>
</tr>
<tr>
<td>LVEDd, mm</td>
<td>3.33 ± 0.06</td>
<td>3.90 ± 0.16*</td>
<td>3.45 ± 0.04</td>
<td>3.44 ± 0.02</td>
<td>4.29 ± 0.15*</td>
<td>3.79 ± 0.11**</td>
</tr>
<tr>
<td>LVEDds, mm</td>
<td>1.84 ± 0.07</td>
<td>2.80 ± 0.17*</td>
<td>1.72 ± 0.05</td>
<td>1.58 ± 0.02</td>
<td>3.28 ± 0.20*</td>
<td>2.51 ± 0.17**</td>
</tr>
<tr>
<td>FS, %</td>
<td>47.9 ± 1.1</td>
<td>30.9 ± 1.8*</td>
<td>50.3 ± 1.0</td>
<td>54.0 ± 0.8</td>
<td>24.3 ± 2.2*</td>
<td>34.7 ± 2.2**</td>
</tr>
<tr>
<td>HR, bpm</td>
<td>607 ± 15</td>
<td>625 ± 16</td>
<td>587 ± 12</td>
<td>574 ± 15</td>
<td>568 ± 12</td>
<td></td>
</tr>
<tr>
<td>BW, g</td>
<td>26.6 ± 0.3</td>
<td>25.1 ± 0.5</td>
<td>27.3 ± 0.4</td>
<td>26.4 ± 0.3</td>
<td>25.8 ± 0.4</td>
<td>25.2 ± 0.2</td>
</tr>
</tbody>
</table>

Values are means ± SE, and each animal was measured 3 separate times. All measurement values are shown in mm, whereas fractional shortening (FS) is shown as a percentage and heart rate (HR) is an absolute value of beats/min (bpm). TAC, transverse aortic constriction; W, weeks; Veh, vehicle; PFD, pirfenidone; IVSth, interventricular septum thickness; PWh, posterior wall thickness, LVEDd, left ventricular end-diastolic diameter; LVEDds, left ventricular end-systolic diameter; BW, body weight. *P < 0.05 vs. Sham groups; †P < 0.05 vs. TAC8w/Veh.
hypertrophy and dysfunction were already recognizable at 4 wk after TAC. At this time point, we began to administer 400 mg/kg of PFD (TAC8w/PFD group, \( n = 16 \)) or vehicle (TAC8w/Veh group, \( n = 13 \)) twice daily for 4 wk (Fig. 1B). The systolic blood pressure and the body weight did not significantly change in the TAC8w/PFD group relative to the TAC8w/Veh group at 8 wk after TAC (Fig. 1A, Table 1), suggesting that long-term administration of PFD would not affect hemodynamics and eating habit of the TAC mice.

At 8 wk after TAC, the HW/TL ratios of both the TAC8w/Veh and TAC8w/PFD groups were significantly increased relative to the sham mice. The TAC8w/PFD group had lower HW/TL and LW/TL ratios relative to the TAC8w/Veh mice although these differences were not statistically significant (Fig. 1, C and D). The wall thicknesses were nearly equivalent in the TAC8w/PFD and TAC8w/Veh groups, but LV dilation was significantly suppressed by PFD administration (Table 1). Moreover, FS was significantly improved in the TAC8w/PFD relative to the TAC8w/Veh group (Fig. 1E, Table 1), suggesting that PFD exerted cardioprotective effects even during the chronic phase of heart failure by regulating cardiac remodeling and systolic function in the pressure-overloaded heart.

**PFD ameliorates cardiac fibrosis and hypertrophy in the TAC heart.** We performed Masson’s trichrome staining to assess cardiac fibrosis. Cardiac fibrosis, especially interstitial fibrosis, was significantly increased in TAC4w hearts and further increased in TAC8w/Veh hearts relative to sham hearts (Fig. 2A). PFD treatment markedly reduced cardiac fibrosis in TAC8w/PFD hearts relative to TAC8w/Veh hearts (Fig. 2A). Quantitative analysis revealed that the fibrosis area of LV in TAC8w/PFD was significantly lower than that in TAC8w/Veh (Fig. 2B).

**PFD alters gene and protein expression in the TAC heart.** The in vivo results of PFD treatment showed ameliorative effects on cardiac dysfunction and fibrotic progression. To determine the expression levels of fibrosis-related genes, quantitative RT-PCR and Western blotting were performed. Quantitative RT-PCR revealed that fibrosis-associated genes such as...
collagen type 1 α1 (Col1a1) and type 3 α1 (Col3a1) were strongly induced in TAC8w/Veh, whereas Col1a1 and Col3a1 were significantly suppressed by PFD treatment (Fig. 3A). Hypertrophic markers such as natriuretic peptide type A (Nppa), natriuretic peptide type B (Nppb), and actin, α1, skeletal muscle (Acta1) were induced in TAC8w/Veh, whereas Acta1 but not Nppa or Nppb was significantly suppressed by PFD treatment (Fig. 3A). Tissue inhibitor of metalloproteinase 1 (Timp1) and matrix metalloproteinase 2 (Mmp2), the markers responsible for maintaining extracellular matrix integrity, were induced only in TAC8w/Veh (Fig. 3A). Timp3, which is reported to promote apoptosis (3), was induced in TAC8w/Veh and significantly suppressed by PFD treatment (Fig. 3A). Fibrosis-related cytokines such as Tgf-β1 and Tgf-β2 were also induced by TAC, and Tgf-β1 but not Tgf-β2 was significantly suppressed by PFD (Fig. 3A). Hypoxia-inducible factor 1α (Hif1α) (11), serine peptidase inhibitor, clade E, member 1 (10) (Serpine 1, also known as PAI1), and Snai1 (19) mRNA expression levels, which were robustly increased by TAC, were significantly suppressed by PFD treatment (Fig. 3A).

Western blotting using the proteins extracted from the LV of the Sham8w/Veh mice, the TAC4w mice, the TAC8w/Veh mice, and the TAC8w/PFD mice revealed that the protein levels of collagen type 1, vimentin, and Snail were highly increased in the LV at 8 wk after TAC, and this upregulated expression was significantly suppressed by a 4-wk PFD administration course (Fig. 3, B and C). Immunohistochemical staining revealed that vimentin (Fig. 4A), peroxisome (Fig. 4B), and Snai1 (Fig. 4C) expression were markedly increased in the interstitial space and in noncardiomyocytes, and all were suppressed by PFD administration. These results indicate that PFD treatment strongly suppresses fibrosis-related protein expression during the chronic phase of heart failure.

PFD regulates adult cardiac fibroblasts and endothelial cells in vitro. To examine the regulatory mechanisms of PFD, we isolated and cultured cardiac fibroblasts and vascular endothelial cells from an adult mouse LV. Quantitative RT-PCR revealed that isolated CD31-positive cardiac endothelial cells strongly expressed endothelial-specific markers such as cadherin 5 (Cdh5, also known as VE-cadherin) and kinase insert domain protein receptor (Kdr, also known as VEGFR2 or Flk1) (Fig. 5, A and B). On the other hand, CD31-negative cells exhibited strong expression of fibroblast markers such as Col1a1 and Col3a1 at the mRNA level and DDR2 protein, but not Cdh5 and Kdr (Fig. 5, A and C). These results suggest that the CD31-negative fraction specifically included cardiac fibroblasts and that the CD31-positive fraction specifically included cardiac vascular endothelial cells. We used these two types of cultured cells to examine the effects of PFD separately.

We stimulated CD31-negative fibroblasts with TGF-β1 in the presence or absence of PFD for 48 h. Western blot analyses revealed that TGF-β1 upregulated collagen type 1 and α-SMA, both of which were significantly suppressed by PFD treatment (Fig. 6, A and B). In cultured cardiac fibroblasts, TGF-β1 activated p38 and Smad2/3 but not p44/42 or JNK, and PFD specifically inhibited the phosphorylation of p38 by TGF-β1 (Fig. 6, A and B).

In CD31-positive endothelial cells, expression of Cldn5, which encodes a tight junction component specifically expressed in vascular endothelial cells that controls cell attachment and vascular permeability (31), was significantly reduced by TGF-β2 and TGF-β1 stimulation, whereas the Cdh5, Kdr, and Acta2 levels were not changed (Fig. 6, C and D). Simultaneous administration of PFD to CD31-positive cells strongly inhibited the Cldn5 downregulation by TGF-β2 stimulation (Fig. 6D).

PFD controls Cldn5 and vascular permeability in TAC hearts. To clarify the in vivo Cldn5 expression levels, we analyzed Cldn5 protein expression in sham and TAC LVs. In sham-operated mice, Cldn5 was abundantly expressed in LVs, whereas its expression levels were strongly reduced in TAC8w/Veh LVs (Fig. 7A). Immunohistochemical analyses further confirmed Cldn5 downregulation in the endothelial cells from both TAC4w and TAC8w/Veh hearts (Fig. 7B). PFD administration significantly restored Cldn5 expression in the TAC8w/PFD hearts (Fig. 7, A and B). To examine LV vascular barrier function, we performed immunostaining of albumin as an indicator of vascular permeability. As shown in Fig. 7C, serum albumin accumulation in the LV interstitial space was observed in TAC4w but not sham hearts and was enhanced in TAC8w/Veh hearts, suggesting the progression of hyperpermeability during the chronic phase of heart failure. Serum albumin accumulation was clearly suppressed by PFD treatment in TAC8w/PFD hearts (Fig. 7C), indicating that PFD suppressed vascular hyperpermeability. Moreover, the number of CD68-positive macrophages in the LV interstitial space, which was increased by the TAC procedure, was significantly lower in TAC8w/PFD than in TAC8w/Veh; the former was equal to that in TAC4w (Fig. 7, D and E).

DISCUSSION

In the present study, we have demonstrated that PFD exerted antifibrotic effects and improved LV systolic dysfunction even at an advanced stage of pressure overload-induced heart failure. PFD has been reported to exert anti-inflammatory, antioxidant, and antifibrotic effects on the heart. In an ischemia-reperfusion rat model, PFD treatment beginning at 1 wk after the procedure prevented cardiac fibrosis, dysfunction, and arrhythmias (20). PFD treatment, when given every 2 days beginning at 10 days after TAC, was also shown to attenuate myocardial fibrosis and inflammatory cytokine expression by inhibiting nod-like receptor

Fig. 3. Assessment of gene and protein expression in the LV. A: quantitation of indicated gene expression in hearts. Open bars indicate Sham8w/Veh; solid bars indicate TAC8w/Veh; shaded bars indicate TAC8w/PFD hearts (n = 5 individual quantitative RT-PCR reactions). *P < 0.05 vs. Sham, †P < 0.05 vs. TAC8w/Veh. Col1a1, collagen type 1 α1; Col3a1, collagen type 3 α1; Nppa, natriuretic peptide type A; Nppb, natriuretic peptide type B; Acta1, actin, α1, skeletal muscle; Tgf-β1, transforming growth factor-β1; Tgf-β2, transforming growth factor-β2; Hif1α, hypoxia-inducible factor 1α; Serpine 1, serine peptidase inhibitor, clade E, member 1 (also known as PAI1); Snai1, snail family zinc finger 1. B and C: Western blotting and quantitation of collagen type 1, vimentin, and Snail using cardiac protein extracts from Sham8w/Veh, TAC4w, TAC8w/Veh, and TAC8w/PFD mice (n = 4). Band intensities were measured and shown in graphs as ratios normalized to GAPDH. *P < 0.05 vs. Sham, †P < 0.05 vs. TAC8w/Veh.
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A

Col1α1

Acta1

Mmp2

Mmp9

Timp1

Col3α1

Nppa

Timp3

Timp4

Tgf-β1

Tgf-β2

Nppb

Relative expression

Sham8w Veh PFD
TAC8w

Relative expression

Sham8w Veh PFD
TAC8w

Relative expression

Sham8w Veh PFD
TAC8w

B

Collagen type 1

GAPDH

Vimentin

GAPDH

Snail

GAPDH

C

Collagen type 1

Vimentin

Snail
pyrin domain-containing 3, which has been associated with inflammasome formation (33). In mice with angiotensin II-induced cardiac hypertrophy, simultaneous PFD administration inhibited cardiac hypertrophy and perivascular and interstitial fibrosis and attenuated mineralocorticoid receptor expression (36). These previous studies showed that PFD could effectively prevent fibrosis if administrated from an early phase of heart failure, but whether PFD could inhibit fibrosis in chronic phase, a particularly important factor in clinical settings, was unknown. We therefore initiated PFD administration at 4 wk after the TAC procedure in this study. Although extracellular matrix accumulation became obvious and began to accelerate during this phase, our results showed that PFD inhibited the cardiac fibrosis progression and preserved cardiac systolic function.

PFD might inhibit cardiac fibrosis by suppressing cardiac fibroblast activation and collagen synthesis. Several studies have reported that an increase in TGF-β production leads to cardiac fibrosis; conversely, inhibition of TGF-β suppresses fibrosis development in many experimental models (27).

Fig. 4. Confocal immunohistochemical analyses of the hearts. A: immunohistochemistry of vimentin (green), troponin T-C (red), and nuclei (blue) was performed in LVs from Sham8w/Veh, TAC4w, TAC8w/Veh, and TAC8w/PFD mice. Vimentin was increased in TAC8w/Veh hearts but suppressed in TAC8w/PFD hearts. Scale bar = 50 μm. B: immunohistochemistry of periostin (green) and VE-cadherin (red) was performed in LVs from Sham8w/Veh, TAC4w, TAC8w/Veh, and TAC8w/PFD mice. Scale bar = 50 μm. C: immunohistochemistry of Snail (green) and CD31 (red) was performed in LV from Sham8w/Veh, TAC4w, TAC8w/Veh, and TAC8w/PFD mice. Snail expression was increased in TAC8w/Veh but suppressed in TAC8w/PFD hearts. Scale bar = 50 μm.
Recent studies reported that PFD blocked TGF-β-induced Smad signaling, but not MAPK signaling, in the human retinal pigment epithelial cell line arising retinal pigment epithelia-19 (4) and exerted antifibrotic effects via the inhibition of TGF-β-induced p38 phosphorylation in the human lung fibroblast cell line MRC-5 (5, 16). These results indicate that PFD might target different signaling pathways in different cell types. In isolated adult cardiac fibroblasts, p38 MAPK and Smad2/3, but not p44/42, activations were induced by TGF-β, and PFD specifically inhibited this p38 phosphorylation as well as collagen synthesis and α-SMA expression.

We clarified that Cldn5 downregulation in the vascular endothelial cells from failing hearts was ameliorated by PFD administration. Using isolated vascular endothelial cells, we also exhibited that TGF-β-mediated Cldn5 downregulation was ameliorated by PFD treatment. Reduced Cldn5 expression was reported in the hearts of patients with end-stage heart failure attributable to both ischemic and nonischemic cardiomyopathy (17, 29), suggesting that Cldn5 plays important but undiscovered roles in the failing heart. Cldn5, which is ubiquitously and strongly expressed in the lung, brain, and heart vasculature (28), is a tight junction protein that plays important roles in the vascular permeability of endothelial cell barriers. Cldn5-deficient mice have been reported to exhibit size-selective permeability of the blood-brain barrier (21). Continuously elevated capillary pressure in the kidney (8) and increased shear stress in pulmonary artery endothelial cells (34) induced albumin leakage and vascular hyperpermeability. With consideration of these findings, Cldn5 downregulation in the vascular endothelial cells of the TAC heart would also increase vascular permeability and induce the leakage of serum factors, including albumin. Several studies have reported that cardiac function is affected by microvascular permeability (6, 7) and that an increase in the interstitial fluid volume of only a few percentage points can impair cardiac function (6).

Downregulation of Cldn5 promotes macrophage infiltration across the blood-brain barrier in patients with HIV encephalitis (25). Leaked water as well as serum factors and inflammatory cells such as macrophages, which are recognized as major TGF-β producers in the wound-healing response (2) and as key factors in cardiac fibrosis, might promote profibrotic responses in failing hearts consequent to Cldn5 downregulation in vascular endothelial cells.

PFD might ameliorate cardiac function and fibrosis by inhibiting the overexpression of fibrosis-related genes as well as inhibiting vascular hyperpermeability. There are two phases of tissue injury and wound healing, acute inflammation with tissue edema and chronically developed fibrosis. In hearts, pressure overload induces a similar stepwise reaction of acute inflammation followed by chronic fibrosis (12, 15). Although acute inflammation and chronic fibrosis are intimately related, chronic fibrosis development might occur independently of the acute inflammatory response in pressure-overloaded hearts. Oku et al. (24) reported that, in an early-phase bleomycin-induced pulmonary fibrosis model, both pulmonary edema and inflammatory cytokine expression were significantly suppressed by prednisolone and PFD treatment; however, PFD but not prednisolone attenuated lung fibrosis during the chronic phase. This finding suggests that specific mechanisms underlie chronic-phase fibrosis development. As PFD could effectively prevent cardiac function impairment and fibrosis in the bleomycin model, PFD might ameliorate the chronic cardiac abnormalities in patients with LV systolic dysfunction.
Fig. 6. In vitro assessment of PFD in cardiac fibroblasts and ventricular endothelial cells. A and B: Western blots for collagen type 1, α-smooth muscle actin (α-SMA), phospho-p38 (P-p38), phospho-p44/42 (P-p44/42), phospho-JNK (P-JNK), and phospho-Smad2/3 (P-Smad2/3) in adult mouse cardiac fibroblasts are shown in A. TGF-β1 and PFD were added to culture media alone or in combination as indicated. Quantitation of these Western blots is shown in B (n = 4 in each group). *P < 0.05 vs. TGF-β1(-)/PFD(-), †P < 0.05 vs. TGF-β1(+)/PFD(-). C: in vitro assessment of gene expression in CD31-positive cardiac endothelial cells isolated from an adult mouse LV. TGF-β1 was added to culture media at a final concentration of 10 ng/ml. After 24 h, quantitative RT-PCR was performed for the expression of indicated genes (n = 4 individual quantitative RT-PCR reactions). *P < 0.05 vs. TGF-β1(-). Acta2, actin, smooth muscle, aorta, also known as α-SMA; Cldn5, claudin 5; Tagln, transgelin. D: quantitative RT-PCR for the expression of indicated genes in adult mouse cardiac vascular endothelial cells. TGF-β2 and PFD were added to culture media alone or in combination as indicated (n = 4 individual quantitative RT-PCR reactions). Cldn5 expression in endothelial cells was significantly suppressed by TGF-β2; however, this suppression was blunted by PFD. *P < 0.05 vs. TGF-β2(-)/PFD(-), †P < 0.05 vs. TGF-β2(+)/PFD(+).
fibrosis even when initiated at 4 wk after TAC, PFD might prevent cardiac fibrosis through mechanisms other than suppressing acute inflammation.

In conclusion, we have demonstrated that PFD treatment ameliorates chronic-phase pressure overload-induced cardiac fibrosis and cardiac dysfunction and suppresses an increase in vascular permeability that is possibly mediated by reduced Cldn5 expression in vascular endothelial cells. Although further investigation will be necessary to reveal the more precise molecular mechanisms about the relationship between cardiac fibrosis and vascular hyperpermeability, PFD may become a novel heart failure treatment.
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DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

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