

## ORIGINAL ARTICLE

# The antifibrotic agent pirfenidone inhibits angiotensin II-induced cardiac hypertrophy in mice

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Pirfenidone (5-methyl-1-phenyl-2-[<sup>1</sup>H]-pyridone) is an effective drug for idiopathic interstitial pneumonia that can prevent and reverse tissue fibrosis in several organs. Therefore, we investigated whether pirfenidone has a potential role in preventing angiotensin II (Ang II)-induced cardiac hypertrophy. A cardiac hypertrophic mouse model was created using an Ang II infusion (200 ng kg<sup>-1</sup> min<sup>-1</sup>) in wild-type mice for 2 weeks. Mice were divided into the following three groups: a saline-infused (control) group, an Ang II infusion (vehicle) group and an Ang II infusion+pirfenidone-treated (PFD) group, which received pirfenidone (300 mg kg<sup>-1</sup> per day) by gastric gavage during the Ang II infusion. At 2 weeks, we assessed hemodynamics and cardiac function and investigated tissue fibrosis of the myocardium histologically and genetically. Blood pressure in the vehicle group was significantly increased compared to the control group. Although blood pressure was not different between the vehicle and PFD groups, heart weight was significantly decreased in the PFD group. Echocardiography revealed that left ventricular hypertrophy was significantly increased in the vehicle group vs. the control group. Interestingly, pirfenidone significantly inhibited this effect. Continuous infusion of Ang II increased the perivascular and interstitial tissue fibrosis, and pirfenidone inhibited these fibrotic changes. Pirfenidone also inhibited Ang II-induced hypertrophy. In the vehicle group, the mRNA expressions of atrial natriuretic peptide, brain natriuretic peptide and transforming growth factor- $\beta$ 1 were increased, which was significantly inhibited by pirfenidone. Furthermore, the expression of mineralocorticoid receptors was attenuated by pirfenidone. These results indicate that pirfenidone might be effective as an antifibrotic drug in the treatment of cardiac hypertrophy induced by hypertension.

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**Keywords:** angiotensin II; cardiac hypertrophy; fibrosis; gene expression; pirfenidone

## INTRODUCTION

Cardiovascular damage due to hypertension represents a growing public health problem. Vascular remodeling is an adaptive response to increased arterial blood pressure. Angiotensin II (Ang II), an important effector of the renin–angiotensin–aldosterone system, is one of the most important factors involved in arterial remodeling.<sup>1</sup> In addition to its role in arterial blood pressure regulation, Ang II induces vascular smooth muscle cell hyperplasia and hypertrophy through the transactivation of epidermal growth factor, platelet-derived growth factor and insulin-like growth factor I receptors.<sup>2</sup> Ang II is also critically involved in the initial stage of inflammation, increased vascular permeability and leukocyte recruitment through the expression of vascular endothelial growth factor and adhesion molecules or chemokines, respectively.<sup>1,3</sup> Ang II also plays a critical role in the genesis of cardiac hypertrophy and fibrosis by producing transforming growth factor (TGF)- $\beta$ , platelet-derived growth factor and endothelin-1, as well as by attenuating interstitial matrix metal-

loproteinase activity, resulting in left ventricular (LV) dysfunction. Most of these effects are mediated by the type 1 Ang II receptor (AT<sub>1</sub>R) rather than the type 2 Ang II receptor. The Ang II/AT<sub>1</sub>R receptor interaction generates an immediate calcium-dependent response and late nuclear factor- $\kappa$ B activation through the activation of different pathways, including protein kinase C, mitogen-activated protein kinase cascades, receptor tyrosine kinases and non-receptor tyrosine kinases.<sup>2,4</sup>

Pirfenidone (5-methyl-1-phenyl-2-[<sup>1</sup>H]-pyridone) has been shown to reduce the fibrosis of different organs (for example, lung<sup>5,6</sup> kidney<sup>7–9</sup> liver,<sup>10</sup> heart<sup>11–13</sup>) and vascular remodeling<sup>14</sup> through its inhibitory effect on fibroblast growth and collagen synthesis, which is achieved by reducing the expression of profibrotic cytokines (for example, TGF- $\beta$ <sup>15,16</sup>). Furthermore, pirfenidone has been successfully used in a double-blind, placebo-controlled trial focused on patients with idiopathic pulmonary fibrosis.<sup>17</sup> However, the role of pirfenidone on hypertensive heart diseases is still unknown.

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In this study, we investigated the effect of pirfenidone on Ang II-induced ventricular dysfunctions, including hypertrophy, fibrosis and coronary arterial remodeling *in vivo*.

## METHODS

### Animals and experimental design

All procedures involving animals were approved by the animal care and use committee of Osaka City University. Experiments were performed with 8- to 9- week-old male BALB/c mice (CLEA Japan, Tokyo, Japan). Pirfenidone was provided as a gift from Shionogi (Tokyo, Japan).

To induce cardiac hypertrophy, Ang II (200 ng kg<sup>-1</sup> min<sup>-1</sup>) was dissolved in saline and continuously infused subcutaneously over 14 days via an osmotic mini-pump (ALZET, Cupertino, CA, USA). The control group received a saline infusion. Ang II-infused mice were divided into two groups. Pirfenidone (300 mg kg<sup>-1</sup> per day, the PFD group) or a 0.5% carboxymethylcellulose solution (the vehicle group) was administered orally by gastric gavage once a day for 14 days (started on the same day as the Ang II infusion). The systolic blood pressure of conscious mice was measured using the tail-cuff method (BP98A; Softron, Tokyo, Japan) before and 13 days after the Ang II infusion.

On day 14 after the infusion, cardiac function was examined using echocardiography as described below. The hearts were immediately excised afterwards, and the ventricle was separated from the atrium and weighed. The ventricle was divided into upper and lower portions. Subsequently, the upper portion of the LV was immediately frozen in liquid nitrogen and stored at -80°C until use. The lower portion was set overnight in 4% paraformaldehyde and was embedded in paraffin.

### Echocardiographic study

Echocardiographic studies were performed on mice using the SONOS 5500 (Philips, Best, the Netherlands) as described previously.<sup>18,19</sup> In brief, mice were lightly anesthetized with tiletamine (10 mg kg<sup>-1</sup> intraperitoneally) and xylazine (10 mg kg<sup>-1</sup> intraperitoneally). A two-dimensional, short-axis view of the LV was obtained at the level of the papillary muscles. Pulsed wave Doppler spectra (E and A waves) of mitral inflow velocities were recorded from the apical four-chamber view with the sample volume placed near the tips of the mitral leaflets and adjusted to the position of maximum velocity and laminar flow pattern. All Doppler spectra were recorded at a paper speed of 200 mm s<sup>-1</sup> and analyzed offline.

### Estimation of cardiac fibrosis

The area of perivascular and interstitial fibrosis was measured as described previously.<sup>19,20</sup> In short, the mouse heart was set with 10% formaldehyde overnight and embedded in paraffin. Five-µm-thick sections were cut and stained with Sirius red stain to measure the interstitial fibrosis area. The interstitial fibrosis area was calculated as the ratio of the sum of the total area of interstitial fibrosis to the sum of the total connective tissue area plus the cardiomyocyte area in all the LV fields of the section that excluded perivascular fibrosis. Perivascular fibrosis was assessed by calculating the ratio of the area of collagen-stained material to the total vessel area, which was defined as the medial plus luminal area. Each field was analyzed with the image-analyzing software (Micro Analyzer, Nihon Poladigital, Tokyo, Japan).

### RNA preparation and analysis

RNA from the LV was isolated using ISOGEN (Nippon Gene, Toyama, Japan).<sup>18</sup> To elucidate the gene expression levels, we subjected the RNA samples to quantitative real-time polymerase chain reaction (ABI Prism 7700; Perkin-Elmer Applied Biosystems, Carlsbad, CA, USA). One-step quantitative real-time polymerase chain reaction reactions were performed using 100 ng of RNA per reaction. TaqMan primers and probes were designed using Primer 3 (v. 0.4.0); see Table 1. For analysis, transcript levels were normalized to glyceraldehyde-3-phosphate dehydrogenase.

### Statistical analysis

All data are presented as means ± s.e.m. Comparisons among groups were made using a one-way analysis of variance followed by Fisher's protected least

**Table 1 Sequences of the qRT-PCR probes and primers used in this study**

Gapd	F	5'-ACTGGCATGGCCTTCGG-3'
	P	5'-TTCTACCCCAATGTGTVVGTCGT-3'
	R	5'-CAGGCGGCACGTCAGATC-3'
Nppa	F	5'-GGGGGTAGGATTGACAGGAT-3'
	P	5'-CCGAAGATAACAGCCAAGGA-3'
	R	5'-GCAGAATCGACTGCCTTTTC-3'
Nppb	F	5'-CCAAGGTGACACATATCTCAAGC-3'
	P	5'-ACAACAACCTCAGTGCCTTACAGCCAA-3'
	R	5'-GAGTCAGAACTGGAGTCTCTG-3'
Tgfb1	F	5'-GAGTCCACCCGCTGCTA-3'
	P	5'-TGTTGGACCGCAACAACGCCATCT-3'
	R	5'-CCGAATGTCTGACGTATTGAAGAAC-3'
Ccl2	F	5'-GGTCCCTGTCATGCTTCTGG-3'
	P	5'-TCAGCCAGATGCAGTTAACGCCCCAC-3'
	R	5'-GATCATCTTGTCTGGTGAATGAGTAG-3'
Iifng	F	5'-GCTTTAACAGCAGCCAGAC-3'
	P	5'-ACAGGCTGTCCCTGAAAGAA-3'
	R	5'-GGAAGCACCAGGTGCAAGT-3'
Ppargc1a	F	5'-AACCACCCCACAGGATCAGA-3'
	P	5'-TGCCATTGTTAAGACCAGAGA-3'
	R	5'-TCTTCGCTTATTGCTCCATGA-3'
Agtr1a	F	5'-ACCAGCTCTGCGGCTCTC-3'
	P	5'-AGCTCTGCTGCTCTCCCGAGCTTAAACA-3'
	R	5'-GCCAGCCATTTATACCAATCTTTC-3'
Nr3c2	F	5'-GGGTAGTGACCTGCGGCA-3'
	P	5'-AGAGCCGTGGAAGGACAACAATCTAT-3'
	R	5'-AGGCAGGACAGTCTTCTTCC-3'

Abbreviations: F, forward primer; qRT-PCR, quantitative reverse transcription-polymerase chain reaction; P, probe; R, reverse primer.

significant difference test using StatView (SAS Institute, Cary, NC, USA). A Pearson's correlation coefficient was used for correlation analysis. Differences were considered to be statistically significant at a value of  $P < 0.05$ .

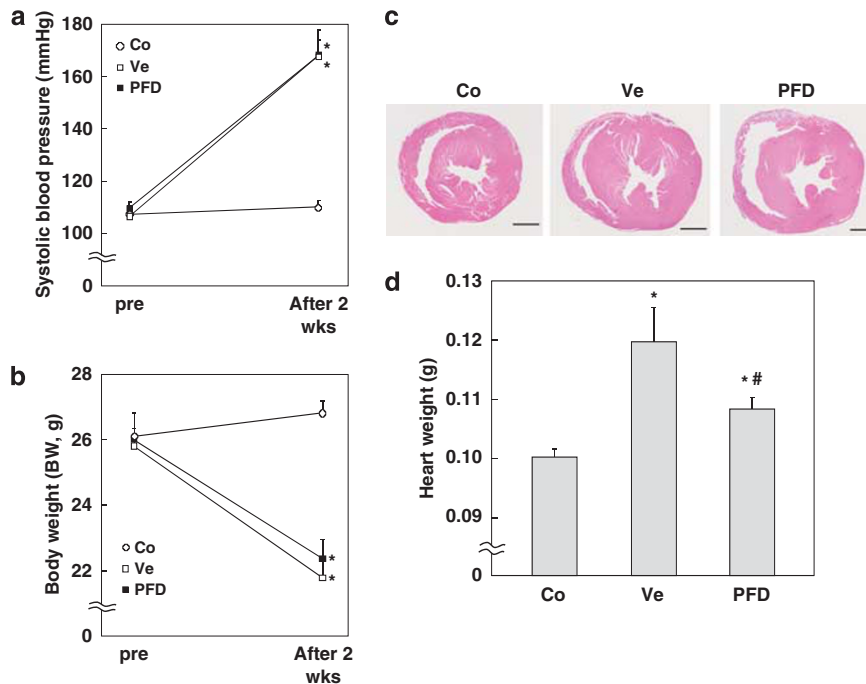
## RESULTS

### Effect of pirfenidone on blood pressure, body weight and heart weight

The blood pressure and body weight of the mice is shown in Figures 1a and b. The systolic blood pressure of the mice receiving a saline infusion (the control group), Ang II infusion (the vehicle group) and Ang II infusion+PFD treatment (the PFD group) were  $110 \pm 2$ ,  $167 \pm 6$  and  $167 \pm 19$  mm Hg, respectively, at day 13. Thus, blood pressure elevation by Ang II was not inhibited by pirfenidone. Body weight was not affected by pirfenidone, although significantly decreased by Ang II.

### Effect of pirfenidone on cardiac hypertrophy in Ang II-infused mice

The effect of pirfenidone on Ang II-induced LV hypertrophy is shown in Figures 1c and d. Ang II infusion for 14 days significantly increased the heart weight compared to the control ( $0.100 \pm 0.002$  vs.  $0.120 \pm 0.006$  g, respectively,  $P < 0.01$ ), and the effect of Ang II was



**Figure 1** Systolic blood pressure (a) and body weights (b) of mice before and after 2 weeks of Ang II administration ( $200 \text{ ng kg}^{-1} \text{ min}^{-1}$ ) and pirlfenidone (PFD) treatment. Co indicates saline-infused control mice; Ve indicates Ang II-infused mice treated with vehicle; and PFD indicates Ang II-infused mice treated with PFD. Values represent means  $\pm$  s.e.m. ( $n=6-10$ ).  $*P<0.05$  vs. control mice at each time point. Representative images of light micrographs of cross-sections of the mid-portion of the heart (c) (bar=1 mm) and heart weight (d) from saline-infused control group (Co), and Ang II-infused groups treated with Ve or PFD. Values represent means  $\pm$  s.e.m. ( $n=6-10$ ).  $*P<0.05$  vs. Co;  $\#P<0.05$  when Ve vs. PFD.

**Table 2** Echocardiographic measurements at 2 weeks after Ang II infusion in mice

	Saline infusion ( $n=10$ )	Ang II infusion ( $n=6$ )	Ang II infusion+ pirlfenidone ( $n=6$ )
LVEDD (mm)	$3.55 \pm 0.11$	$3.22 \pm 0.16^*$	$3.44 \pm 0.13$
LVESD (mm)	$2.08 \pm 0.09$	$1.98 \pm 0.25$	$2.05 \pm 0.22$
IVS (mm)	$0.66 \pm 0.02$	$0.91 \pm 0.03^*$	$0.71 \pm 0.02^\ddagger$
PW (mm)	$0.62 \pm 0.02$	$0.92 \pm 0.04^*$	$0.72 \pm 0.03^{*\ddagger}$
FS (%)	$41.5 \pm 1.7$	$39.5 \pm 4.8$	$40.9 \pm 5.0$
EF (%)	$70.0 \pm 1.9$	$68.4 \pm 1.8$	$67.8 \pm 3.9$
E wave ( $\text{cm s}^{-1}$ )	$75.9 \pm 1.3$	$50.4 \pm 1.7^*$	$54.4 \pm 3.2^*$
A wave ( $\text{cm s}^{-1}$ )	$38.3 \pm 1.8$	$42.4 \pm 2.0$	$40.8 \pm 4.2$
E/A	$1.98 \pm 0.11$	$1.19 \pm 0.06^*$	$1.34 \pm 0.11^*$

Abbreviations: Ang II, angiotensin II; E/A, the ratio of E wave to A wave; EF, ejection fraction; FS, fractional shortening; IVS, intraventricular septum; LVEDD, left ventricular end-diastolic dimension; LVESD, left ventricular end-systolic dimension; PW, posterior wall.  
 $*P<0.05$  vs. saline-infused mice;  $^\ddagger P<0.05$  between Ang II infusion + pirlfenidone-treated mice vs. Ang II-infused mice.

significantly inhibited by pirlfenidone ( $0.108 \pm 0.002 \text{ g}$ ). Intraventricular septum and posterior wall thickness estimated by echocardiography were also significantly larger in the vehicle and the PFD groups than in the control group (Table 2). The increase in thickness was significantly smaller in the PFD group than the vehicle group. LV end-diastolic and -systolic dimension, fractional shortening and ejection fraction were not significantly different among the groups.

#### Effects of pirlfenidone on perivascular fibrosis in Ang II-infused mice

Perivascular fibrosis of large and small coronary arteries in the LV of mice was larger than that of the control mice (Figures 2a and d).

Perivascular fibrosis caused by Ang II was attenuated by pirlfenidone treatment.

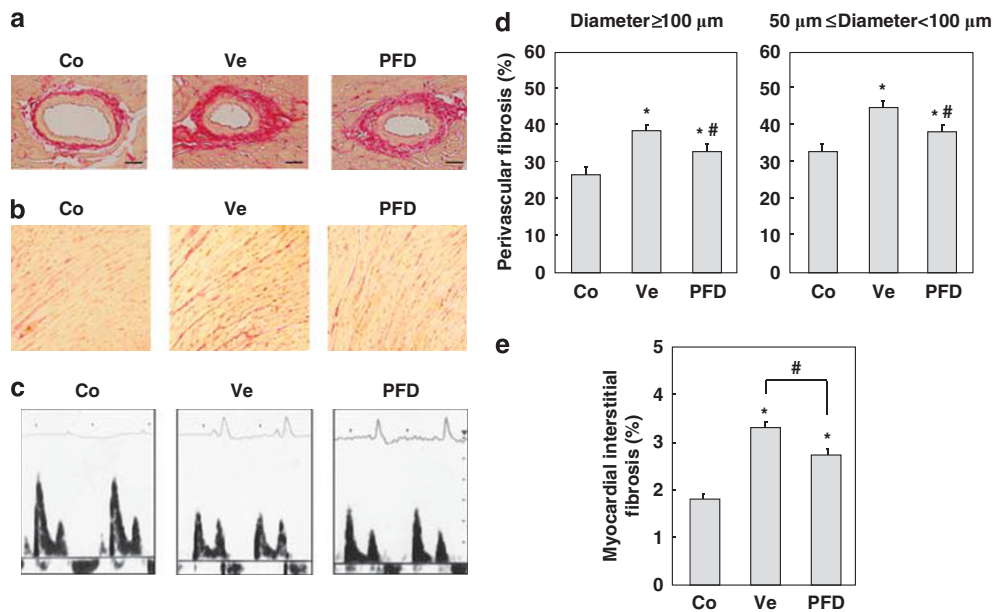
#### Effects of pirlfenidone on myocardial interstitial fibrosis and LV diastolic function in Ang II-infused mice

Ang II infusion for 2 weeks significantly increased the area of myocardial interstitial fibrosis in mice by 1.8-fold ( $P<0.01$ ), whereas Ang II-induced fibrosis was significantly diminished by pirlfenidone (Figures 2b and e).

The mitral inflow pattern in each group, as estimated by echocardiography, is shown in Figure 2c and Table 2. The ratio of the early rapid filling wave (E wave) velocity to the atrial contraction (A wave) velocity (E/A ratio) in the vehicle group was expressed as an abnormal pattern. Ang II lessened the E wave velocity and E/A ratio. Pirlfenidone tended to improve them.

#### Effect of pirlfenidone on cardiac gene expressions

The LV mRNA levels measured by quantitative real-time polymerase chain reaction in each group of mice are shown in Figure 3. LV mRNA expression levels for atrial natriuretic peptide and brain natriuretic peptide, which are closely related to cardiac hypertrophy, were increased by 9.1- and 3.0-fold ( $P<0.01$ ), respectively, following Ang II infusion. The upregulation of these mRNAs by Ang II was significantly decreased by pirlfenidone. The mRNA levels for LV TGF- $\beta$ 1 and monocyte chemoattractant protein-1 (MCP-1), which are closely related to cardiac fibrosis and remodeling, were increased by 1.8- and 2.2-fold ( $P<0.01$ ), respectively, following Ang II infusion. Pirlfenidone significantly attenuated the increased TGF- $\beta$ 1 expression. The MCP-1 expression level, which was increased by Ang II, tended to be suppressed with the treatment of pirlfenidone. The expression level of interferon- $\gamma$ , which has several potential antifibrotic actions, was



**Figure 2** Perivascular fibrosis and myocardial interstitial fibrosis in control, Ang II-infused and pirfenidone (PFD)-treated mice. Representative images of light micrographs of cross-sections of the coronary artery (a) and myocardial interstitial fibrosis (b) on left ventricular free wall sections. Original magnification: a,  $\times 400$  (bar=50  $\mu\text{m}$ ) and b,  $\times 200$ . Co indicates saline-infused control mice; Ve indicates Ang II-infused mice treated with vehicle; and PFD indicates Ang II-infused mice treated with PFD. (c) Representative pulse-wave Doppler spectra of mitral inflow pattern from the saline-infused control group (Co), and Ang II-infused groups treated with Ve or PFD. Quantitative results of perivascular fibrosis (d) and relative area of interstitial fibrosis (e). Co indicates saline-infused control mice; Ve indicates Ang II-infused mice treated with vehicle; and PFD indicates Ang II-infused mice treated with PFD. Values represent means  $\pm$  s.e.m. ( $n=6-10$ ). \* $P<0.05$  vs. Co; # $P<0.05$  when Ve vs. PFD.

significantly reduced by Ang II and increased with pirfenidone treatment. Peroxisome proliferator-activated receptor  $\gamma$  coactivator-1 $\alpha$  (PGC-1 $\alpha$ ), which is closely related to mitochondrial function, was also downregulated by Ang II, and pirfenidone tended to reverse this effect.

In addition, we investigated the role of pirfenidone on the gene expressions of Ang II type 1a receptor (AT<sub>1</sub>aR) and mineralocorticoid receptor (MR) in the mouse heart. Ang II significantly reduced the expression levels of AT<sub>1</sub>aR and MR. Pirfenidone did not affect the AT<sub>1</sub>aR expression. However, the MR expression in the PFD group was further decreased compared to that in the vehicle group. The expression of type 1 Ang II receptor in each group was too low to compare (data not shown).

#### Correlation of effects of cardiac TGF- $\beta$ 1 expression and perivascular fibrosis or myocardial interstitial fibrosis

We analyzed the correlation of the effects of cardiac TGF- $\beta$ 1 expression and LV hypertrophy, perivascular fibrosis or myocardial interstitial fibrosis. A correlation between TGF- $\beta$ 1 expression level and heart weight was not found (data not shown). However, there was a positive correlation between perivascular or myocardial interstitial fibrosis and TGF- $\beta$ 1 expression level (Figure 4). These data suggest that the expression level of TGF- $\beta$ 1 parallels the perivascular and myocardial interstitial fibrotic changes.

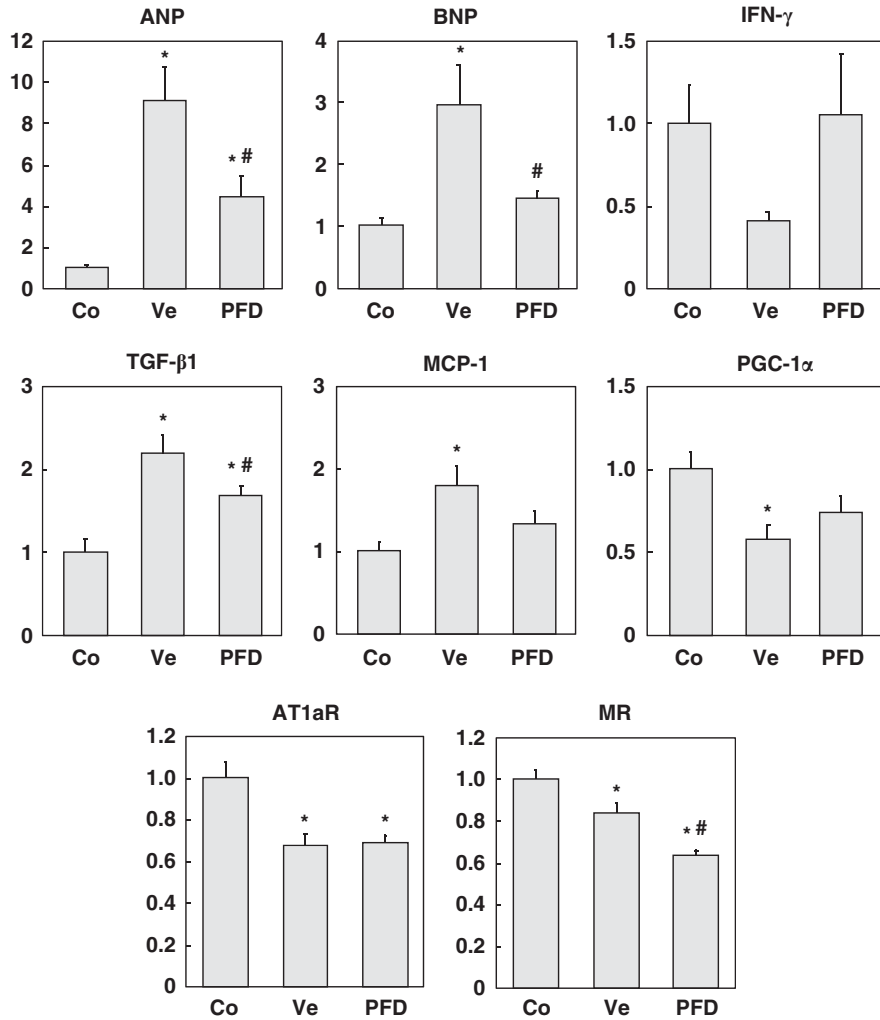
#### DISCUSSION

Accumulating evidence suggests that TGF- $\beta$  plays a causal role in LV hypertrophy induced by pressure overload, which leads to LV systolic and diastolic dysfunction.<sup>21</sup> Cardiac hypertrophy results in an increased expression of the mRNA for the extracellular matrix protein collagen.<sup>22</sup> Eghbali *et al.*<sup>23</sup> have demonstrated that TGF- $\beta$ 1 regulates collagen biosynthesis, and, subsequently, the actual deposition of

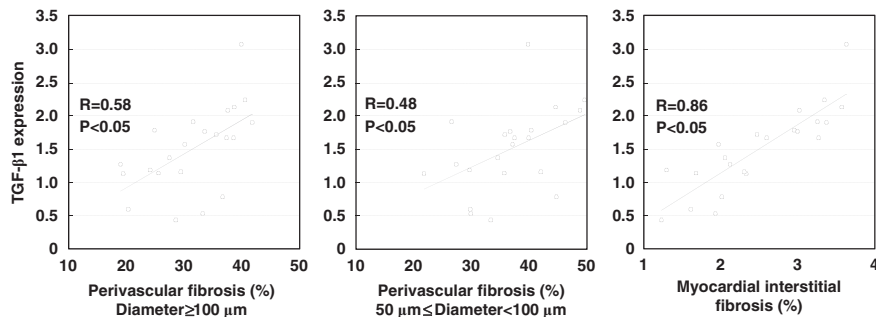
collagen proteins in cultured cardiac fibroblasts. Furthermore, Villarreal *et al.*<sup>24</sup> have reported that the mRNA level of TGF- $\beta$ 1 was increased in cardiac hypertrophy induced by thoracic aorta banding in rats. These results suggest that TGF- $\beta$ 1 is one of the major regulators of extracellular matrix protein deposition. Another study has reported that TGF- $\beta$ 1 appears to play an important role directly or in part via increased  $\beta$ -adrenergic signaling during the transition from compensated to decompensated cardiac hypertrophy.<sup>25</sup> Thus, blocking TGF- $\beta$ 1 pathways might be a pharmacological intervention in cardiac remodeling involving cardiac hypertrophy.

Pirfenidone has been reported to downregulate the gene expression and protein production of TGF- $\beta$ ,<sup>6</sup> although its mechanisms of action are not well understood. The antifibrotic actions of pirfenidone have been shown in several organs, including bleomycin-induced fibrotic lung in hamsters,<sup>26</sup> remnant kidney with collagen accumulation by partial nephrectomy in rats,<sup>27</sup> dimethylnitrosamine-induced fibrotic liver in rats,<sup>28</sup> chlorhexidine gluconate and ethanol-induced sclerosing peritonitis in rats<sup>29</sup> and intimal thickening after angioplasty of the carotid artery in rats.<sup>14</sup>

There are a few reports concerning the effect of pirfenidone on heart diseases. Lee *et al.*<sup>30</sup> investigated the effects of pirfenidone on arrhythmogenic atrial remodeling with ventricular tachypacing-induced chronic heart failure in canines. In their study, pirfenidone treatment resulted in a significant reduction of atrial fibrosis. Mirkovic *et al.*<sup>11</sup> reported that pirfenidone reverses and prevents cardiac remodeling as well as increased cardiac stiffness in deoxycorticosterone acetate-salt hypertensive rats. Those results suggest a broad effect of pirfenidone on potent profibrotic mediators.<sup>31</sup> However, there has been no evidence regarding the role of pirfenidone in cardiac hypertrophy and remodeling or its genetic analysis *in vivo*. In this study, we determined the role of pirfenidone in hypertrophy and remodeling that is induced by Ang II infusion. Mice were infused with



**Figure 3** Gene expressions in control, Ang II-infused and pirfenidone (PFD)-treated mice. The bar graph shows each mRNA value, corrected for the glyceraldehyde-3-phosphate dehydrogenase mRNA value. Mean values in the control mice group are represented as 1. Co indicates saline-infused control mice; Ve indicates Ang II-infused mice treated with vehicle; and PFD indicates Ang II-infused mice treated with PFD. Values represent means  $\pm$  s.e.m. ( $n=6-10$ ). \* $P < 0.05$  vs. Co; # $P < 0.05$  when Ve vs. PFD.



**Figure 4** The correlation between the TGF- $\beta$ 1 expression level and the ratio of perivascular fibrosis (left and middle panels) or myocardial interstitial fibrosis (right panel).

Ang II, which caused a gradual elevation of blood pressure and gradual development of cardiac hypertrophy; this finding is similar to the physiological condition in humans.

In this study, we first showed that pirfenidone significantly inhibited Ang II-induced LV TGF- $\beta$ 1 expression and LV hypertrophy

(Figures 1 and 3). Furthermore, there was a positive correlation between perivascular or myocardial interstitial fibrosis and TGF- $\beta$ 1 expression level. These results suggest that LV TGF- $\beta$ 1 is directly suppressed by pirfenidone (Figure 4). TGF- $\beta$  signaling is thought to be a therapeutic target for fibrotic diseases.<sup>32</sup> Iyer *et al.*<sup>6</sup> suggested that

pirfenidone may directly act on both macrophages and epithelial cells and fibroblasts to compromise their ability to synthesize and release TGF- $\beta$ 1. However, we could not testify the role of these cells because this study was based on whole-heart analysis *in vivo*. Further studies are needed to elucidate this point.

As estimated by LV weight, perivascular and interstitial fibrosis, echocardiographic measurements, and LV atrial natriuretic peptide and brain natriuretic peptide mRNA expression levels, cardiac hypertrophy induced by Ang II was significantly attenuated in pirfenidone-treated mice independently of blood pressure. These data strongly support the hypothesis that pirfenidone could improve the progression of cardiac hypertrophy and remodeling induced by Ang II. Furthermore, Doppler echocardiographic analysis of the LV inflow pattern revealed that Ang II induced an 'abnormal relaxation pattern', and pirfenidone returned it to a 'normal pattern' (Figure 2c); however, it was difficult to evaluate the LV inflow pattern quantitatively because the heart rates were not equalized adequately in the echocardiographic study. Therefore, pirfenidone treatment effectively prevents an increase in LV relaxation and a decrease in the passive elastic properties.

In addition to Ang II-induced LV TGF- $\beta$ 1 expression, we also examined some LV mRNA expression levels. MCP-1 is one of the major inflammatory mediators. Pressure overload-induced perivascular and cardiac fibrosis are mediated through MCP-1 induction and macrophage accumulation.<sup>33</sup> In addition, MCP-1 expression is increased in the aortic tissues of hypertensive rats<sup>34</sup> and in human atherosclerotic lesions.<sup>35</sup> Thus, MCP-1 plays an important role in the process of cardiac hypertrophy and fibrosis. In this study, LV MCP-1 expression was significantly increased by Ang II in accordance with our previous report,<sup>36</sup> and the increase tended to be smaller in the LV of pirfenidone-treated mice. It was not obvious whether pirfenidone could inhibit cardiac MCP-1 expression in this study.

The expression of PGC-1 $\alpha$ , which is a critical transcriptional regulator of energy metabolism, is decreased with the development of cardiac dysfunction and heart failure. Accordingly, PGC-1 $\alpha$  is thought to be a novel therapeutic target for heart failure.<sup>37</sup> In our model, Ang II decreased the level of PGC-1 $\alpha$  expression, and pirfenidone reversed this effect. Thus, pirfenidone may inhibit the development of cardiac dysfunction partially via PGC-1 $\alpha$ .

It is well-known that Ang II induces cardiac remodeling via not only type I Ang II receptor but also MR.<sup>38</sup> Therefore, we investigated whether pirfenidone affected gene expressions of AT<sub>1</sub>aR and MR in the mouse heart (Figure 3). Excessive administration of Ang II reduced the expressions of both AT<sub>1</sub>aR and MR. Pirfenidone decreased the MR expression more, suggesting that it could prevent Ang II-induced cardiac remodeling partially via the inhibition of aldosterone signaling pathways.

In conclusion, our present work provided *in vivo* evidence that the antifibrotic agent pirfenidone prevented the progression of Ang II-induced cardiac hypertrophy and fibrosis. One of the major mechanisms of pirfenidone may be mediated via TGF- $\beta$ 1 expression and, partially, via MR expression.

## CONFLICT OF INTEREST

The authors declare no conflict of interest.

## ACKNOWLEDGEMENTS

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