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4-PBA prevents pressure overload-induced myocardial hypertrophy and interstitial fibrosis by attenuating endoplasmic reticulum stress

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Abstract

Our previous study indicated that attenuation of endoplasmic reticulum (ER) stress by administration of 4-phenylbutyric acid (4-PBA) could prevent cardiac rupture and remodeling in a mouse model of myocardial infarction (MI). However, whether 4-PBA is protective in hypertrophic heart disease is unclear. Thus, we tested the therapeutic effect of 4-PBA on pressure-overload induced myocardial hypertrophy. Transverse aortic constriction (TAC) was used to create myocardial hypertrophy in C57BL/6 male mice for 4 weeks. Immediately after surgery, the mice were administrated either 4-PBA (20 mg/kg/day) or 0.9% NaCl by intraperitoneal injection. At the end of 4 weeks, the mice underwent high-resolution echocardiographic imaging. Our results showed that both the left ventricular posterior wall thickness at end systole (LVPWs) and diastole (LVPWd) were increased in the TAC group, compared to control. 4-PBA administration attenuated hypertrophy and decreased the heart weight over body weight ratio. Masson's trichrome staining showed that myocardial interstitial fibrosis and collagen deposition were also decreased by 4-PBA. We next detected the ER stress response in the heart tissues of TAC mice in different time points. Western blotting showed that the expression of ER stress marker, GRP78, CHOP and phosphor-PERK, were persistently increased 4 weeks after TAC. The treatment of 4-PBA inhibited the expression of ER stress markers. We also demonstrated that the 4-PBA at 20 mg/kg/day had no effect on histone 3 deacetylation inhibition, while attenuating ER stress and TAC-induced hypertrophy. These findings suggest that 4-PBA may be a therapeutic strategy to consider in preventing pressure-overload induced myocardial hypertrophy and interstitial fibrosis by selectively attenuating ER stress.

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Conflict of interest

The authors declare that there are no conflicts of interest.

Keywords

Myocardial hypertrophy; fibrosis; ER stress; 4-Phenylbutyric acid

1. Introduction

Endoplasmic reticulum (ER) is an organelle that is mainly responsible for the synthesis, folding and modification of proteins in eukaryotic cells [1]. The properly folded and modified proteins are then transported to Golgi apparatus, fused with the plasma membrane and released into the extracellular space. Alterations in the intracellular environment can interrupt the protein processing and result in the accumulation of unfolded proteins in the ER as well as activate the unfolded protein response (UPR) [2]. Triggers for this stress response include ischemia, hypoxia, glucose deprivation and oxidative stress, among many. While the UPR initially aims to restore ER functions and promote cell survival, when ER stress persists, it can activate apoptosis and cell death [3].

The activation of ER stress in the heart is associated with myocardial apoptosis [4,5], hypertrophy [6,7], and fibrosis [8,9], the pathological processes common in development of ischemic and hypertrophic heart diseases. Therefore, inhibiting ER stress may be an important consideration in developing treatments for ischemic or hypertrophic cardiomyopathy. To that effect, our previous study has indicated that attenuation of ER stress by administration of 4-Phenylbutyric acid (4-PBA) at 20 mg/kg/day in a mouse model of myocardial infarction (MI) prevents cardiac rupture and remodeling by inhibiting cardiac apoptotic and fibrotic signal pathways [10]. However, little is known whether the administration of 4-PBA is effective on mitigating hypertrophic heart disease.

As an inhibitor of ER stress, 4-PBA directly targets mutant and/or misfolded proteins in cells and aids proper protein trafficking, thus eliminating intracellular UPR [11–16]. The underlying mechanisms by which 4-PBA attenuates ER stress are associated with modulation of several molecules, including inhibition of GRP78, protein kinase RNA-like ER kinase (PERK), c-Jun NH2-terminal kinase (c-JNK) phosphorylation, X-box-binding protein 1 (XBP1) splicing, and CCAAT/enhancer-binding protein homologous protein (CHOP) expression [17]. Some of the ER stress indicators, in particular, GRP78 and CHOP, are significantly upregulated during the pathogenesis of left ventricular hypertrophy (LVH) [6]. Therefore, inhibiting ER stress using 4-PBA may be a promising therapeutic approach to mitigate hypertrophic heart disease.

Current research shows that orally administering 4-PBA daily for a week at a high dosage (100 mg/kg/day) plays a protective role against cardiac hypertrophy and fibrosis in mice [18]. However, whether the protective effect by 4-PBA may be sustained chronically at a lower dosage is unclear. In the present study, we tested the long term therapeutic effect of 4-PBA on pressure-overload induced myocardial hypertrophy at a much lower dosage. Our results showed that 4-PBA when administrated for 4 weeks intraperitoneally at 20 mg/kg/day, was effective in preventing pressure overload hypertrophy and interstitial fibrosis of the heart by inhibiting ER stress.

2. Materials and methods

2.1 Animal

All procedures were performed in accordance with our institutional guidelines for animal research that conforms to the *Guide for the care and Use of Laboratory Animals* (NIH Publication No. 85-23, revised 1996).

Briefly, 8-week-old adult C57/BL6 male mice (18–22 g) were anesthetized with a mixture of ketamine (100 mg/kg) and xylazine (5 mg/kg) by intraperitoneal injection. After successful endotracheal intubation (tidal volume: 0.3 ml; respiratory rate: 110 per minute), the chest cavity was opened in the second intercostal space at the left upper sternal border through a small incision. When the aortic arch was exposed, transverse aortic constriction (TAC) was then performed between brachiocephalic artery and left common carotid artery by tying a 7-0 nylon suture ligature against a 27-gauge needle to yield a narrowing 0.4 mm in diameter when the needle was removed and a reproducible TAC of 65–70% (see the diagram in Figure 1A). At the end of the procedure, the chest was closed by sutures.

After recovery from the surgery, the mice were intraperitoneally injected with 4-PBA (Sigma–Aldrich, St. Louis, MO) daily for 4 weeks at the dosage of 20 mg/kg/day. 4-PBA was first dissolved by DMSO then diluted in 0.9% NaCl. The animals were housed in the 12-12 cycle environment, and checked three times each day. The dosage of 4-PBA was determined according to our previous study [10]. At the end of 4 weeks, the surviving animals were subjected to echocardiographic imaging, then euthanized for autopsy, and histological analysis, and western blotting.

2.2 Echocardiographic imaging

At the end of 4 weeks after TAC, the mice were anesthetized with a mixture of ketamine and xylazine as described above and placed on a warmed platform. Then transthoracic echocardiographic imaging was performed using Siemens ultrasonic instrument (Acuson Sequoia 512) with Siemens Acuson 15L8 probe. The hearts were scanned using M-mode at the levels of papillary muscle for cardiac function analysis.

2.3 Autopsy and Histology

After mice were sacrificed, the hearts were rapidly excised, rinsed with PBS, fixed in 4% paraformaldehyde and embedded in paraffin. Then, 5μm-sections at the levels of papillary muscle were cut for histology. To evaluate the extent of cardiac fibrosis, each heart section was stained with Masson's trichrome kit (#HT15, Sigma-Aldrich). The extent of vascular fibrosis and interstitial fibrosis were determined as the ratio of the Masson's trichrome-stained area to the total vascular area and total left ventricular area, respectively.

2.4 Western blot analysis

The heart samples in the sham and TAC group with or without 4-PBA were excised and homogenized. The following antibodies were used for western blotting: anti-GRP78 (sc-376768, Santa Cruz), anti-CHOP (#2895, Cell Signaling Technology), anti-β-actin (sc-47778, Santa Cruz), anti-phospho-PERK (sc-32577, Santa Cruz), anti-PERK (sc-13073,

Santa Cruz), anti-Acetyl-Histone 3 (#9649, Cell Signaling Technology) and anti-Histone 3 (#4499, Cell Signaling Technology). Samples containing equal amounts of protein were separated by SDS-PAGE and transferred onto PVDF membranes. The membranes were blocked with 5% skim milk at room temperature for 2 h and then incubated overnight at 4°C with the primary antibody. After being incubated with anti-mouse secondary antibody (#7076S, Cell Signaling Technology) or anti-rabbit secondary antibody (#7074S, Cell Signaling Technology) for 1 h at room temperature, the blots were detected in the dark room using autoradiography film (Denville Scientific, Inc) and quantified by densitometry using the Image J Analysis software (National Institutes of Health).

2.5 Statistical analysis

All data are expressed as mean ± SEM, and $P< 0.05$ was considered to be statistically significant. Statistical differences were evaluated by one-way ANOVA followed by Bonferroni's multiple comparison exact probability test. All analyses were performed using SPSS 13.0 software (SPSS Inc, Chicago, IL, USA).

3. Results

3.1 ER stress response persists in the development of myocardial hypertrophy

As illustrated in Fig. 1A, we induced myocardial hypertrophy in C57BL/6 male mice by using the classic TAC method. After surgery, the aortic rupture and congestive heart failure were occasionally observed within the 4 weeks of pressure overload post TAC (Fig. 1B). The death rate in the TAC-treated mice was 20%. Of the 10 TAC-treated mice, 1 died from aortic rupture in the first 2 weeks and another died from congestive heart failure in the second half of the 4 week period. The dead mice were excluded from the experiment. The surviving animals developed pressure overload hypertrophy of the heart post TAC. As shown in Fig. 1C, the heart weight over body weight ratio (HW/BW) in the TAC group was significantly increased, compared with the sham group at 4 weeks. After the establishment of the TAC model, we investigated the temporal relationship of the ER stress response in the development of myocardial hypertrophy. The TAC mice were sacrificed at different time points, and the ER stress markers (GRP78, CHOP and phosphor-PERK) in the heart were examined by western blotting. As shown in Fig. 1D, the protein levels of GRP78, CHOP and phosphor-PERK were persistently increased in the entire period up to 4 weeks after TAC. These results suggest that ER stress remains activated in the pathogenesis of cardiac hypertrophy.

3.2 Low-dose 4-PBA selectively attenuates ER stress response while unaltering histone H3 acetylation

Because 4-PBA is an inhibitor of both ER stress [19] and histone deacetylase (HDAC) [20], we investigated whether 4-PBA had differential effects on ER stress and histone 3 deacetylation during development of pressure-load hypertrophy. In our TAC model, GRP78, CHOP, phosphor-PERK, and acetyl-histone 3 were all significantly increased post TAC. However, the treatment of 4-PBA at the dosage of 20 mg/kg/day for 4 weeks decreased ER stress response, while it did not affect histone 3 acetylation (Fig. 2A and B). Of note, when the 4-PBA dosage was increased to 100 mg/kg/day, the histone 3 acetylation was enhanced

both in the sham and TAC group (Fig. 2C). These findings suggest that 4-PBA at the lower dose of 20 mg/kg/day is effective at selectively inhibiting ER stress response while leaving histone H3 acetylation unaffected.

3.3 4-PBA ameliorates pressure-overload hypertrophy of the left ventricle

Based on our findings that ER stress was elevated in the development of cardiac hypertrophy and that 4-PBA efficiently inhibited the ER stress response, we tested if 4-PBA mitigated pressure overload hypertrophy *in vivo*. 4-PBA (20 mg/kg/day) was intraperitoneally injected into the TAC-treated mice for 4 weeks, followed by echocardiographic imaging. At the end of 4 weeks, the left ventricular posterior wall thickness at end systole (LVPWs), left ventricular posterior wall thickness at end diastole (LVPWd) and fractional shortening in percentage (FS%) in the TAC-treated mice were significantly increased, as expected, compared to the sham group. Administration of 4-PBA significantly inhibited this hypertrophic response while preserving the systolic function (Fig. 3A, B, C and D). Of note, 4-PBA did not affect the heart rate between the two treatment groups (Fig. 3E). Consistent with the echocardiographic data, findings from autopsy demonstrated that the HW/BW was lower in the TAC+4-PBA group, compared to the TAC-treated group without 4-PBA (Fig. 3F).

3.4 4-PBA inhibits TAC-associated myocardial interstitial fibrosis

We further examined whether 4-PBA had impact on the extent of cardiac fibrosis. As shown in Fig. 4A, we found extensive peri-vascular and interstitial fibrosis in the hearts of the TAC-treated mice at the end of 4 weeks. The administration of 4-PBA did not change the extent of peri-vascular fibrosis (Fig. 4B) but significantly decreased the interstitial fibrosis (Fig. 4C).

4. Discussion

Our present study supports that ER stress is an important factor in the pathogenesis of pressure overload hypertrophy of the heart. First, we demonstrated *in vivo* that the ER stress response (manifested by increased protein levels of GRP78, CHOP and p-PERK) was persistent 4 weeks after TAC. We also demonstrated that TAC-induced ER stress was inhibited by long-term administration of 4-PBA at a low dose of 20 mg/kg/day. Furthermore, 4-PBA attenuated TAC-induced LVH and decreased the extent of interstitial fibrosis in the myocardium.

Cardiac hypertrophy and fibrosis accompany many forms of heart disease. While the etiology behind the hypertrophy and fibrosis is diverse, they may intersect in a shared cellular mechanism in the form of the ER stress response. For example, in cultured neonatal rat cardiomyocytes, application of ER stress inducer thapsigargin (TG) produced cellular hypertophy in a dose- and time-dependent manner [7]. TG also triggered the UPR and accumulation of intracellular procollagen in cultured rat cardiac fibroblasts [21]. Furthermore, accumulating in-vivo data from the pressure overload model implicates ER stress as a central player in the development of cardiac hypertrophy [22–24]. In this study, we shed light on the temporal relationship between ER stress and LVH, demonstrating that

the ER stress response persists up to 4 weeks after TAC. Although mechanisms by which ER stress modulates myocardial hypertrophy and interstitial fibrosis remain unclear, some suggest involvement of CaN-MEF2c signaling pathways in cardiomyocytes [7] and TGFβ1-Smad2/3 in cardiac fibroblasts [10]. Further investigations are needed to detail the putative mechanisms.

Interestingly, although ER stress response was reported to be responsible for proliferation, migration and collagen synthesis in vascular smooth muscle cells (VSMCs) in atherosclerosis and vascular injury [25,26], we did not observe significant modulation of peri-vascular fibrosis by 4-PBA in the hearts of TAC-treated mice in the present study. It is possible that 4-PBA, at 20 mg/kg/day, may be insufficient to mitigate the fibrotic signaling transduction in VSMCs under mechanical stress created by pressure overload or hypertension.

4-PBA, also called sodium phenylbutyrate or Buphenyl, is a low-molecular-weight fatty acid and a non-toxic pharmacological compound that was originally used as a nitrogen-scavenging medication for chronic management of urea cycle disorders [27]. In the last decade, it was also shown to be a histone deacetylase inhibitor (HDACI) and chemical chaperone, leading to its use in clinical trials and preclinical research studying various conditions, including sickle cell anemia [28], beta thalassemia [29], cirrhosis and hepatic encephalopathy [30], acute myeloid leukemia [31], solid tumors [32] and various motor neuron disorders [33]. It should be noted that most of these studies used 4-PBA at much higher dosages (>100 mg/kg/day) [34]. On the contrast, our study showed that the in-vivo administration of low dose 4-PBA at 20 mg/kg/day selectively attenuated ER stress response and reduced LVH associated with pressure overload while having no effect on histone 3 acetylation. When the dosage was increased to 100 mg/kg/day, the acetylation of histone 3 was upregulated. Though HDACI actions of 4-PBA have been linked to attenuation of hypertension or pressure overload-induced cardiac hypertrophy and heart failure [35,36], our data show that 4-PBA selectively dosed as an ER stress inhibitor may be behind and/or overlap with the HDACI effect on its anti-hypertrophic actions.

4-PBA metabolizes to phenylacetate (PAA) by β-oxidation in the liver. Then PAA is conjugated with glutamine to form phenylacetylglutamine (PAG), which is eliminated in the urine. There are few studies reporting pharmacokinetics of 4-PBA in animal models. In clinical trials, when 4-PBA was intravenously administrated in patients with refractory solid tumors for 5 days at 410 mg/kg/day, the plasma concentration of 4-PBA and PAA was maintained at 500 μmol/liter and 1000 μmol/liter, respectively, throughout the duration of the infusion [34]. The high concentration of 4-PBA was required to influence histone deacetylation and antitumor effects. Our current study shows that the low dosed 4-PBA effectively inhibited TAC-triggered ER stress and LVH, while histone deacetylation remained unaffected.

In conclusion, our study is the first to demonstrate that (1) cardiac ER stress response is sustained during the development of myocardial hypertrophy and fibrosis up to 4 weeks after the onset of pressure overload, (2) the inhibitory effect of 4-PBA on ER stress can be differentiated from that on HDAC by selective dosing, and (3) attenuation of ER stress by 4-

PBA significantly reduces pressure-induced LVH and associated myocardial fibrosis. These novel findings may provide a potential target for innovate treatments of hypertensive heart disease.

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Abbreviations

4-PBA	4-Phenylbutyric acid
ER	endoplasmic reticulum
UPR	unfolded protein response
MI	myocardial infarction
PERK	protein kinase RNA-like ER kinase
c-JNK	c-Jun NH2-terminal kinase
XBP1	X-box-binding protein 1
CHOP	CCAAT/enhancer-binding protein homologous protein
TAC	transverse aortic constriction
LVH	left ventricular hypertrophy
LVPWs	left ventricular posterior wall thickness at end systole
LVPWd	left ventricular posterior wall thickness at end diastole
HDAC	histone deacetylase
HDACI	histone deacetylase inhibitor
TG	thapsigargin
VSMCs	vascular smooth muscle cells
PAA	phenylacetate
PAG	phenylacetylglutamine

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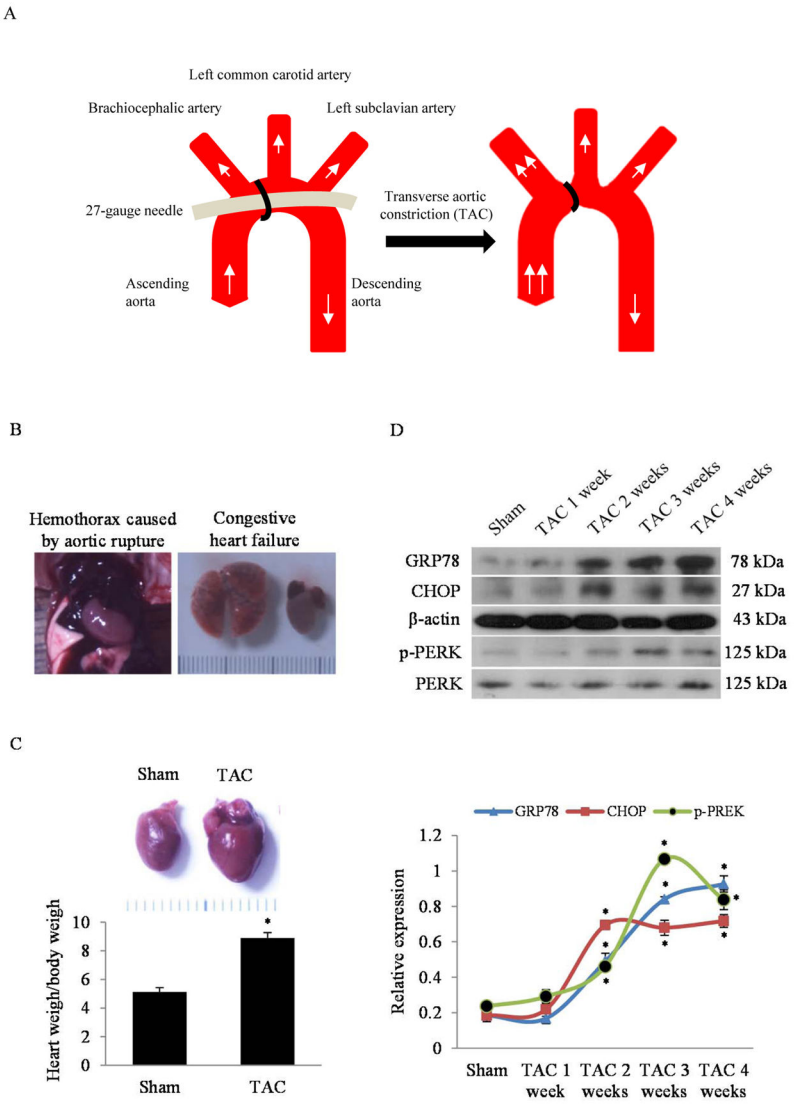

Fig. 1. Cardiac hypertrophy and ER stress response in mice following TAC

(A) The diagram of transverse aortic constriction (TAC) in mouse. (B) The representative autopsy pictures of aortic rupture and congestive heart failure within the 4 weeks of TAC. (C) Representative autopsy pictures of the normal heart and hypertrophic heart induced by pressure overload for 4 weeks and the corresponding quantitative analysis of heart weight over body weight ratio. $^{*}P<0.05$ vs. Sham; n=8 in each group. (D) Western blots and quantitative analysis of ER stress response markers (GRP78, CHOP and p-PERK) in the whole heart homogenates of mice in designated time points after TAC. $^{*}P<0.05$ vs. Sham; n=3.

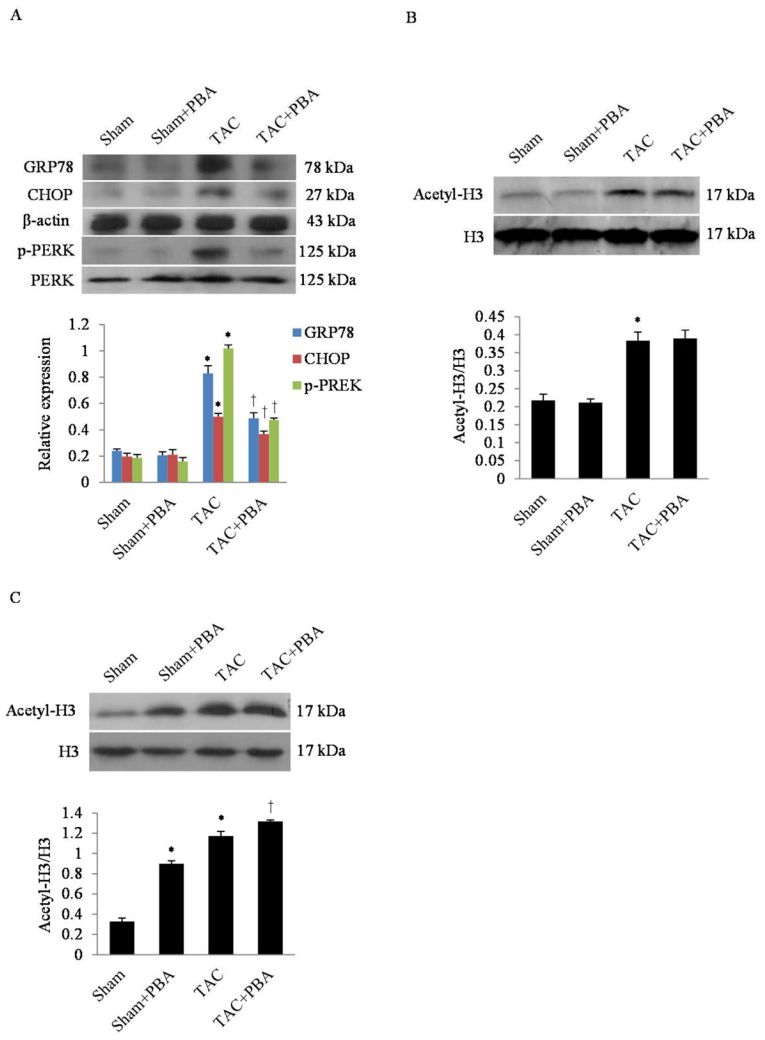

Fig. 2. Effects of 4-PBA treatment on ER stress response and histone acetylation

(A) Representative western blots and quantitative analysis of ER stress response markers (GRP78, CHOP and p-PERK) in the whole heart homogenates of mice with or without 4-PBA at 4 weeks after TAC. $^{*}P<0.05$ vs. Sham; $^{\dagger}P<0.05$ vs. TAC; n=3. (B) Western blots and quantitative analysis of Histone 3 acetylation in the whole heart homogenates of mice with or without 4-PBA (20 mg/kg/day) after 4 weeks of TAC. $^{*}P<0.05$ vs. TAC; n=3. (C) Western blots and quantitative analysis of Histone 3 acetylation in the whole heart homogenates of mice with or without 4-PBA (100 mg/kg/day) after 4 weeks of TAC. $^{*}P<0.05$ vs. Sham; $^{\dagger}P<0.05$ vs. TAC; n=3.

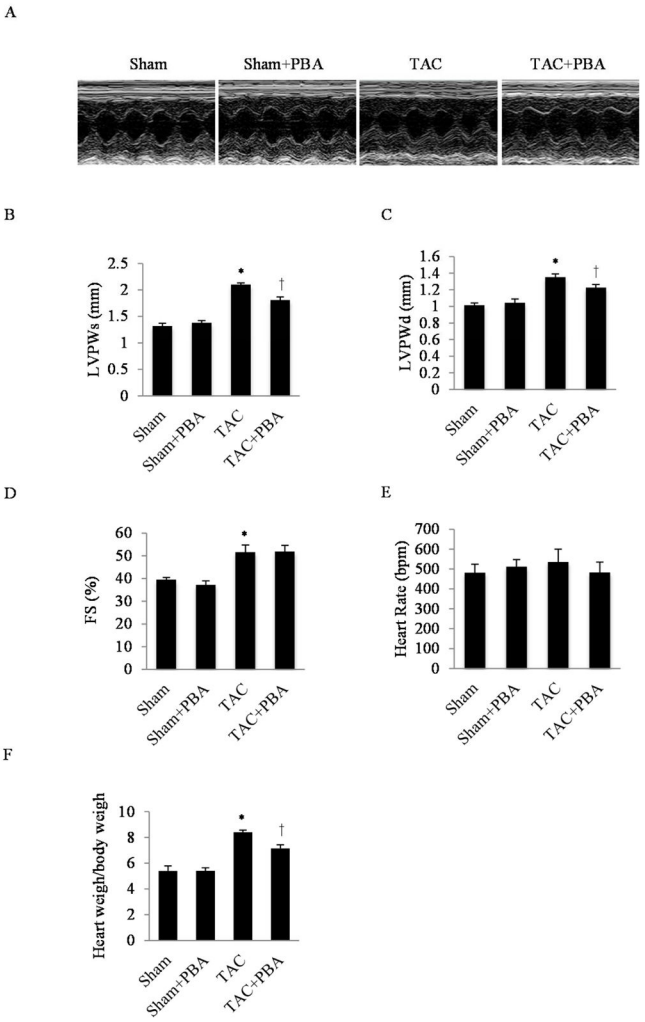

Fig. 3. Effects of 4-PBA treatment on cardiac hypertrophy

(A) Representative pictures of M-mode echocardiographic imaging in mice after 4 weeks of TAC. (B) Quantitative analysis of left ventricular posterior wall thickness at systole (LVPWs). (C) Quantitative analysis of left ventricular posterior wall thickness at diastole (LVPWd). (D) Quantitative analysis of left ventricular fractional shortening (FS%). (E) Quantitative analysis of heart rate. (F) Quantitative analysis of the heart weight over body weight ratio (HW/BW). $^{*}P<0.05$ vs. Sham; $^{\dagger}P<0.05$ vs. TAC; n=5 in each group.

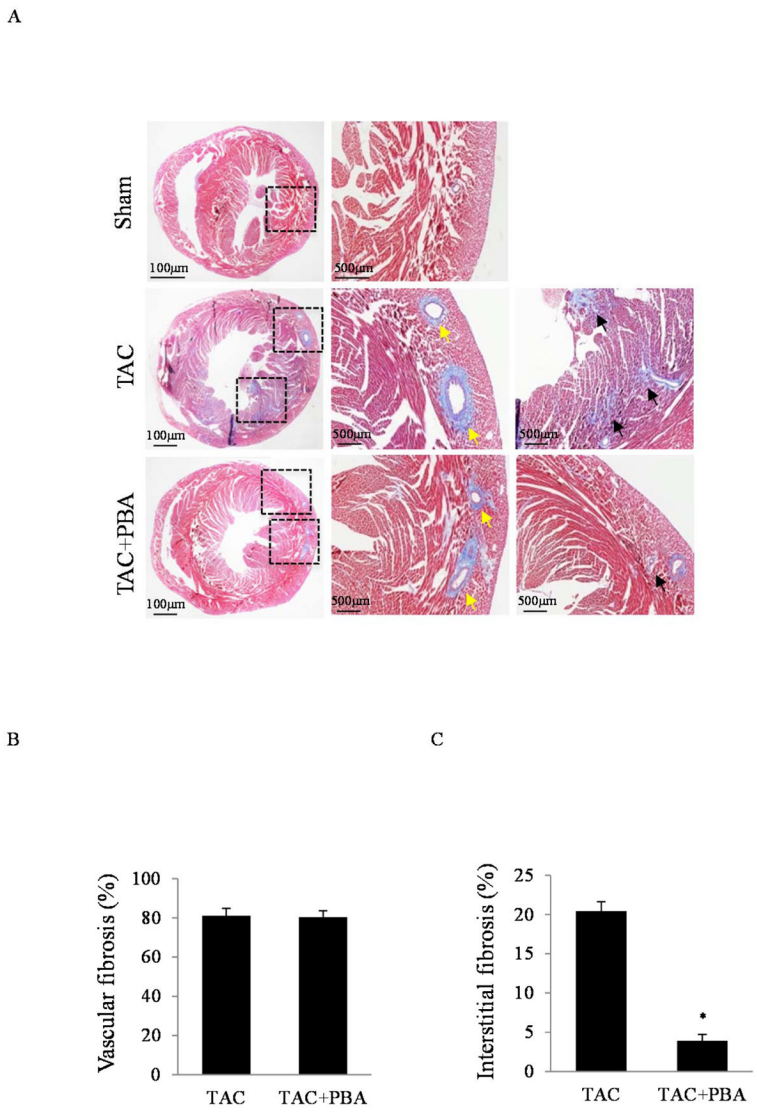

Fig. 4. Effects of 4-PBA treatment on cardiac fibrosis

(A) Masson staining of cardiac fibrosis from the hearts of mice with or without 4-PBA administration at the end of 4 weeks after TAC. Perivascular fibrosis (yellow arrow) and interstitial fibrosis (black arrow) were identified. (B) Quantitative analysis of vascular fibrosis. (C) Quantitative analysis of interstitial fibrosis. $^{*}P<0.05$ vs. TAC.