

Original Paper

Cardiac-Specific PID1 Overexpression Enhances Pressure Overload-Induced Cardiac Hypertrophy in Mice

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Key Words

PID1 • Cardiac hypertrophy • Akt • MAPK

Abstract

Background/Aims: PID1 was originally described as an insulin sensitivity relevance protein, which is also highly expressed in heart tissue. However, its function in the heart is still to be elucidated. Thus this study aimed to investigate the role of PID1 in the heart in response to hypertrophic stimuli. **Methods:** Samples of human failing hearts from the left ventricles of dilated cardiomyopathy (DCM) patients undergoing heart transplants were collected. Transgenic mice with cardiomyocyte-specific overexpression of PID1 were generated, and cardiac hypertrophy was induced by transverse aortic constriction (TAC). The extent of cardiac hypertrophy was evaluated by echocardiography as well as pathological and molecular analyses of heart samples. **Results:** A significant increase in PID1 expression was observed in failing human hearts and TAC-treated wild-type mouse hearts. When compared with TAC-treated wild-type mouse hearts, PID1-TG mouse showed a significant exacerbation of cardiac hypertrophy, fibrosis, and dysfunction. Further analysis of the signaling pathway *in vivo* suggested that these adverse effects of PID1 were associated with the inhibition of AKT, and activation of MAPK pathway. **Conclusion:** Under pathological conditions, over-expression of PID1 promotes cardiac hypertrophy by regulating the Akt and MAPK pathway.

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Introduction

Cardiac hypertrophy is a common response of the myocardium to a variety of pathological stimuli including hypertension, valve disease, myocardial ischemia, and genetic

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mutations, characterized by an increase in the size of individual cardiac myocytes and whole organ enlargement [1, 2]. Although hypertrophic response is initially compensatory elicited by an increased workload, prolonged cardiac hypertrophy eventually leads to functional and histological deterioration of the myocardium, fibrosis, inflammation, and altered cardiac gene expression and then congestive heart failure, arrhythmia, and sudden death [3-5].

Many researchers in recent decades have attempted to elucidate the underlying mechanisms of pathological hypertrophy and have sought to reverse its maladaptive consequences. Despite that many signaling pathways involved in the hypertrophic process have been identified [6], the underlying molecular mechanisms for cardiac hypertrophy are still poorly understood, which hampers the efficient treatment of this cardiac condition in the clinical practice. Thus, it is important to define and modulate the specific signaling mechanism activated by each hypertrophic stimulus to enable the discovery of novel molecular targets for suppressing maladaptive hypertrophy.

Phosphotyrosine interaction domain containing 1 (PID1, also named as NYGGF4) was a novel gene which was isolated and characterized in obese subjects initially. It is a 1527-bp cDNA, containing 753 nucleotides of an ORF (open reading frame) predicting 250 amino acids with a molecular mass of 28.27 kDa [7]. Amino acid sequence analysis revealed PID1 has a phosphotyrosine-binding (PTB) domain, which was initially identified based on the ability to recognize phosphorylated tyrosine residues. The PTB domains are found in many cytoplasmic signaling proteins, allowing them to bind specifically to other polypeptides that are tyrosine-phosphorylated in response to growth factor stimulation [8].

Northern blot analysis revealed PID1 is expressed primarily in adipose tissue, heart, and skeletal muscle but not in any other tissue examined [7]. PID1 is highly expressed in the heart, suggesting a possible function for PID1 in cardiac disease. Previously studies indicated that PID1 acts directly on the IRS1/PI3K/AKT insulin pathway to reduce glucose uptake and transport and impairs mitochondrial function in adipose tissue [9]. Whether energy metabolism abnormality or mitochondrial dysfunction is thought to be tightly associated with the development of cardiac hypertrophy [10]. However, the role of PID1 in cardiac hypertrophy has not yet been fully investigated. Given that PID1 is an important signaling protein in AKT signaling [9], which is key players in cardiac hypertrophy, we hypothesized that PID1 may be involved in the cardiac hypertrophic process. In the present study, we employed cardiac specific PID1-overexpressing transgenic (TG) mice to determine the role of PID1 in murine hearts in response to transverse aortic constriction (TAC) and the related molecular mechanisms.

Materials and Methods

Human heart samples

Samples of human failing hearts were collected from the left ventricles of dilated cardiomyopathy (DCM) patients undergoing heart transplants. Control samples were obtained from the left ventricles of normal heart donors who died in accidents but whose hearts were not suitable for transplantation for non-cardiac reasons. The samples were obtained after informed consent and with approval of the institutional review boards (Chinese Academy of Medical Sciences and Nanjing Medical University Ethics Committee, Nanjing, China). This study conforms to the principles outlined in the Declaration of Helsinki.

Generation of α -MHC-PID1 transgenic (PID1-TG) mice

All of the animal protocols were approved by the Animal Care and Use Committee at the Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences and Nanjing Medical University Ethics Committee. Full-length human PID1 cDNA was cloned downstream of the cardiac myosin heavy chain (MHC) promoter. The α -MHC-PID1 construct was microinjected into fertilized mouse embryos (C57BL/6J background) to produce transgenic mice. Three independent transgenic lines were established and studied. Tail genomic DNA was used to identify transgenic mice by PCR analysis.

Animal models

We used 7- to 8-week-old male mice with cardiac-specific expression of human PID1 and their control littermates. Transverse aortic constriction (TAC) was performed as described previously [11]. Age- and sex-matched WT and TG mice were anesthetized with isoflurane. A 7.0 nylon suture ligature was tied against a 27-gauge needle at the transverse aorta to produce a 65–70% constriction following removal of the needle. Echocardiology was performed to analyze the cardiac function after TAC surgery. Animals were anesthetized for echocardiology and euthanized 4 weeks after surgery. Echocardiography was performed via a MyLab 30CV ultrasound (BiosoundEsaote Inc.) with a 10 MHz linear array ultrasound transducer. The LV was assessed in both parasternal long-axis and short-axis views at a frame rate of 120 Hz. Endsystole or end-diastole was defined as the phase in which the smallest or largest area of LV, respectively, was obtained. LV end-systolic diameter (LVESD), LV end-diastolic diameter (LVEDD), and LV wall thickness were measured from the LV M-mode tracing with a sweep speed of 50 mm/s at the mid-papillary muscle level. Per cent fractional shortening (%LVFS) and ejection fraction (%LVEF) were calculated. After assessed by echocardiology, the hearts of the euthanized mice were dissected and weighed to examine HW/BW (in milligrams per grams).

Histological and morphometric analysis

Hearts were excised, placed immediately in a 10% potassium chloride solution to ensure that they were stopped in diastole, washed with saline solution, and placed in 10% formalin. The hearts were cut transversely close to the apex to visualize the left and right ventricles. The samples were then dehydrated in an ethanol gradient, rinsed in xylene and embedded in paraffin. Finally, paraffin blocks were cut into 5µm sections. The paraffin sections were stained in hematoxylin-eosin (H&E) for histopathology and Masson for collagen deposition and then visualized by light microscopy. To determine the cross-sectional area (CSA) of the myocytes, the HE-stained sections were used. A single myocyte was measured using a quantitative digital analysis imaging system (Image-Pro Plus 6.0). Between 100 and 150 myocytes in the left ventricles were outlined in each group.

Real-time quantitative RT-PCR

Total RNA was isolated using TRIzol reagent (Invitrogen) following the manufacturer's protocol. First-strand cDNA was made using reverse transcriptase (New England Biolabs). Real-time PCR with SYBR green (Takara) was used to examine the relative levels of selected mRNAs. All data were normalized to the internal standard (GAPDH). Each sample was measured in triplicate, and the gene expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method. Sequences for real-time PCR primer pairs are listed in Table 1. The primer was designed by Primer-3 software, and then its efficiency was determined through the main peak by RT-qPCR.

Western blotting

Cardiac tissues were lysed in RIPA lysis buffer. Protein was measured using a protein assay kit (Thermo), and equal amounts of samples (50µg per lane) were separated using PAGE with 4-12% Bis-Tris gels (Invitrogen), and subsequently transferred to PVDF membranes. Immunoblots were incubated overnight at 4°C with the antibody (CST, Santa Crus, Sigma, Abcam). After washing, the immunoblots were

Table 1. The primers sequence in this study

Gene name	Forward 5'-3'	Reverse 5'-3'
PID1	ATGTTTCAGCCTGCCCTTAG	GCCATCATCGGATTCGAATTC
ANP	ACCTGCTAGACCACCTGGAG	CCTTGCTGTTATCTTCGGTACCGG
BNP	GAGGTCACCTCTATCCTCTGG	GCCATTTCTCCGACTTTTCTC
β-MHC	TTCATCCGAATCCATTTTGGGG	GCATAATCGTAGGGGTTGTTGG
Col1agen I	AGGCTTCAGTGGTTTGGATG	CACCAACAGCACCATCGTTA
Col1agen III	CCCAACCCAGAGATCCCATT	GAAGCAGAGCAGGTGTAGA
CTGF	TGACCCCTGCGACCCACA	TACACCGACCCACCGAAGACACAG
Fibronectin	ACCGAAGCCGGGAAGAGCAA	ACCGAAGCCGGGAAGAGCAA
GAPDH	ACTCCACTCACGGCAAATTC	TCTCCATGGTGGTGAAGACA

incubated with secondary IgG antibodies. Immunoblots were scanned using the Odyssey Infrared Imaging System (LI-COR), and all determined values were normalized to GAPDH.

Statistical analysis

Data analysis was performed with SPSS (Statistical Package for the Social Sciences) version 3.0 (SPSS, Inc., Chicago, IL). The data are presented as the mean \pm SD. Paired data were compared using a Student's t-test. Differences among groups were determined using a 1-way or 2-way analysis of variance (ANOVA) with repeated measures, followed by the Bonferroni post hoc test. A probability value of 0.05 was considered significant.

Results

PID1 expression is up-regulated in human failing hearts and murine hypertrophic hearts

To investigate the potential role of PID1 in cardiac hypertrophy, we first examined PID1 expression in the left ventricles of DCM patients undergoing heart transplantation because of end-stage heart failure. Real-time RT-PCR analysis showed that PID1 mRNA levels were increased, accompanied with increased mRNA levels of foetal genes ANP in failing hearts, compared with donor hearts. Accordingly, results of western blotting revealed that protein levels of PID1 were increased in human failing samples relative to normal donor hearts (Fig. 1A and C). Similarly, level of the PID1 protein was increased in murine hearts over 4 weeks of TAC (Fig. 1B and D). These data implicate the possible involvement of PID1 in cardiac hypertrophy and cardiomyopathy.

Characterisation of cardiac-specific PID1 overexpression mice

To define the role of PID1 cardiac hypertrophy *in vivo*, we generated a transgenic (TG) mouse model with cardiac-specific overexpression of PID1 using the α -myosin heavy chain promoter (Fig. 2A). Western blot analysis demonstrated that PID1 was successfully overexpressed in hearts from three TG mouse lines (Fig. 2B). We then selected the mouse line (TG3) that expressed the highest levels of PID1 the heart for the following studies. The PID1 protein was robustly expressed in the heart but not in other organs, compared with WT mice, the PID1 expression level in heart tissue was significantly increased in TG mice, indicating cardiac specificity of the transgene expression (Fig. 2C).

Overexpression of PID1 facilitate pressure overload-induced cardiac hypertrophy in vivo

The results of the gross heart and HE staining displayed an exaggerated hypertrophic effect of PID1 overexpression on cardiac remodeling after TAC (Fig. 3A). It is important to note here that, under basal conditions, PID1-TG mice did not show any pathological/physiological alterations in their heart morphology and contractile function (data not shown). However, 4 weeks after TAC, wild-type (WT) mouse hearts exhibited a significant enlargement, wherein the size of cardiomyocytes was increased, as well as increased ratios of heart weight/body weight (HW/BW), compared with those of sham-operated mice. Remarkably, however, these parameters were more pronounced in PID1-TG hearts upon 4-week TAC, relative to TAC-treated WT samples (Fig. 3B). Consistently, the mRNA levels of several hypertrophy markers including ANP, BNP, and β -MHC, were significantly increased in TG hearts, compared with WTs after 4-week TAC (Fig. 3C). Moreover, PID1-TG mice showed an aggravated cardiac dilation and dysfunction compared with WT mice in response to chronic pressure overloading, as evidenced by echocardiographic parameters (Fig. 4, Table 2). To further determine the effect of PID1 overexpression on maladaptive cardiac remodeling, we examined cardiac fibrosis, a classical feature of developing pathological cardiac hypertrophy, in these pressure-overloading hearts. Fibrosis was quantified by visualizing the total amount of collagen present in the interstitial spaces of the heart and by measuring the collagen volume. Our results showed that 4 week TAC dramatically produced interstitial fibrosis in WT hearts, which was further aggravated in TG hearts. Accordingly, TG hearts exhibited significant

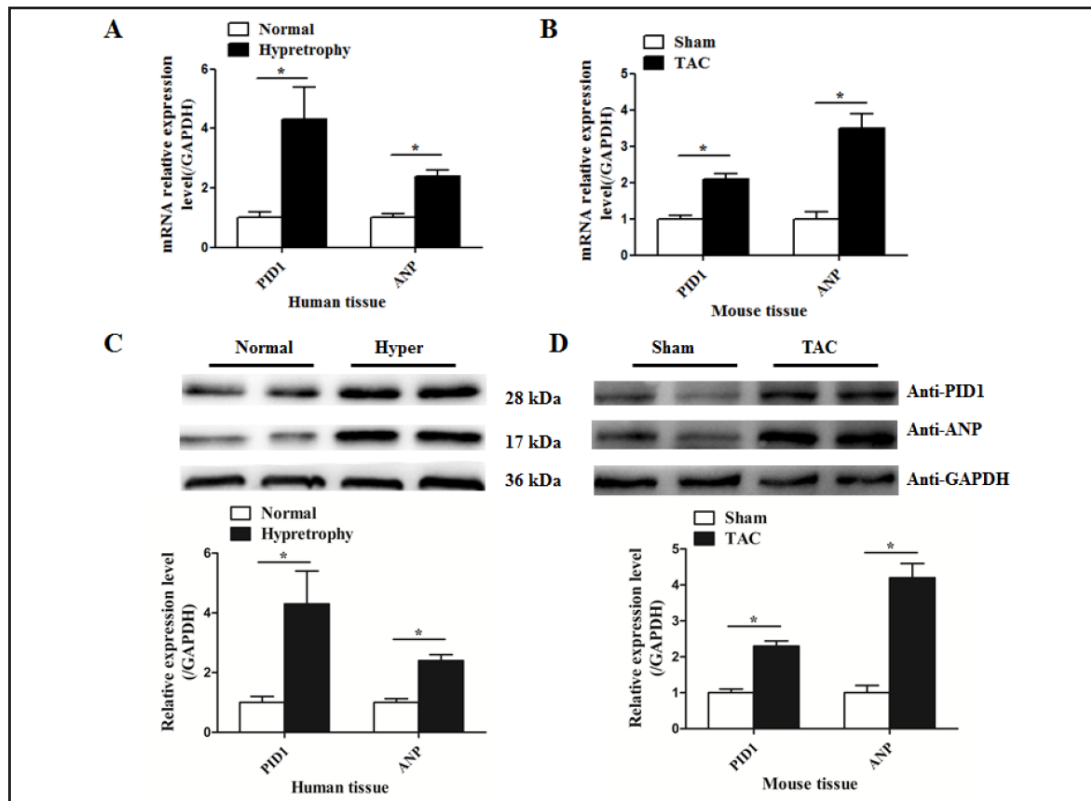


Fig. 1. PID1 expression in failing human hearts and experimental hypertrophic models. (A) Real-time PCR analysis of PID1 and ANP expression in human failing (n=4) and donor hearts(n=4). (B) Real-time PCR analysis of PID1 and ANP expression in WT hearts after TAC at the indicated time points (n=6). (C) Representative Western blots of PID1 in the hearts of normal donors (n=4) and patients with heart failure (n=4). (D) Representative Western blots of PID1 WT hearts after TAC at the indicated time points (n=6). *P<0.05 vs. normal donors or sham.

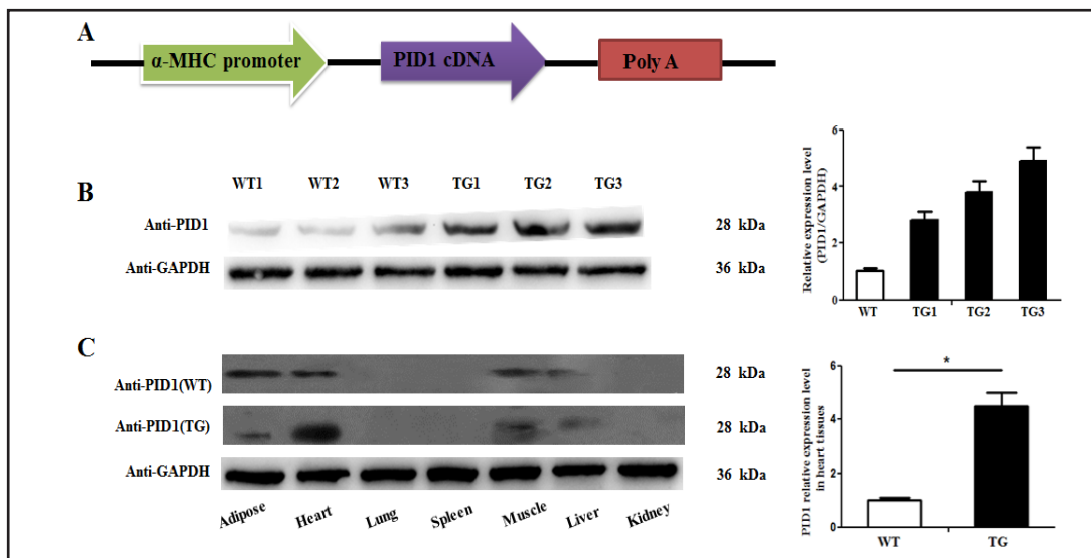


Fig. 2. α -MHC-PID1 transgene construction (A) Schematic diagram of the α -MHC-PID1 transgene construct. (B) Representative western blots of the PID1 protein in heart tissues from three TG lines and WT mice. (C) Representative western blots of the PID1 protein in various tissues from the TG and WT mice. Compared with WT mice, the PID1 expression level in heart tissue was significantly increased in TG mice.

Fig. 3. (A) Gross hearts and HE staining 4 weeks after sham or TAC surgery, magnification is 10x (upper and middle) and 40x (lower) respectively (B) Statistical results of the myocyte CSA and HW/BW of the indicated groups. (n=12–14). (C) The TAC-induced expression of hypertrophic markers (ANP, BNP, and β -MHC) was determined by real-time PCR analysis in WT and TG mice. (n=7). *P<0.05 vs. WT/sham; #P<0.05 vs. WT/TAC.

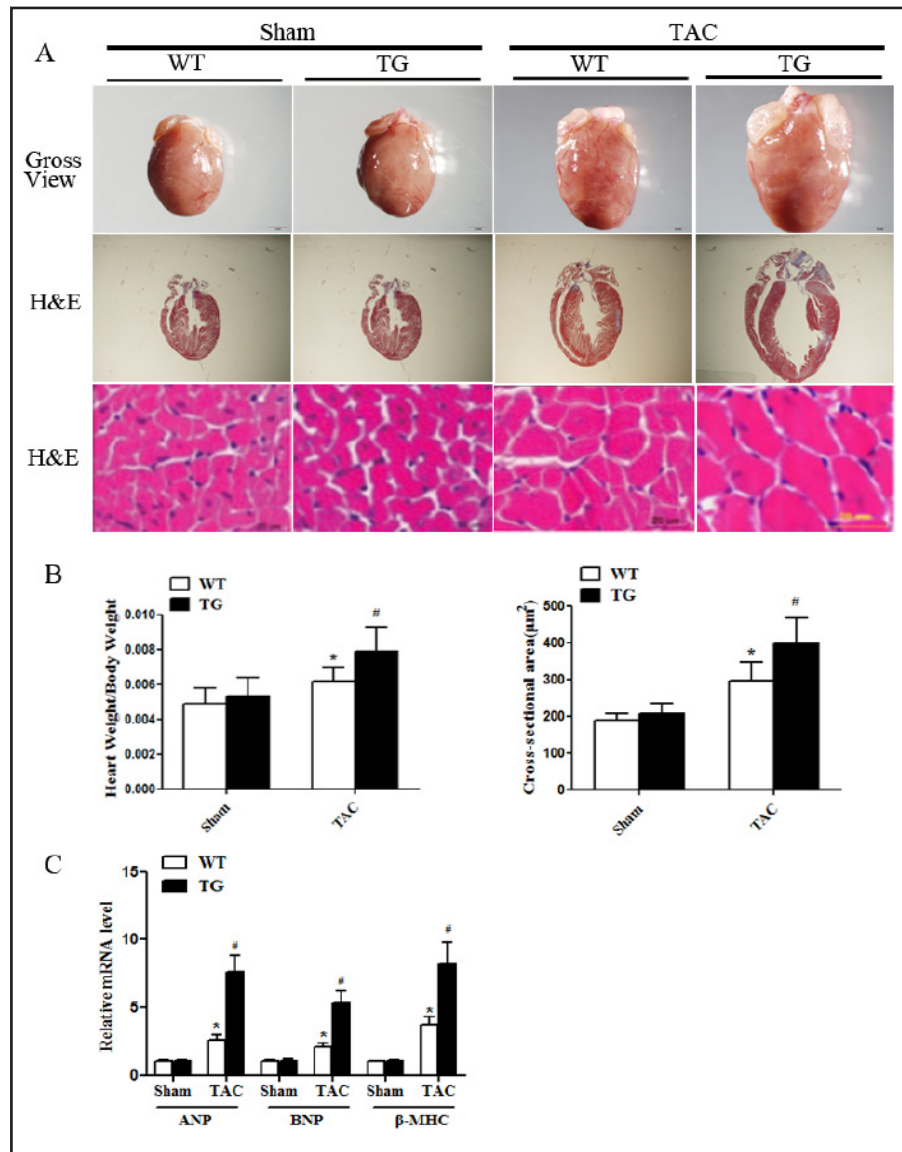
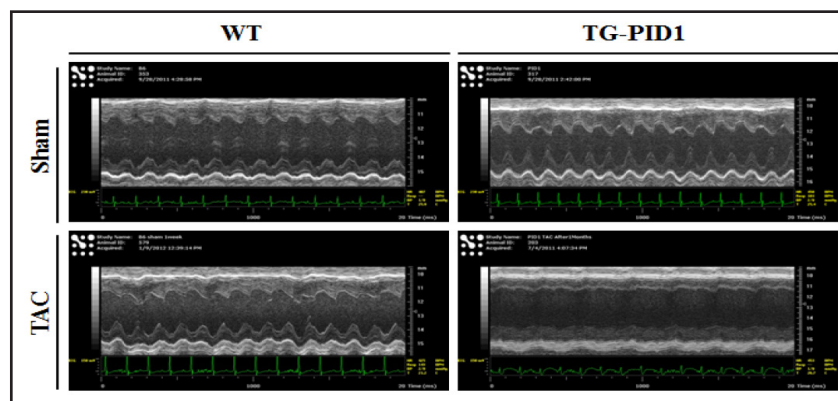


Fig. 4. Representative echocardiographic M-mode pictures in PID1 TG mice and WT littermates at 4 weeks after sham operation or TAC.



increases in total collagen volume (Fig. 5A and B), and mRNA levels of collagen I, collagen III, CTGF and fibronectin, known mediators of fibrosis (Fig. 5C), compared with WT in response to 4-week TAC. Collectively, these results provide first *in vivo* evidence that overexpression of PID1 in the heart would contribute to maladaptation to chronic pressure overloading.

Table 2. Echocardiographic parameters in PID1 TG mice and WT littermates at 4 weeks after sham operation or TAC. All values are mean \pm SEM. BW, body weight; HR, heart rate; LVEDD, left ventricular end diastolic diameter; LVESD, left ventricular end systolic diameter; PWT, posterior wall thickness; IVSd, left ventricular septum diastolic; EF, ejection function.* P< 0.05 vs. WT sham operation. # P<0.05 vs. WT TAC after 4 weeks

Parameters	WT-Sham	PID1-Sham	WT-TAC	PID1-TAC
Number	9	9	9	9
BW (g)	25.7 \pm 0.5	25.9 \pm 0.7	26.2 \pm 0.6	27.5 \pm 0.6
HR (bpm)	485 \pm 43	496 \pm 46	493 \pm 45	488 \pm 51
IVS; d (mm)	0.77 \pm 0.3	0.78 \pm 0.1	0.88 \pm 0.2*	0.99 \pm 0.1#
LVEDd (mm)	3.61 \pm 0.4	3.69 \pm 0.3	4.16 \pm 0.6*	4.76 \pm 0.4#
LVESd (mm)	2.35 \pm 0.4	2.30 \pm 0.3	2.90 \pm 0.4*	3.2 \pm 0.5#
PWT (mm)	0.70 \pm 0.07	0.74 \pm 0.05	0.88 \pm 0.08*	0.97 \pm 0.09#
EF (%)	66.9 \pm 8.1	68.8 \pm 6.3	56.2 \pm 6.4*	45.9 \pm 14.2#
FS (%)	40.4 \pm 4.6	42.2 \pm 3.8	35.2 \pm 5.3*	30.1 \pm 3.7#

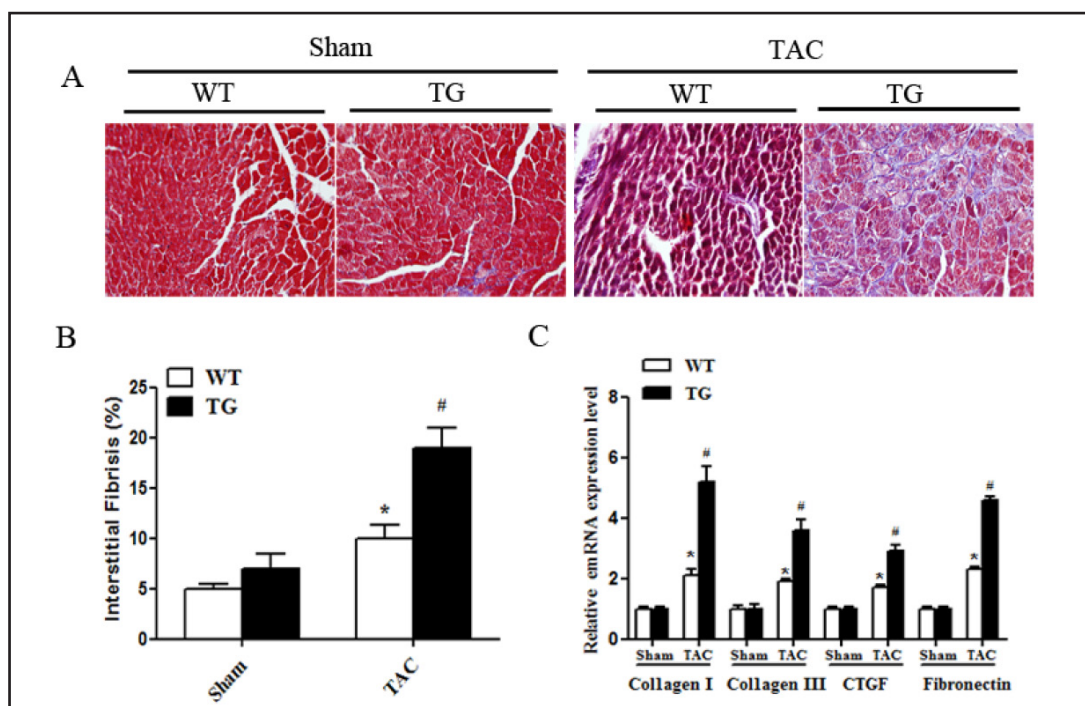


Fig. 5. (A) Masson staining of histological LV sections was performed on the indicated groups 4 weeks after TAC, magnification is 20x (B) Fibrotic areas in the histological sections were quantified using an image analysis system. (n=6). (C) A real-time PCR analysis was performed to determine mRNA expression levels of collagen I, collagen III, CTGF and fibronectin in the indicated mice. (n=6), *P<0.05 vs. WT/sham; #P<0.05 vs. WT/TAC.

PID1 regulates Akt and MAPK signaling pathway in hypertrophic hearts

To dissect the possible mechanisms by which PID1 enhanced cardiac remodeling after TAC, we next investigated the Akt signaling and mitogen-activated protein kinases (MAPK) signaling, two pathways known to be involved in pathological cardiac hypertrophy. Our western blotting results showed that TAC caused a similar degree of activation of the AKT signaling molecules (i.e. GSK3 β , mTOR and P70S6K) in both TG and WT hearts. Moreover,

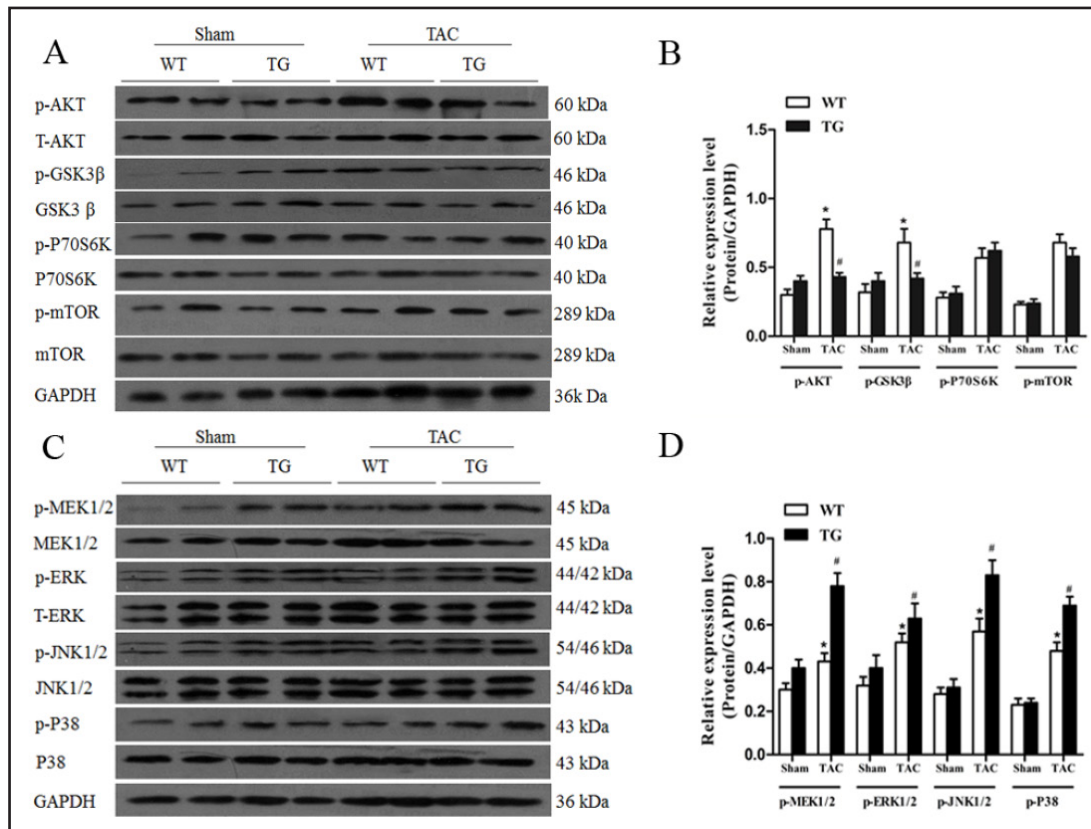


Fig. 6. Effects of PID1 on the AKT and MAPK signaling pathway. (A) Representative western blots and quantitative results showing the phosphorylation and total protein levels of AKT, GSK3 β , mTOR, and P70S6K at 4 weeks after Sham or TAC surgery in TG and WT mice, (n=3), *P<0.05 vs.WT/sham; #P<0.05 vs. WT/TAC. (B) Statistical analysis of Western Blot. GAPDH was used as a loading control. (C) Representative western blots and quantitative results showing the phosphorylation and total protein levels of MEK1/2, JNK1/2, ERK1/2 and P38 at 4 weeks after Sham or TAC surgery in WT and TG mice, (n=3), *P<0.05 vs.WT/sham; #P<0.05 vs. WT/TAC. (D) Statistical analysis of Western Blot. GAPDH was used as a loading control.

although AKT and GSK3 β phosphorylation was partially blocked by PID1-overexpression, elevation of the phosphorylated P70S6K and mTOR was similar between PID1-TGs and WTs (Fig. 6A and B). These data indicate that hypertrophic effects of PID1 may not be entirely associated with the AKT-GSK3 β -mTOR axis. Therefore, we further examined the MAPK pathway and excitingly observed that TAC-induced activation of the MAPK pathway was more pronounced in TG mice than in WT mice, as evidenced by a significant increase of MEK1/2, ERK, JNK1/2 and p38 phosphorylation levels in TG hearts, compared with WTs (Fig. 6C and D). These results suggest that PID1 may exert its hypertrophic effects by promoting MAPK signaling activation. Taken together, these data indicate that PID1-mediated exacerbation of pathological cardiac hypertrophy is associated with activation of the MAPK signaling pathway.

Discussion

It has been well documented that cardiac hypertrophy is an important cause of heart failure that reciprocally reinforces the development of heart failure [6, 12]. However, our understanding of the pathogenesis of cardiac hypertrophy at the molecular level remains extremely limited. The molecular mechanisms that modulate the transition from

compensated hypertrophy to dysfunctional heart failure need further to be expounded. In the present study, we identify PID1 as a positive hypertrophic factor that enhances maladaptive remodeling and the transition to heart failure. The major findings of this study are as follows: (1) A significant increase in PID1 expression was observed in the hearts of patients with hypertrophic cardiomyopathy; (2) PID1 expression is significantly increased in mice failing hearts that display severe cardiac hypertrophy; (3) cardiac-specific overexpression of PID1 markedly enhances cardiac hypertrophy and left ventricular dysfunction in mice in response to TAC; and (4) PID1 was involved in AKT and MAPK signaling pathways. Together, we demonstrate that PID1 plays a critical role in enhancing cardiac hypertrophy.

PID1 expression was significantly up-regulated in failing human hearts and hypertrophic mouse hearts. Based on our findings that the transgenic overexpression of PID1 in the heart exaggerated TAC-induced cardiac hypertrophy and fibrosis, the increased expression of endogenous PID1 observed in human failing hearts is likely maladaptive. These results strongly suggest that PID1 plays an important role in the maladaptive response to stress in the heart.

The hypertrophic effect of PID1 is intriguing. However, the mechanism by which PID1 mediates its effects during the transition from cardiac hypertrophy to failure remains elusive. It is generally accepted that a mechanical signal induced by pressure overload will initiate a cascade of biological signaling transduction pathways that increase collagen synthesis and cardiomyocyte growth [13-17]. Previous studies have demonstrated that the AKT pathway is crucial in the process of cardiac hypertrophy [18-21]. Moreover, our group indicated that PID1 could inhibit the IRS1/PI3K/AKT insulin pathway in adipose tissue [22]. Therefore, in this study, we first examined the AKT signaling pathway (AKT-GSK3 β -mTOR). It has been reported that the activation of AKT and mTOR via phosphorylation produces pro-hypertrophic effects in cardiomyocytes [23]. GSK-3 β is a negative regulator of the calcineurin/nuclear factor in activated T cell signaling and cardiac hypertrophy, and it is inactivated by phosphorylation upon hypertrophy stimulation [24]. However, although AKT and GSK3 β phosphorylation was partially blocked by PID1-overexpression, elevation of the phosphorylated P70S6K and mTOR was similar between PID1-Tgs and WTs. These data indicate that hypertrophic effects of PID1 may not be entirely associated with the AKT-GSK3 β -mTOR axis. So far, there is controversy regarding the regulatory role of AKT in cardiac hypertrophy. Some researchers found that the cardiac-specific overexpression of AKT regulates downstream targets and promotes cardiac hypertrophy, which is associated with impaired contractile function and interstitial fibrosis [18-20]. Nevertheless, additional experiments have produced different results. For example, Condorelli et al. [25] observed that cardio-selective AKT overexpression markedly increased cardiomyocyte cell size and LV hypertrophy, which was associated with increased cardiac contractility. DeBosch et al. [21] reported that AKT-deficient mice have similar heart weights and cardiac function compared with WT mice. Therefore, the inhibition of AKT activity in this study seemed cannot explain the enhanced effects of hypertrophy in PID1 overexpression heart.

As well known, cross-talk between numerous signaling pathways are common in physiological and pathological process. Previous studies demonstrated Akt signaling can exert an inhibitory effect on the ERK and JNK MAP kinase pathways [26, 27], both of which strengthen growth in the myocardium [28, 29]. Therefore, we then examined the MAPK signaling pathway, an additional key contributor to the development of cardiac hypertrophy. The MAPK cascade comprises a sequence of successive kinases, including p38, JNKs, and ERKs. These kinases directly modify transcription regulatory factors, regulate cardiac gene expression, and induce cardiac hypertrophy [30]. Our results demonstrated MEK-ERK-JNKs1/2 and P38 activation was promoted by cardiac-specific PID1 overexpression in response to chronic pressure overload. Therefore, MEK-ERK1/2 signaling may be a crucial pathway through which PID1 enhanced the effect of cardiac hypertrophy.

In conclusion, this investigation is the first study to define the role of PID1 in cardiac remodeling. Our findings indicate that PID1 functions as a novel positive modulator of cardiac hypertrophy and failure in response to pressure overload. The underlying mechanism of the

promoting role of P1D1 in the development of cardiac hypertrophy appears to involve the activation of MEK-ERK signaling. Therefore, the present study also provides novel insights into the molecular mechanisms of pathological cardiac remodeling. Based on these findings, P1D1 may represent a new therapeutic target for suppressing the onset of heart failure.

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Disclosure Statement

None declared.

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