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Mechanism of Profilin-1 in regulating eNOS/NO signaling pathway and its role in hypertensive myocardial hypertension

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ABSTRACT

Objective: To explore the mechanism of Profilin-1 in regulating eNOS/NO pathway and its role in the development of myocardial hypertrophy. **Methods:** Spontaneously hypertensive rats (SHR) aged 5 weeks were injected with different adenovirus vectors to induce Profilin-1 expression knockdown (SHR-I) or over express (SHR-H) or to use as control (SHR-C). All these treatment were compared with Wistar-Kyoto rats (SKY) treated with control adenovirus vectors (WKY-C). The same injection was executed at the sixth week during the experiment of 12 weeks. After experiment, the left ventricular weight-to-heart weight ratio (LVW/HW) and left ventricular long axis (LVLA) were measured. Meanwhile, NO contents in blood and myocardium, Profilin-1, eNOS and Caveolin-3 mRNA and protein levels and phosphorylated eNOS (P-eNOS) protein level in myocardium were determined. **Results:** Compared with WKY-C group, the SHR-C group was statistically higher in LVW/HW (0.79 ± 0.03), LVLA (11.82 ± 0.58 mm) and Profilin-1 mRNA and protein level ($P < 0.05$), but lower in NO content [$(18.63 \pm 6.23) \mu\text{mol/L}$] in blood and [$(2.71 \pm 0.17) \mu\text{mol/L}$] in myocardium, eNOS activity and Caveolin-3 expression ($P < 0.05$). The over expressing Profilin-1 led SHR-H group to a higher value of LVW/HW [(0.93 ± 0.03) mm and LVLA (14.17 ± 0.69) mm] in comparison with SHR-C group ($P < 0.05$), and to a lower value of NO content (in myocardium), eNOS activity and Caveolin-3 expression ($P < 0.05$); however, this phenomenon was reversed by the knockdown Profilin-1 expression (SHR-I group). **Conclusions:** Profilin-1 expression, being negative in regulating Caveolin-3 expression and eNOS/NO pathway activity, promotes the development of myocardial hypertrophy which can be reversed by Profilin-1 silencing.

1. Introduction

According to WHO, 25% of global adult population are suffering from hypertension which has been reported to be a common risk factor for the development of left ventricular hypertrophy (LVH) [1–3]. For patients with essential hypertension, LVH can stimulate the

risk of cardiovascular disease and even cause death of patients[4,5]. Nitric oxide (NO) is a signaling molecule synthesized by endothelial nitric oxide synthase (eNOS) and this compound is considered to have cardioprotective effects by being responsible for multiple functions such as the regulation of vascular tone and relevant gene expression[6]. As reported, eNOS/NO signaling pathway is related to many cardiovascular diseases including myocardial hypertrophy. It is found that angiotensin type II (AT₂) receptor can be activated by the expression of cardiac eNOS and therefore produce an antihypertrophic effect[7]. In the diabetic heart, the maintenance of Akt/eNOS/NO signaling is suggested to attenuate cardiac diastolic dysfunction[8]. Polhemus *et al* reported that the increase in the phosphorylation of eNOS and the bioavailability of NO could improve vascular density to attenuate left ventricular remodeling in

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H₂S-treated hearts[9]. Profilin-1, having a molecular weight of 12-15 kD, belongs to the family of actin-binding proteins. The activation of a hypertrophic signaling cascade by this protein can regulate actin polymerization and cytoskeleton remodeling[10]. Profilin-1 has been found to function in eNOS uncoupling by activating Akt/Erk signaling pathway[11] and its molecular expression and protein phosphorylation exert a regulatory mechanism in the development of cardiovascular diseases[12]. As mentioned above, Profilin-1 and eNOS/NO signaling pathway are related with cardiovascular diseases, while the information in this regard is elusive in patients with essential hypertrophy and hypertension. In this study, the constructed adenovirus vectors were used to induce the overexpression or silence of Profilin-1 in spontaneously hypertensive rats. The relationship between Profilin-1 and eNOS/NO pathway and their role in myocardial hypertrophy were analyzed and this study may provide reference for the therapy of this disease through possible Profilin-1 regulation.

2. Materials and methods

2.1. Materials

Experimental animals: Spontaneously hypertensive rats (SHR) and Wistar-Kyoto rats (WKY) with all aged four weeks were used in the present study. The SPF male rats were purchased from Yaoming Kangde new drug development co., LTD. Shanghai [SYXK (Hu) 2010-0102]. All rats were acclimated at room temperature of (23±1) °C with a constant diurnal rhythm and well ventilation. Prior to experiments initiation, an acclimation of one week was allowed.

Reagents: rhIL-2 (Shenyang 3SBio Inc.); RPMI-1640 and M19 medium (Gibco, USA); fetal bovine serum (Beijing Solarbio Science & Technology Co., Ltd., China); NKC culture kit (Jiangsu Vanvo Bio-medical Technology Co., Ltd.); antibody CD31 and VEGF (Invitrogen, USA); DNA marker (Promega, USA).

Instruments: Synergy 2 multimode reader (Biotek, USA); SHELLAB CO₂ incubator (SHELLAB, USA); Stratadigm flow cytometry (Stratadigm, USA); Biometra gradient PCR amplifier (Biometra, Germany); rodent ventilator (Kent, USA); DYY-2C electrophoresis apparatus (Beijing Liuyi Instrument Plant, China); BioSpectrum-UVP Gel Imaging System (UVP, USA).

2.2. Experimental procedures

Adenovirus vectors of Profilin-1 silence (pAd-miR-profilin-1) and overexpression (Ad-profilin-1-IRES-EGFP) were established respectively.

SHR rats of 60 were randomly divided into three groups (20 rats in each group) as following: SHR-C (treated with control vector), SHR-I (treated with pAd-miR-profilin-1) and SHR-H (treated with Ad-profilin-1-IRES-EGFP). At the same time, 20 WKY rats were treated with control vector and named WKY-C group. All treatments were conducted by tail intravenous injection at a dose of 3×10^9 PFU/rat, which was done again at sixth week during the experiment of 12 weeks (*ie.* rats grew from 5 to 17 weeks old).

2.3. Sample collection

After the experiment of 12 weeks, all rats were rendered unconscious with 2% isoflurane. Blood sample was then withdrawn from hepatic portal vein and centrifuged for 10 min at 4 °C and 3000 *g*. Supernatants were stored at -80 °C until the analysis of NO content. The hearts were excised and weighted (heart weight, HW). Then, left free ventricular wall and interventricular septum were excised and weighted to obtain the left ventricular weight (LVW). The left ventricular long axis (LVLA) from the atrial valve to apex cordis was measured with a vernier caliper.

After measurement, left ventricle sample was excised, rinsed with ice-cold saline and dried with blotting paper. After that, the sampled was placed into a microtube, flash frozen in liquid nitrogen and finally stored at -80 °C for the determination of the expression of Profilin 1, eNOS and caveolin 3 and the phosphorylation of eNOS (P-eNOS).

2.4. Determination of NO in blood and myocardium

Frozen myocardial tissue was powdered with mortar and pestle under liquid nitrogen and a certain amount of powder was homogenized in 9 volumes of 0.85% saline. After centrifugation of 10 min at 1 000 *g* and 4 °C, supernatants were collected for the determination of NO content in myocardium with commercial NO test kits (nitrate reductase method) according to the manufacturers' instructions (Nanjing Jiancheng Bioengineering Institute, China).

Table 1

Sequences of Profilin 1, eNOS, Caveolin 3 and β -actin primers.

| Genes | Sense primer | Reverse primer |
|-------------------|-----------------------------|----------------------------------|
| <i>Profilin 1</i> | 5'-ATGCAGGGATGATGTTCTGG-3' | 5'-GTGCAAAAGCCAAAGGGAG-3' |
| <i>eNOS</i> | 5'-TGGGCAGCATCACCTACGATA-3' | 5'-GGAACCACTCCTTTTGATCGAGTTAT-3' |
| <i>Caveolin 3</i> | 5'-GCTGCACCAGGGAACACTAT-3' | 5'-CCCTATTTATGCCAGCAAA-3' |
| β -actin | 5'-CTCCATCCTGGCCTCGCTGT-3' | 5'-GCTGTCACCTTCACCGTTCC-3' |

PCR system (20 μ L): 2.5 master solution 8 μ L, 20 SYBR Green 1 μ L, sense (10 μ M) and reverse primers (10 μ M) 0.25 mL respectively, cDNA 1.5 μ L, ddH₂O 9 μ L.

PCR amplifying conditions: 95 °C 1 min; 95 °C 5 s, 58 °C 15 s, 68 °C 20 s (containing 35 cycles); 65 °C 7 min, 4 °C.

The absorbance of final reaction system at 550 nm was read and used to calculate NO concentration (μ mol/L). Blood NO content was also measured with the same kit.

2.5. Determination of mRNA levels of Profilin 1, eNOS and caveolin 3

The mRNA levels of Profilin 1, eNOS and caveolin 3 were determined with the RT-PCR method. Myocardium tissue was powdered under liquid nitrogen and mixed with Trizol reagent. Total RNA was extracted using a commercial kit (Invitrogen, USA) followed by the detection of RNA content (A_{260}) and purity (A_{260}/A_{280}).

Total RNA of 1 μ g was reverse transcribed into the first strand of cDNA and the product was PCR amplified after addition of Profilin 1, eNOS and caveolin 3 primers respectively. β -actin was used as the reference gene. Primers sequences were listed in Table 1. The amplification results were analyzed using the $2^{-\Delta\Delta Ct}$ method.

2.6. Determination of mRNA levels of Profilin 1, eNOS, P-eNOS and caveolin 3

Western-blot method was applied for the determination of protein levels of Profilin 1, eNOS, P-eNOS and caveolin 3. Total protein was extracted from 50 mg myocardium tissue after addition of 500 μ L RIPA. After 20 min on ice, the mixture was centrifuged for 10 min at 12 000 g and 4 $^{\circ}C$. Collected supernatants were mixed with buffer solution, boiled for 5 min and stored at -20 $^{\circ}C$.

Total protein concentration was measured with the Bicinchoninic Acid (BCA) method (562 nm) using a commercial kit (Sangon Biotech (Shanghai) Co.,Ltd., China). Sample protein was separated by sodium dodecyl sulfate (SDS)-polyacrylamide gels and transferred onto polyvinylidene fluoride (PVDF) membranes followed by blocking with skimmed milk powder. After incubation overnight at 4 $^{\circ}C$ with corresponding primary antibodies of Profilin 1, eNOS, P-eNOS and caveolin 3, membranes were washed with 1 TBST. Then membranes were mixed with corresponding secondary antibodies and incubated at room temperature for 1 h. After another washing with 1 TBST, the color reagent of ECL-plus was added and protein levels was analyzed with Quantity One biological gel electrophoresis image analysis system. Using β -actin

as the control, the relative optical density was used to represent the relative protein level of Profilin 1, eNOS and caveolin 3, while for P-eNOS, total protein level was used as the control. All primary and secondary antibodies were purchased from Beijing Zhongshan Golden Bridge Biotechnology Co., Ltd.

2.7. Statistical analysis

Data in the present study were expressed as mean \pm SD and analyzed with SPSS 13.0 software. Differences between treatments were determined using Student's t -test. P -values $<$ 0.05 were considered statistically significant for all variance tests.

3. Results

3.1. Effects of Profilin-1 on LVW/HW and LVLA

The LVW/HW and LVLA were used as two indicators to reflect myocardial hypertrophy, which were shown in table 1. With respect to LVW/HW, SHR-C group (0.79 ± 0.03) was statistically higher than WKY-C group (0.63 ± 0.04) ($t=0.08$, $P<0.01$) and SHR-I group (0.68 ± 0.05) ($t=0.05$, $P<0.05$) and lower than SHR-H group (0.93 ± 0.03) ($t=0.07$, $P<0.05$). A similar difference between treatments was also observed in LVLA. The SHR-C group was significantly higher in LVLA (11.82 ± 0.58) than WKY-C group (9.13 ± 0.49) ($t=1.28$, $P<0.01$) and SHR-I group (9.52 ± 0.51) ($t=1.09$, $P<0.01$) and lower than SHR-H group (14.17 ± 0.69) ($t=1.16$, $P<0.01$).

3.2. Effects of Profilin 1 on NO content in myocardium and blood

As shown in Figure 1A, WKY-C group had a higher blood NO content (31.50 ± 7.52) than SHR-C group (18.63 ± 6.23) and the difference was significant ($t=6.23$, $P<0.01$). However, no significant difference was detected between SHR-C group and SHR-I group (17.52 ± 5.65) or SHR-H group (16.47 ± 5.12) ($P>0.05$).

For NO content in the myocardium (Figure 1B), WKY-C group (3.67 ± 0.19) was significantly higher than SHR-C group (2.71 ± 0.17) ($t=0.43$, $P<0.05$). Compared to the NO level of SHR-C group, SHR-I group (3.45 ± 0.25) was significantly higher ($t=0.35$, $P<0.05$) while

Table 2

Effects of Profilin 1 on LVW/HW and LVLA ($n=20$).

| Index | Treatments | | Treatments | | t value | P value |
|-----------|------------|-----------------|------------|-----------------|-----------|-----------|
| LCW/HW | SHR-C | 0.79 ± 0.03 | WKY-C | 0.63 ± 0.04 | 0.08 | 0.003 |
| | | | SHR-I | 0.68 ± 0.05 | 0.05 | 0.021 |
| | | | SHR-H | 0.93 ± 0.03 | 0.07 | 0.015 |
| LVLA (mm) | SHR-C | 11.82 ± 0.58 | WKY-C | 9.13 ± 0.49 | 1.28 | 0.005 |
| | | | SHR-I | 9.52 ± 0.51 | 1.09 | 0.000 |
| | | | SHR-H | 14.17 ± 0.69 | 1.16 | 0.018 |

Note: t and P values indicate the difference between the treatment in the same row and SHR-C group.

SHR-H group (1.85 ± 0.21) significantly lower ($t=0.46, P<0.01$).

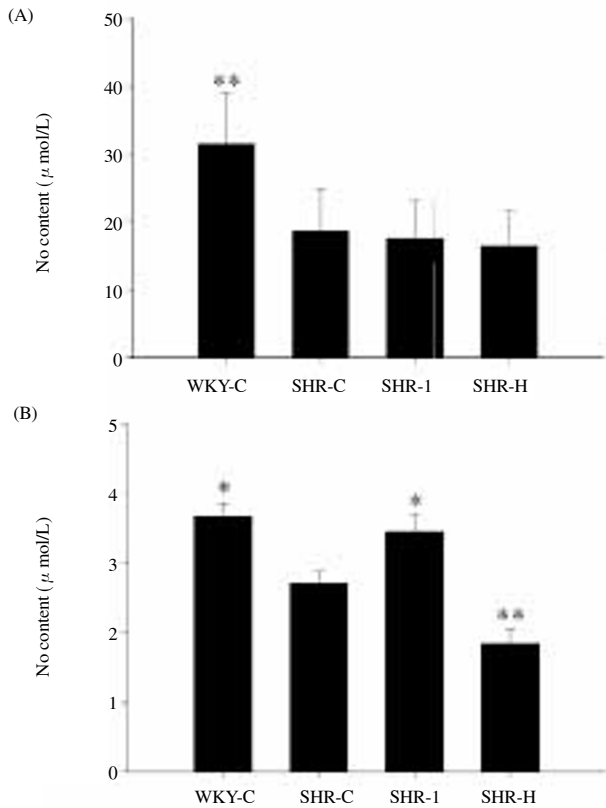


Figure 1. Content of NO in serum (A) and cardiac muscle (B) of different treatments. Note: “*” indicates a significant difference compared to SHR-C group ($P<0.05$), while “***” indicates an extremely significant difference ($P<0.01$).

3.3. Effects of Profilin 1 on mRNA levels of related genes in myocardium

Figure 2 shows that WKY-C group was lower in Profilin 1 mRNA level compared to SHR-C group and a significant difference was detected ($t=0.76, P<0.01$). The Profilin 1 mRNA level was reduced in SHR-I group ($t=0.41, P<0.05$) and elevated in SHR-H group ($t=0.38, P<0.05$) compared to SHR-C group.

With regard to eNOS mRNA level, no significant difference was detected between treatments ($P>0.05$).

Caveolin mRNA expression of SHR-C group was reduced to 51.42% of WKY-C group and a statistical difference was detected ($t=0.24, P<0.05$). In comparison with SHR-C group, caveolin expression was significantly elevated in SHR-I group ($t=0.16, P<0.05$) and reduced in SHR-H group ($t=0.13, P<0.01$).

3.4. Effects of Profilin 1 on protein levels of related genes in myocardium

Figure 3 shows the protein levels of Profilin 1, eNOS, P-eNOS and Caveolin-3. WKY-C group showed a significantly lower protein level of Profilin 1 than SHR-C group ($t=0.09, P<0.05$), and SHR-C group was lower than SHR-H group ($t=0.15, P<0.05$) and higher

than SHR-I group ($t=0.13, P<0.01$).

With regard to eNOS protein level, no significant difference was observed between treatments ($P>0.05$). However, significant differences were observed between treatments in the P-eNOS protein level: it was higher in WKY-C group and SHR-I group but lower in SHR-H group when compared to that in SHR-C group.

Compared to SHR-C group, the caveolin 3 protein level was elevated in WKY-C group and the difference was statistically significant ($t=0.15, P<0.01$). SHR-C group was significantly lower in caveolin 3 protein level than SHR-I group ($t=0.09, P<0.01$) and significantly higher than SHR-H group ($t=0.05, P<0.01$).

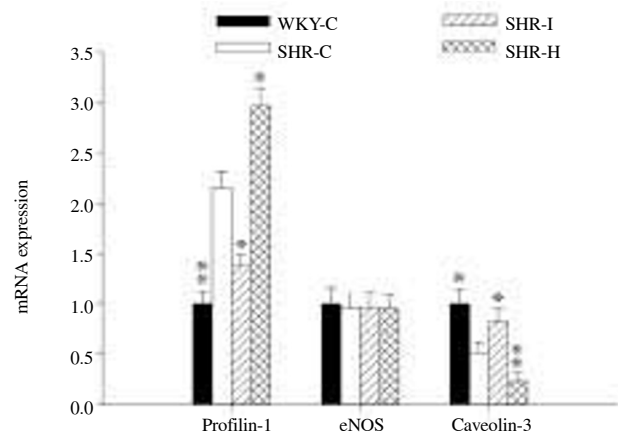


Figure 2. mRNA expression levels of Profilin 1, eNOS and Caveolin-3 in different treatments. Note: “*” indicates a significant difference compared to SHR-C group ($P<0.05$), while “***” indicates an extremely significant difference ($P<0.01$).

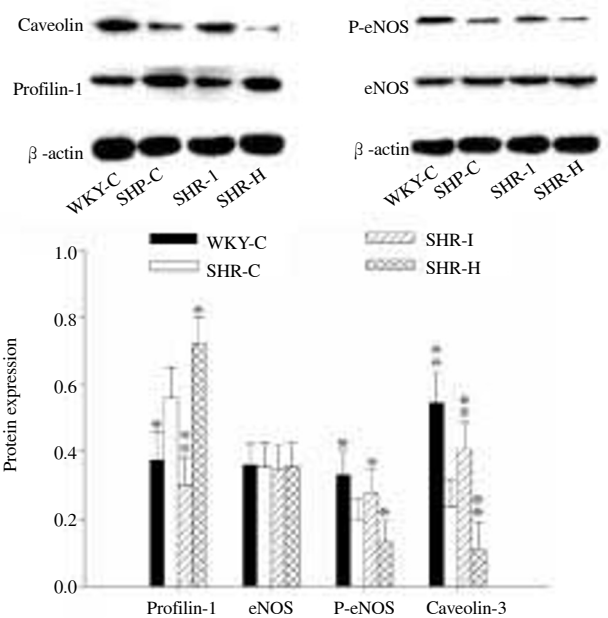


Figure 3. Protein expression levels of Profilin-1, eNOS, P-eNOS and Caveolin-3 in different treatments. Note: “*” indicates a significant difference compared to SHR-C group ($P<0.05$), while “***” indicates an extremely significant difference ($P<0.01$).

4. Discussion

Hypertension is a common chronic disease related with human health and also a cause of many diseases including cardiovascular diseases. eNOS/NO signaling pathway is reported to exert a protective effect on cardiovascular system by regulating NO level[6]. Profilin 1 is an important participator in the regulation of multiple signaling pathways and consequently influences the development of cardiovascular diseases[12,13]. In the present study, we explored the relationship between Profilin 1 and eNOS/NO signaling pathway and further studied the effect of silence and overexpression of Profilin 1 on hypertensive cardiac hypertrophy. This study aimed to provide reference for the potential value of Profilin 1 in hypertrophy therapy.

4.1. Effects of Profilin 1 on eNOS/NO signaling pathway

NO is a bioactive molecule involved in the regulation of blood pressure[14,15] and its release into vascular vessel can inhibit platelet and leukocyte adhesion to the vascular wall[6]. In the present study, we detected NO level in myocardium and blood of rats with Profilin 1 silence or overexpression. A significant change in NO level was only observed in myocardium of SHR rats, indicating that this gene might function locally in myocardial tissue rather than influence organismal NO level through systemic response. In cells, the elevation of eNOS activity and its product-NO contribute to the elimination of reactive oxygen species (ROS) [16] and the maintenance of vascular function[17]. According to the detection of eNOS mRNA and protein expression level, we found that overexpressed Profilin 1 could reduce eNOS activity and NO content in myocardium, resulting in a weaker capability of scavenging ROS[14] and a consequent declined protection effect to cardiovascular system. As a result, an enlarged LVW/HW and LVLA was observed in myocardium. In contrast, silencing Profilin 1 activated eNOS activity and elevated NO level, which are believed to be good to stabilize blood pressure and therefore to attenuate myocardial hypertrophy.

Caveolae are sacs with diameter of 50-100 nm which are formed by invagination of cell membranes and their principal components are caveolin, sphingolipid and cholesterol[18]. Caveolin plays a central role in extracellular signals enrichment and intracellular signals transduction[18]. As one of the three components of caveolin (i.e. caveolin 1, 2 and 3), caveolin 3 is only found in muscle and can interact with eNOS to regulate multiple signal transduction pathways[18,19]. The expression of caveolin 3 has been reported by many studies to be conducive to the attenuation of myocardial hypertrophy. Horikawa *et al* found that overexpression of caveolin 3 could improve myocardial hypertrophy in transgenic mice by increasing the natriuretic peptide expression and signaling[20]. Feiner *et al* reported that the selective decrease in the expression

of caveolin 3 could lead to left ventricular dysfunction[21]. In the present study, caveolin 3 expression and eNOS/NO pathway activity was lower in SHR rats than in WKY rats, while this situation was reversed by silencing Profilin 1. This suggests that Profilin 1 has an inhibitory effect on caveolin 3 expression, which may be due to that the disturbed actin polymerization influences caveolae function and structure and eNOS activity[18,19].

4.2. Relationship between Profilin 1 and myocardial hypertrophy

The development of myocardial hypertrophy was regulated by many factors including Profilin 1[22]. The close relationship between Profilin 1 and eNOS/NO pathway is may be related with the role of the former in actin polymerization and cytoskeleton remodeling[10,23]. As an important actin binding protein, the normal expression of Profilin 1 can help maintain the structure and function of actin microfilaments, while dysfunctional Profilin 1 expression usually break the balance. The study of Caglayan *et al* demonstrated the role of Profilin 1 expression in atherosclerosis, making this gene become a potential target in disease treatment[24]. Zhong *et al* reported that the expression of Profilin 1 was elevated in aorta of SHR rats and telmisartan could inhibit its expression to attenuate aortic hypertrophy[25]. It was found in the present study that SHR rats possessed a higher value of LVW/HW and LVLA than WKY rats and correspondingly the former had a higher Profilin 1 expression level. Through overexpressing or silencing Profilin 1 in SHR rats, our study demonstrated the positive relationship between Profilin 1 expression and hypertensive cardiac hypertrophy. According to our results, Profilin 1 silence significantly improved hypertensive cardiac hypertrophy, which provided further evidence that Profilin 1 expression is not good to maintenance of cardiovascular structure and function.

It should be noticed that no valid evidence could be provided concerning the role of Profilin 1 in the direct regulation of eNOS/NO pathway. In fact, genes like IL-18[19] and ACE2[10,11] also play a role in the development of myocardial hypertrophy. What's more, how other factors related with Profilin 1 and eNOS/NO pathway influence hypertensive cardiac hypertrophy remains elusive. Accordingly, a further study is needed to find out the synergistic function of these factors in myocardial hypertrophy.

As discussed above, Profilin 1 expression could inhibit the expression of caveolin 3 and the activity of eNOS/NO pathway, exerting a promotion effect to the development of hypertensive cardiac hypertrophy. However, this situation can be reversed by silencing Profilin 1 and myocardial hypertrophy is therefore improved. Results of this study not only enrich the study of the regulatory mechanisms of myocardial hypertrophy but also may be a scientific reference for its clinical therapy through silencing Profilin 1.

Conflict of interest statement

We declare that we have no conflict of interest.

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