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Chronic inhibition of farnesyl pyrophosphate synthase attenuates cardiac hypertrophy and fibrosis in spontaneously hypertensive rats

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ABSTRACT

Farnesyl pyrophosphate synthase (FPPS), an essential enzyme in the mevalonate pathway, was reported to be upregulated in young spontaneously hypertensive rats (SHR) when compared with Wistar–Kyoto (WKY) rats, and this was accompanied by development of left ventricular hypertrophy. Five-week-old rats were daily gavaged with vehicle or an FPPS inhibitor (alendronate, 1 or 10 mg/kg) and blood pressures was monitored by the tail-cuff method every other week. Twelve weeks of alendronate treatment attenuated the left ventricular weight to body weight ratio (LVW/BW), hydroxyproline content, collagen deposition in the interstitia, and gene expression of atrial natriuretic peptide, B-type natriuretic peptide, and procollagen type I/III in the SHR left ventricle, all of which were significantly higher in SHRs than in WKY rats. Furthermore, long-term treatment with an FPPS inhibitor significantly reduced RhoA activation, ERK phosphorylation, and TGF-β1 expression in the SHR left ventricle, all of which were upregulated more in SHRs than in WKY rats. In conclusion, chronic treatment with an FPPS inhibitor attenuates the development of cardiac hypertrophy and fibrosis, and the suppression of ERK1/2 phosphorylation and TGF-β1 expression with inhibition of RhoA activation may be an important mechanism.

1. Introduction

Cardiac hypertrophy and fibrosis are key aspects of heart remodeling secondary to hypertension [1]. The prevalence of these phenotypes is relatively high and is associated with an increased risk of cardiovascular morbidity and mortality [2,3]. Although the exact mechanisms underlying cardiac hypertrophy and fibrosis are complicated and remain unclear, a large body of evidence indicates that mitogen-activated protein kinases (MAPK), including the extracellular-regulated kinases 1/2 (ERK1/2), play causal roles in the development of cardiac hypertrophy [4–6]. Moreover, cardiac fibrosis is thought to be partially mediated by transforming growth factor beta 1 (TGF-β1), a potent stimulator of collagen-producing cardiac fibroblasts [7,8].

It has been known for several years that the ERK cascade is activated by the small GTPases in many cells, including cultured cardiac myocytes [9,10]. In addition, previous studies showed that the Rho family plays an important role in the TGF-β-collagen cascade [11,12]. Two important isoprenoid intermediates of the mevalonate pathway are farnesyl pyrophosphate (FPP) and geranylgeranyl pyrophosphate (GGPP), which are needed for protein isoprenylation of the small GTPases for their activation [13,14]. Therefore, we reasoned that the mevalonate pathway is an important cellular metabolic pathway in modulating cardiac hypertrophy and fibrosis, but direct evidence in support of this hypothesis is insufficient.

In previous studies, we found that the expression of key enzymes in the mevalonate pathway, including farnesyl pyrophosphate synthase (FPPS), are significantly upregulated in the spontaneously hypertensive rat (SHR) [15,16]. FPPS is a key enzyme in the mevalonate pathway and directly catalyzes the synthesis of FPP and geranyl pyrophosphate (GPP; two molecules of GPP condense to GGPP) [17], which are required for isoprenylation of small GTPases. On the other hand, the SHR has served for decades as one of the preferred animal models of left ventricular hypertrophy and fibrosis [18–21]. Moreover, enhanced activity of ERK1/2 [22,23] and increased expression of TGF-β1 [24] occur in the early stage of development of left ventricular hypertrophy in SHRs. Thus, we can assume that increased synthesis of FPP and GGPP contribute to the cardiac hypertrophy and fibrosis observed in SHRs. However, it remains to be tested whether the decreased synthesis of FPP and GGPP by an FPPS inhibitor leads to a regression of ventricular remodeling in SHRs.

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Therefore, the present study was designed to determine whether chronic inhibition of FPPS reverses the cardiac hypertrophy and fibrosis present in SHRs. For this purpose, we used alendronate, an inhibitor of FPPS in the mevalonate pathway [25–27].

2. Materials and methods

2.1. Animals and reagents

Five-week-old male SHRs and normotensive Wistar–Kyoto (WKY) rats were purchased from the Shanghai Laboratory Animal Center, Chinese Academy of Sciences, and housed in a pathogen-free laboratory at the First Affiliated Hospital of Zhejiang University. The investigation conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996) and the guidelines of the Animal Care and Use Committee of Zhejiang University.

Eighteen male SHRs were randomly divided into three groups consisting of the distilled water group (SHR-C), the low-dose (1 mg/kg/d) alendronate group (SLA), and the high-dose (10 mg/kg/d) alendronate group (SHA). Six male age- and weight-matched WKY rats were housed as controls (WKY-C). Alendronate (Fosamax, a gift from Merck, NJ, USA) was administered every day for 12 weeks by the intragastric route.

Reagents and antibodies were purchased as follows: rabbit anti-Rho A monoclonal antibody (67B9), rabbit anti-p44/p42 MAPK (ERK1/2) polyclonal antibody, and rabbit anti-phospho-p44/p42 MAPK (ERK1/2) (Thr202/Tyr204) monoclonal antibody were from Cell Signaling Technology (CST, USA); the Rho pull-down kit was from Cytoskeleton Co. (Germany). The remaining reagents were from Sigma Chemical Co. (St. Louis, MO, USA) unless specified otherwise.

2.2. Blood pressure

Systolic blood pressure (SBP) was measured every other week by the tail-cuff method as previously described [28] while the rats were conscious. Data were averaged for 5–10 consecutive cardiac cycles.

2.3. Hemodynamic measurements

After 12 weeks of drug intervention, hemodynamic studies were performed using methods previously described [29]. Briefly, each rat was anesthetized by intraperitoneal injection of chloral hydrate (400 mg/kg body weight). A polyethylene tube (PE50, Becton–Dickinson) was introduced via the right carotid artery to measure the mean arterial pressure (MAP), and then further advanced into the left ventricle to measure the heart rate (HR), left ventricular end-diastolic pressure (LVEDP), and maximum rate of left ventricular pressure increase/reduction ($\pm dp/dt_{max}$).

2.4. Lipid measurements

At the end of the hemodynamic studies, a 5 mL blood sample was taken, the serum isolated, and the levels of total cholesterol (TC), high-density lipoprotein cholesterol (HDL-C), and low-density lipoprotein cholesterol (LDL-C) were measured with commercially available enzymatic kits. At the same time, hearts were collected immediately and stored at −80 °C for subsequent analysis.

2.5. Histological analysis

The weight of the whole heart (WHW) and the left ventricle (LVW) were measured, and the ratios of WHW or LVW to body weight (WHW/BW, LVW/BW) were calculated as an index of cardiac hypertrophy. Sections of left ventricular free wall and septum were fixed in 10% neutral formalin, embedded in paraffin, and stained with Masson's trichrome for microscopy to show the area of interstitial fibrosis. Collagen volume fraction was detected and calculated by ImageJ software.

2.6. Hydroxyproline content assay

Hydroxyproline (Hyp) was assayed using a procedure described previously [30]. The apex of the left ventricle (about 100 mg) was defatted and hydrolyzed with 1 mL alkaline lysis liquid at 95 °C for 20 min, then centrifuged at 3500 rpm for 10 min. One millilitre of supernatant was used to determine the Hyp content relative to a Hyp standard at a wavelength of 550 nm.

2.7. Western blot analysis

The procedure for Western blot analysis was performed as described in our previous report [15]. The expression of ERK and phospho-ERK was detected using their specific antibodies: anti-ERK (diluted 1:1000) and anti-phospho-ERK (1:500). To ensure equal protein loading, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an endogenous control.

2.8. RhoA pull-down assay

The RhoA pull-down assay was performed using a RhoA activation assay Biochem kit (Cytoskeleton, USA) according to the manufacturer's instructions. Briefly, left ventricle (about 100 mg) was homogenized in Mg^{2+} lysis buffer. Equal total proteins of left ventricular lysates (about 200 μg) were incubated with GST-tagged Rhotekin-Rho binding domain (RBD) agarose beads. GTP-bound Rho protein was detected by Western blotting using a polyclonal antibody against RhoA (diluted 1:1000). Western blotting of the total amount of RhoA was performed for comparison with the RhoA activity (level of GTP-bound RhoA) in the same samples.

2.9. Real-time polymerase chain reaction

The mRNA expression levels of TGF-β1, procollagen types I and III, CTGF, ANP, BNP, and the reference gene GAPDH were analyzed by the real-time polymerase chain reaction (RT-PCR). Total RNA was isolated from homogenized left ventricular myocardium using a Trizol kit (Invitrogen Life Technologies). The reverse transcription reaction was performed with a TaKaRa High Fidelity RNA PCR kit (Takara Bio Co., Tokyo, Japan). PCR was carried out with an LC Fast Start DNA Master SYBR Green kit (Roche Applied Science, Mannheim, Germany) using 1 μL of cDNA, corresponding to 25 ng of total RNA in a 25 μL final volume, 1.5 mmol/L $MgCl_2$ and 0.4 μmol/L of each primer (Table 1). The quantitative PCR was performed using a Light-Cycler (Roche Applied Science) and the amplification specificity was checked using a melting curve following the manufacturer's instructions. Data were normalized by the abundance of GAPDH mRNA and then expressed relative to the mean value for the WKY-C group.

2.10. Statistical analysis

Results are expressed as mean ± SEM. One-way analysis of variance (ANOVA) followed by the Bonferroni *post hoc* test was used to determine significant differences among groups. Repeated measures ANOVA followed by the Bonferroni *post hoc* test was used in the results of blood pressure obtained by the tail-cuff method every

Table 1
Sequences of primers used for real-time polymerase chain reaction.

	Sequence	Product (bp)
TGF-β1	F: 5′-GCTGAACCAAGGAGACGGAATA-3′ R: 5′-ACCTCGACGTTTGGGACTGA-3′	115
ANP	F: 5′-TGAGCCGAGACAGCAAACA-3′ R: 5′-TCCAGGTGGTCTAGCAGGTT-3′	186
BNP	F:5′-CTGTGACGGGCTGAGGTTGT-3′ R: 5′-TGGCAAGTTTGTGCTGGAAG-3′	195
CTGF	F: 5′-CAGGGAGTAAGGGACACGA-3′ R: 5′-ACAGCAGTTAGGAACCCAGAT-3′	127
Procollagen I	F: 5′-GAGCCTAACCATCTGGCATCT-3′ R: 5′-AGAACGAGGTAGTCTTTCAGCAAC-3′	219
Procollagen III	F:5′-GAGCGGAGAATACTGGGTTGAT-3′ R: 5′-GGTATGTAATGTTCTGGGAGGC-3′	293
GAPDH	F: 5′-AAGAAGGTGGTGAAGCAGGC-3′ R: 5′-TCCACCACCCTGTTGCTGTA-3′	203

TGF-β1, transforming growth factor beta 1; ANP, atrial natriuretic peptide; BNP, B-type natriuretic peptide; CTGF, connective tissue growth factor; GAPDH, glyceraldehyde phosphate dehydrogenase; F, farward; R, retroward.

other week. Differences were considered statistically significant at a value of $p < 0.05$.

3. Results

3.1. Alendronate reduced SBP and improved hemodynamics in SHRs

As expected, before treatment, the SBP of SHR-C was already higher than that of WKY-C (120.3 ± 3.2 mmHg versus 115.0 ± 2.8 mmHg, $P < 0.01$), and the difference persisted as time progressed (Fig. 1). Over the 12-week period, SBP further increased in the SHR-C group compared to the WKY-C group (226.5 ± 5.5 mmHg versus 122.5 ± 3.4 mmHg, $P < 0.01$, at the end of the study). Twelve-week administration of high-dose alendronate (10 mg/kg/d) slightly

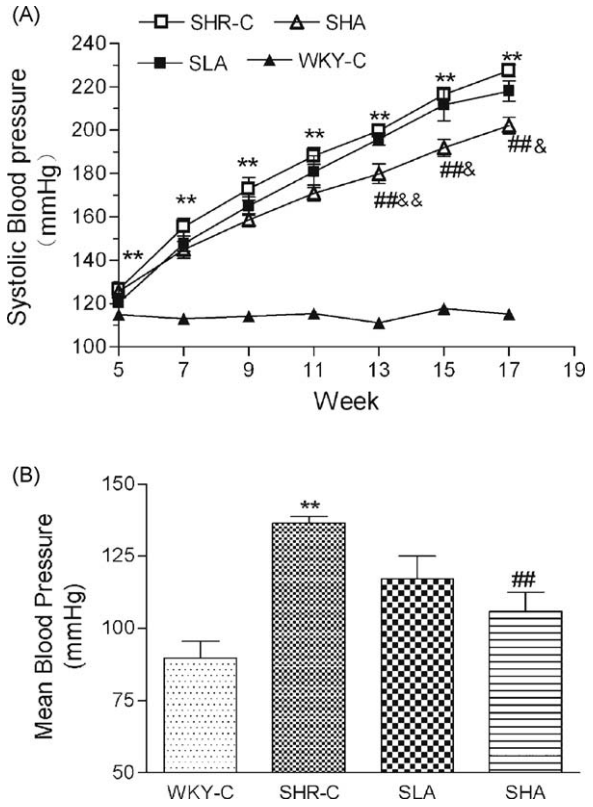

Fig. 1. Effect of alendronate treatment on systolic blood pressure (SBP) and mean arterial pressure (MAP). SBP was measured by the tail-cuff method (A) every other week and MAP was recorded by carotid catheter at the end of the study (B). SHRs were gavaged daily with distilled water (SHR-C, $n = 6$), 1 mg/kg/d alendronate (SLA, $n = 6$), or 10 mg/kg/d alendronate (SHA, $n = 6$), and WKY rats were gavaged with distilled water as control (WKY-C, $n = 6$). These results are expressed as mean ± SEM. $^{*}P < 0.05$ and $^{**}P < 0.01$ SHR-C compared with WKY-C group, $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ compared with SHR-C group, $^{\&}P < 0.05$ and $^{\&\&}P < 0.01$, SHA compared with SLA group.

Table 2
Effects of alendronate on hemodynamic parameters by left ventricular catheterization.

	WKY-C	SHR-C	SLA	SHA
HR (beat/min)	347.2 ± 25.2	412.9 ± 22.1	376.4 ± 20.6	383.7 ± 15.3
$+dp/dt_{max}$ (mmHg/s)	6953 ± 484	7002 ± 388	7245 ± 394	7272 ± 343
$-dp/dt_{max}$ (mmHg/s)	7174 ± 398	7502 ± 470	7947 ± 571	7761 ± 569
LVEDP (mmHg)	9.97 ± 0.91	22.03 ± 2.17*	13.17 ± 1.54#	6.43 ± 1.16#

These results are expressed as mean ± SEM, $n = 6$. The hemodynamic data were obtained from the left ventricle of unconscious rats. WKY-C, WKY control group; SHR-C, SHR control group; SLA, SHR rats treated with 1 mg/kg/d alendronate; SHA, SHR rats treated with 10 mg/kg/d alendronate; HR, heart rate; LVEDP, left ventricular end-diastolic pressure.
* $P < 0.01$ compared with WKY-C.
# $P < 0.01$ compared with SHR-C.

Table 3
Effects of alendronate on cardiac hypertrophy index, lipid profile, and hydroxyproline (Hyp) content in WKY and SHR at 17 weeks.

	WKY-C	SHR-C	SLA	SHA
BW (g)	300.6 ± 7.4	286.8 ± 5.8	288.8 ± 4.2	282.3 ± 4.4
WHW (mg)	866 ± 32.4	1302 ± 38.6**	1211 ± 26.9#	1029 ± 18.3##
LVW (mg)	673 ± 28.9	1056 ± 30.7**	946 ± 18.2##	835 ± 16.6##
WHW/BW (mg/g)	2.88 ± 0.04	4.54 ± 0.07**	4.19 ± 0.09##	3.65 ± 0.06##
LVW/BW (mg/g)	2.23 ± 0.05	3.68 ± 0.05**	3.28 ± 0.06##	2.95 ± 0.04##
HDL-C (mmol/L)	0.27 ± 0.03	0.22 ± 0.03	0.17 ± 0.01	0.17 ± 0.03
LDL-C (mmol/L)	1.15 ± 0.05	0.85 ± 0.07**	0.83 ± 0.03	0.70 ± 0.07#
TC (mmol/L)	1.42 ± 0.06	1.09 ± 0.08*	1.00 ± 0.03	0.87 ± 0.09#
Hyp (μg/mg wet weight)	0.17 ± 0.00	0.26 ± 0.01**	0.18 ± 0.01##	0.18 ± 0.01##

These results are expressed as mean ± SEM, $n = 6$. WKY-C, WKY control group; SHR-C, SHR control group; SLA, SHR rats treated with 1 mg/kg/d alendronate; SHA, SHR rats treated with 10 mg/kg/d alendronate; BW, body weight; WHW, whole heart weight; LVW, left ventricular weight; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; TC, total cholesterol; Hyp, hydroxyproline.
* $P < 0.05$ compared with WKY-C group.
** $P < 0.01$ compared with WKY-C group.
# $P < 0.05$ compared with SHR-C.
$P < 0.01$ compared with SHR-C.

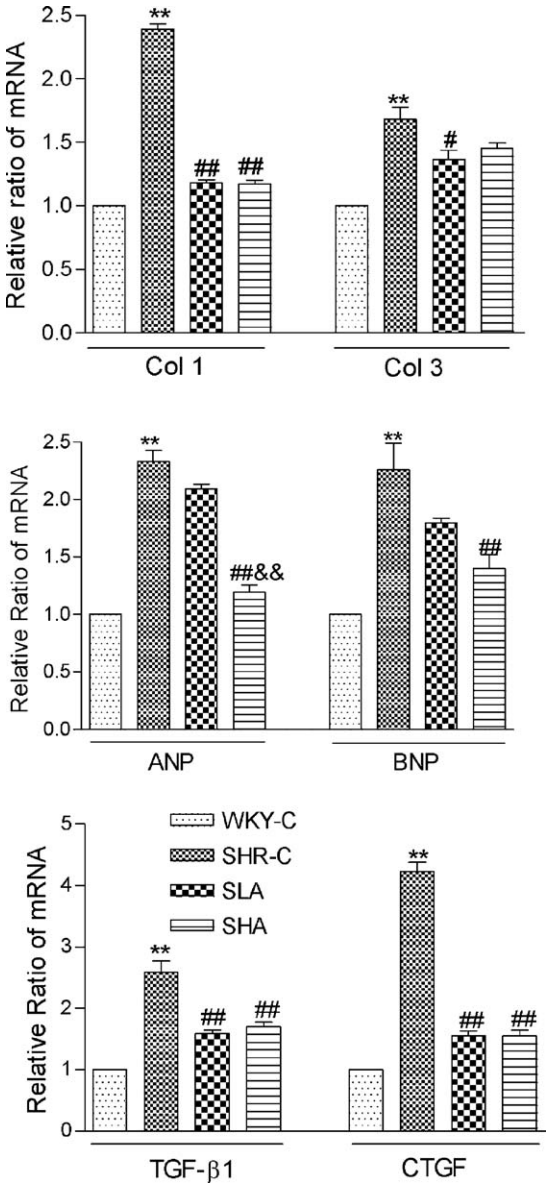

Fig. 2. mRNA expression of pro-hypertrophy and pro-fibrosis genes in left ventricle of rats by real-time polymerase chain reaction. SHRs were gavaged daily with distilled water (SHR-C), 1 mg/kg/d alendronate (SLA), or 10 mg/kg/d alendronate (SHA), and WKY rats were gavaged with distilled water as control (WKY-C). Data were normalized by the abundance of GAPDH mRNA and then expressed relative to the mean value for the WKY-C group. Data expressed as mean ± SEM ($n = 6$). $^{*}P < 0.05$ and $^{**}P < 0.01$ compared with WKY-C group, $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ compared with SHR-C group, $^{\&}P < 0.05$ and $^{\&\&}P < 0.01$, SHA compared with SLA group. Col 1, procollagen I; Col 3, procollagen III; ANP, atrial natriuretic peptide; BNP, B-type natriuretic peptide; TGF-β1, transforming growth factor beta 1; CTGF, connective tissue growth factor.

reduced the SBP in SHRs, and this effect reached statistical significance from the 13th week. However, the SBP-lowering effect was not found with low-dose alendronate (1 mg/kg/d) treatment of SHRs. As to MAP (Fig. 1B), SHRs had higher values than WKY rats (142.6 ± 5.2 mmHg versus 93.6 ± 4.8 mmHg, $P < 0.01$). MAP was also reduced in the SHR group treated with high-dose alendronate compared with that in the SHR-C group (118.7 ± 4.8 mmHg versus 142.6 ± 5.2 mmHg, $P < 0.01$).

SHR-C, compared with the WKY-C group, showed a higher LVEDP, but no differences in HR and $\pm dp/dt_{max}$ (Table 2). Twelve-week treatment with alendronate dose-dependently decreased the LVEDP in hearts from the SLA and SHA groups.

3.2. High-dose alendronate decreased the lipid profiles levels

Serum total cholesterol and LDL-C levels were significantly lower in SHR-C than in WKY-C. Twelve-week treatment with high-dose alendronate noticeably reduced the serum concentrations of total cholesterol and LDL-C in SHRs, but did not influence the levels of HDL-C. In addition, no significant effect of low-dose alendronate on lipid levels was found in SHRs (Table 3).

3.3. Alendronate ameliorated cardiac hypertrophy and fibrosis in SHRs

Compared to WKY-C, SHR-C had higher values for WHW, LVW and the ratios of WHW/BW and LVW/BW at the end of treatment (Table 3). Long-term treatment with alendronate dose-dependently reduced both the weights and the ratios without a change in BW. The mRNA expression of GADPH showed no differences among the ventricles of the four groups. Also, SHR-C showed increased mRNA expression of ANP and BNP, which are considered to be tissue markers for cardiac hypertrophy and fibrosis, and their reduction may be a sensitive index of cardiac hypertrophy regression [31]. After 12-week treatment, the low-dose alendronate slightly decreased the mRNA expression of ANP and BNP without statistical difference, while the high-dose alendronate statistically reduced the mRNA expression of ANP and BNP in the left ventricle (Fig. 2).

More collagen deposition (green staining) in the interstitia was found in the left ventricle of SHR-C, in contrast to that of WKY-C (Fig. 3), as well as more mRNA expression of procollagen type I/III, TGF-β1, and connective tissue growth factor (CTGF) (Fig. 2), and greater content of the collagen, Hyp (Table 3). Both low- and high-dose alendronate administrations significantly attenuated the collagen deposition, the mRNA expression of procollagen type I, TGF-β1, and CTGF, and the content of Hyp in the left ventricle of SHRs.

3.4. Alendronate suppressed Rho activation and phosphorylation of ERK in SHRs

Rho function depends on its conversion from the GDP- to the GTP-bound state. The levels of the GTP-bound active form of RhoA in hearts were determined by an affinity pull-down assay using Rhotekin GST-RBD. The levels of activated RhoA were higher in the SHR-C group than in the WKY-C group (Fig. 4, $P < 0.01$). Treatment with alendronate dose-dependently decreased GTP-RhoA levels in left ventricles from the SLA group ($P < 0.01$ versus SHR-C and SHA groups). However, there were no significant changes in the expression levels of total RhoA between SHR and WKY, and between alendronate-treated and -untreated SHR.

Meanwhile, SHR-C showed 2.46 ± 0.24-fold higher phosphorylated ERK1/2 expression than WKY-C (Fig. 4). Chronic treatment with alendronate notably suppressed the ERK1/2 phosphorylation, even at the low dose. Also, there were no significant changes in the expression levels of total ERK1/2 among the four groups.

4. Discussion

This study was designed to investigate the effects of an FPPS inhibitor on cardiac hypertrophy and fibrosis, and to explore its possible mechanism of action in SHRs undergoing cardiac remodeling. We have now, for the first time, shown that long-term treatment with alendronate improved left ventricular hypertrophy and fibrosis, suppressed ERK cascades and TGF-β1 signalling pathways, and inhibited RhoA activation in spontaneously hypertensive rats.

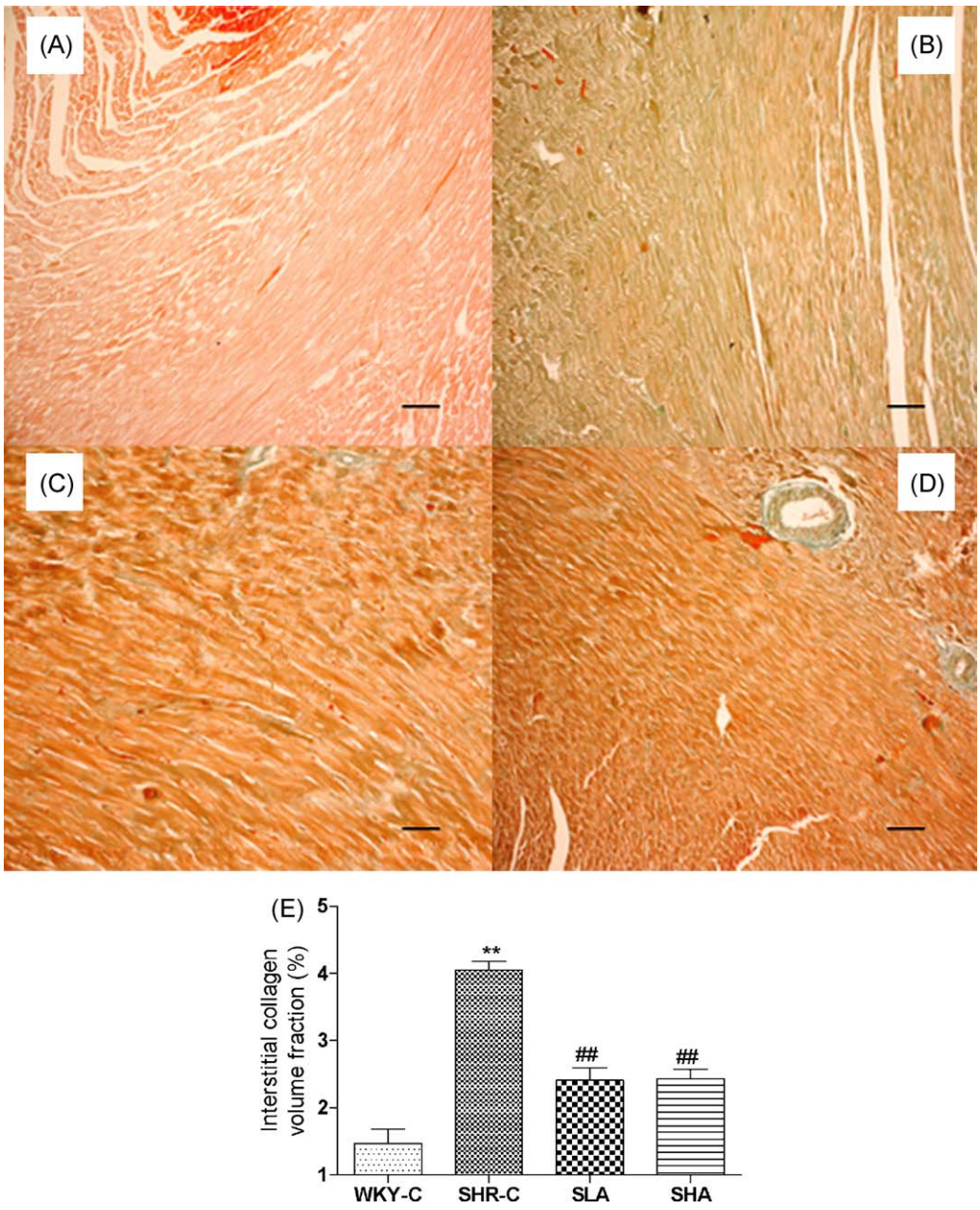

Fig. 3. Alendronate inhibited collagen deposition in the interstitia (A–D). Left ventricles embedded in paraffin were stained with Masson's trichrome to evaluate tissue fibrosis (green): (A–D) representative images and (E) collagen volume fraction. (A) WKY-C group; (B) SHR-C group; (C) SHR treated with low-dose alendronate; (D) SHR treated with high-dose alendronate. (A–D) 100× magnification. Data expressed as mean ± SEM, $n = 6$. $^{*}P < 0.05$ and $^{**}P < 0.01$ compared with WKY-C group, $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ compared with SHR-C group. Bars = 200 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

MAPK is a widely distributed group of enzymes ending in three terminal MAPK branches: p38-MAPK, ERK1/2, and c-Jun NH_2-terminal kinases (JNK). There is general agreement that MAPK cascades are involved in features of cardiac hypertrophy [32,33]. For example, ERK1/2 MAPK activation leads to a concentric form of hypertrophy with enhanced cardiac function [5]. In our study, alendronate treatment dose-dependently suppressed the expression of phosphorylated ERK1/2, which was increased in the SHR left ventricle, supporting the hypothesis that the mechanisms for cardiac hypertrophy regression induced by alendronate include the ERK cascades.

TGF-β1 is a locally generated cytokine that has been implicated as a major contributor to tissue fibrosis in various organ systems [34]. Recent studies in humans and experimental models have shown increased myocardial TGF-β1 expression during cardiac hypertrophy and fibrosis [35,36]. Connective tissue growth factor (CTGF) is responsible for mediating some of the effects of TGF-β1 and has been implicated in the onset and progression of fibrosis in most tissues [37–39]. In the present study, the increase of cardiac fibrosis in SHRs was accompanied by marked increases of TGF-β1, CTGF and procollagen I/III expression in the left ventricle. All these increases were inhibited by chronic low- and high-dose alendronate administration. Our data suggested that the mechanisms for cardiac fibrosis regression induced by alendronate might include the TGF-β1 signalling pathways.

Neonatal ventricular myocytes transfected with mutant active Ras, specifically in the heart, over-express ANP [40]. Likewise, the hypertrophy in myocytes is inhibited by transfection with dominantly negative Ras [40] gene or Rho [41]. These findings demonstrated that the small GTPase pathway, at least in part,

mediates myocyte hypertrophy. In our study, we found a higher level of GTP-bound active RhoA in left ventricles from SHRs than those from WKY rats, but a comparable level of total RhoA, and these results are consistent with several recent investigations [42,43]. Furthermore, chronic administration of alendronate in SHRs dose-dependently decreased the level of active RhoA but had no effect on total RhoA expression levels. Importantly, these results demonstrated that the FPPS inhibitor only affects the process of activation RhoA, and this may be mediated by inhibiting the synthesis of the isoprenoid intermediates, FPP and GGPP. Alendronate is used as an FPPS antagonist for the treatment of bone resorption and cancer by inhibiting Rho activity.

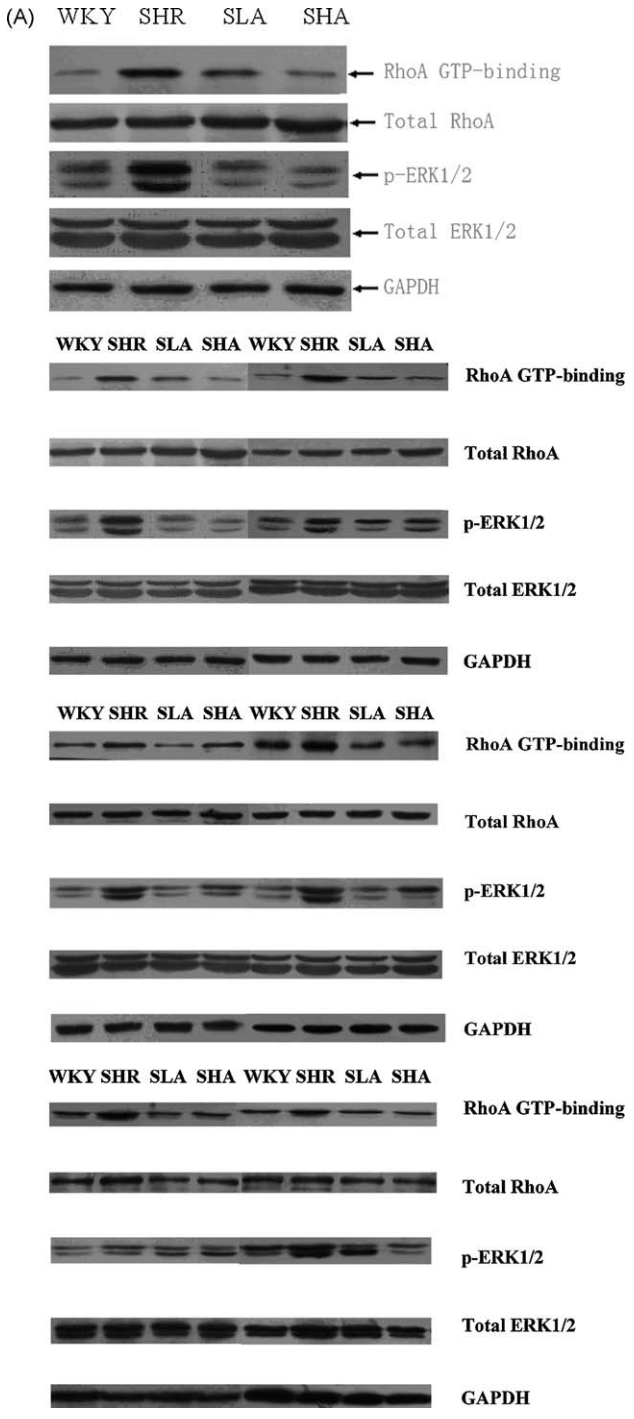

Fig. 4. Inhibitory effects of alendronate on RhoA activation and ERK1/2 phosphorylation in left ventricle. SHRs were gavaged daily with distilled water (SHR-C), 1 mg/kg/d alendronate (SLA), or 10 mg/kg/d alendronate (SHA), and WKY rats were gavaged with distilled water as control (WKY-C): (Panel A) representative images of Western blots and (Panel B) graphical representation. Relative activated ERK1/2 is represented as the ratio between phosphorylated ERK1/2 and total ERK1/2. RhoA activation is expressed as the ratio between GTP-binding and total RhoA protein. Data expressed as mean ± SEM, $n = 6$. $^{*}P < 0.05$ and $^{**}P < 0.01$ compared with WKY-C group, $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ compared with SHR-C group.

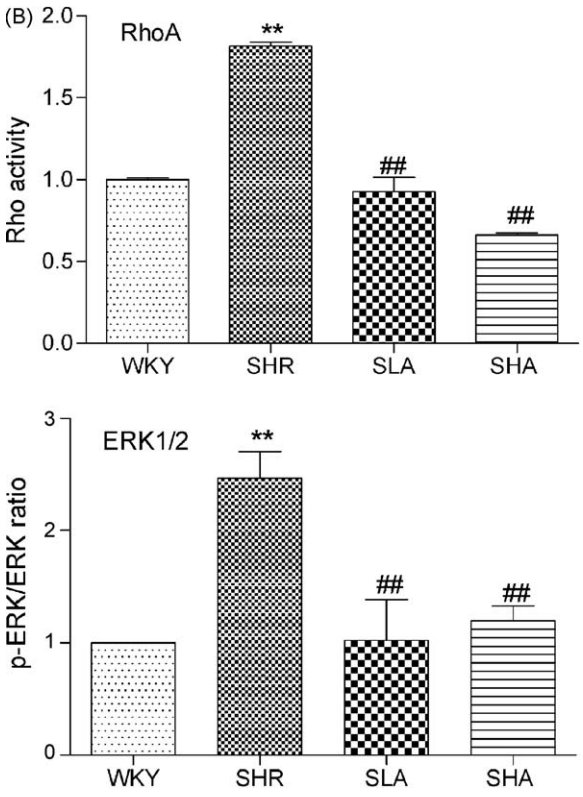

Fig. 4. (*Continued*).

It has been reported that small GTPase participates in the activation of the ERK pathway [9,10,44,45] and the TGF-β-collagen cascade [11,12], although these cascades can be triggered by several different agents. Moreover, Rho participates in ERKs phosphorylation in the cardiac myocytes. Aikiwa et al. revealed that mechanical stress-induced phosphorylation of ERKs is strongly suppressed by over-expression of Rho-GDI (Rho guanine nucleotide-dissociation inhibitor, a protein binding with the inactive Rho) or pretreatment with the C3 exoenzyme (specific inhibitor of Rho) in cardiac myocytes [45]. Rho-kinase (ROCK), an effector and downstream of the Rho, plays a very important role in cardiac remodeling, which is activated by binding the active form of Rho. Thus, decreased expression of active small GTPase by inhibition of isoprenylation with alendronate, leads to decreased Rho/ROCK binding and inhibits TGFβ expression. Ishikawa et al. used Rho-kinase inhibitor decreased mRNA expression of TGFβ in renal tissue of malignant hypertensive rats [11]. Rikitake et al. found that cardiac fibrosis induced by angiotensin II infusion or other treatments, e.g. N^{G}-nitro-L-arginine methyl ester administration, myocardial infarction, and transaortic constriction, was attenuated in ROCK1$^{+/-}$ haploinsufficient mice. The expression level of ROCK1 in ROCK1$^{+/-}$ heart was approximately half of that in wildtype heart. Further, the elevated mRNA expression of TGFβ and CTGF induced by angiotensin II was markedly decreased in ROCK1$^{+/-}$ mice, compared with wildtype mice [46]. Thus, we assumed that Rho/ROCK signaling participated in cardiac fibrosis, and inhibition of Rho/ROCK decreased the TGFβ expression. Maybe that's the reason for the attenuation of cardiac fibrosis induced by alendronate.

It was unexpected that treatment with high-dose alendronate did not further down-regulate the expression of TGF-β1, CTGF and collagen as well as decrease the phosphorylation of ERK in SHRs than low-dose treatment. Perhaps increased expression of some cytokines was induced by high-dose alendronate in a complicated and multi-regulated cytokine circuit, which led to no further decrease in expression of the TGFβ1 pathway and phosphorylated ERK regardless of further decreases in activation of RhoA in SHA. Thus, we assume that the mechanisms for cardiac hypertrophy and fibrosis regression induced by alendronate might include the Rho-ERK1/2 cascade, the TGF-β1 pathway, and some unknown process. Moreover, in this study chronic treatment with low-dose alendronate slightly decreased the mRNA expression of ANP and BNP without statistical difference, and only high-dose alendronate statistically reduced the mRNA expression of ANP and BNP in the left ventricle. In the previous report, Laufs proved that pharmacological and genetic inhibition of RhoA only partially decreased the angiotensin II-induced ANP over-expression in cardiomyocytes. Rac1, another member of Rho family, plays an important role in the regulation of ANP [47]. Thus, we assumed that low-dose alendronate may partially inhibit the Rac1, which lead to insufficient downregulation of ANP and BNP expression, in despite of markedly inhibition of RhoA activity.

In the present study, chronic treatment with low-dose alendronate decreased the left ventricular hypertrophy and fibrosis in SHRs, as with high-dose alendronate, while no changes were found in blood pressure by the tail-cuff and carotid catheter methods or in lipid profiles by serum assay. Moreover, previous studies on pressure-induced left ventricular hypertrophy revealed that hydralazine, a vasodilator, decreases blood pressure, even normalized blood pressure, but fails to decrease mRNA and protein expression of ANP and collagen in hypertensive rat models [48,49]. Furthermore, the SHR is considered to be a model of hypertension and left ventricular hypertrophy with lower lipid profile levels [15,16,30]. Therefore, we assumed that the regression of left ventricular hypertrophy induced by alendronate treatment was independent of blood pressure and cholesterol in SHRs, and the amelioration of LVEDP in the SLA group was attributable to the regression of cardiac remodeling, not to the blood pressure reduction.

It should be pointed out that, in general, the doses of various drugs used in rat studies are 50 to 100 times higher than those used in humans [50]. Alendronate is a nanomolar inhibitor of FPPS in liver extract [27]. In studies of osteoporosis and cancer metastasis, the regular dose used for oral administration is 1–3 mg/kg/d for rats [51,52] and 10 mg/d for humans [53,54]. The low dose was chosen according to osteoporosis study in rats, and the high dose was still in the range of 50–100 times higher than the human dose. Moreover, Peter and Rodan showed that no adverse developmental effects were noted at doses up to 25 mg/kg/d in rats [55]. This finding suggested that the doses used in this study were still in the regular range and the alendronate-induced effects in this dose range were below its adverse effects.

In conclusion, we provide the first experimental evidence that chronic treatment with an FPPS inhibitor attenuates the development of cardiac hypertrophy and fibrosis, and these effects are accompanied by the inhibition of RhoA activation, ERK1/2 phosphorylation, and TGF-β1 expression. We further suggest that the cardiac protective effects of alendronate are attributable, at least in part, to inhibition of RhoA activation, suppression of ERK1/2 phosphorylation and TGF-β1 expression.

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