Original Article

Angiotensin-Converting Enzyme Inhibitor Suppresses Activation of Calcineurin in Renovascular Hypertensive Rats

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Accumulating evidence suggests an important role of the calcineurin signaling pathway in mediating the development of cardiac hypertrophy. It has also been reported that angiotensin-converting enzyme inhibitors (ACEIs) regressed cardiac hypertrophy in some animal and human models. In this study, we investigated the possible role of calcineurin in the regression of cardiac hypertrophy induced by the ACEI perindopril in rats with renovascular hypertension. The effect of the calcineurin inhibitor cyclosporine A (CsA) was also studied. Starting from 2 months after a two-kidney one-clip (2K1C) procedure, the rats that had developed progressive left ventricular (LV) hypertrophy were daily administered perindopril (1 mg/kg per day) or CsA (20 mg/kg per day) until 3 months. At the end of either treatment, the LV gravimetric, morphometric and histological measurements revealed the regression of LV hypertrophy; and the enzymatic assay, Western blotting and reverse transcription–polymerase chain reaction (RT-PCR) showed that both calcineurin activity and the calcineurin protein and mRNA expression levels were significantly decreased compared with untreated 2K1C rats, but that LV systolic performance was unchanged by either treatment. These data suggest that the cardiac hypertrophy regression induced by the ACEI perindopril is likely mediated, at least in part, through inhibition of the calcineurin signaling pathway. (*Hypertens Res* 2007; 30: 1247–1254)

***Key Words*: calcineurin, cardiac hypertrophy, perindopril, hypertension**

Introduction

Cardiac hypertrophy is not only an adaptive response of the heart to volume and pressure overload but also a leading predictor of progressive heart disease and morbidity. Epidemiological studies have shown that cardiac hypertrophy causes ischemic heart disease, arrhythmia and sudden death (*1*). It is thus of paramount importance to elucidate the mechanism of the development of cardiac hypertrophy. Since cardiac myocytes lose their proliferative ability after birth, the development of cardiomyocyte hypertrophy is the only available means of responding to external stresses. Cardiac hypertrophy is induced by a variety of stimuli, including hemodynamic overload, ischemic disease, neurohumoral factors, or intrinsic defects in cardiac structural protein genes (*2*). The intracellular Ca^{2+} level is elevated by various hypertrophic stimuli (*3*), and Ca^{2+} has been reported to play a critical role in the development of cardiac hypertrophy (*4*). Calcineurin is a calcium/calmodulin-activated cytoplasmic protein phosphatase that transduces hypertrophic stimuli to regulate transcriptional control of myocyte transformation. Studies have established that the calcium-calcineurin signaling pathway plays a critical role in the development of cardiac hypertrophy (*5–8*).

Evidence suggests that angiotensin II (Ang II) is a potent stimulator of cardiac hypertrophy. Angiotensin-converting enzyme inhibitors (ACEIs) or angiotensin II type 1 (AT1)

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Table 1. Sequence of Oligonucleotide Primers (Forward and Reverse) Used for RT-PCR

Target	Sequence (5′ to 3′)	Location	Length (bp)
Calcineurin			
Forward	AGCTTGACTTGGACAACTCT	151–170	400
Reverse	ATATCTAGGCCACCTACAAC	531–550	
GAPDH			
Forward	GGAGGCCATGTAGGCCATGAGGTC	522–545	555
Reverse	AATGCATCCTGCACCACCAACTGC	1053–1076	

RT-PCR, reverse transcription–polymerase chain reaction.

receptor blockers prevent the development of left ventricular (LV) hypertrophy both in animal models and in hypertensive patients (*9–11*). However, there has been little research into the effects of ACEIs on calcineurin signaling *in vivo*, and the issue of whether suppression of calcineurin induces the regression of cardiac hypertrophy in renovascular hypertensive rats once cardiac hypertrophy develops remains to be explored. Recent evidence suggests that hypertrophy is not required for the maintenance of systolic performance under all conditions of homodynamic stress. Mice subjected to surgical banding of the thoracic aorta maintain normal systolic performance despite elimination of hypertrophy by calcineurin inhibition (*12*). We considered that it would be of interest to examine whether LV systolic performance is preserved when cardiac hypertrophy is reversed.

The main purpose of the present study was to investigate whether blockade of the RAS with perindopril, an ACEI, induces the regression of cardiac hypertrophy in rats with renovascular hypertension, and if so, whether this drug also inhibits calcineurin activation *in vivo* and whether cardiac systolic function is preserved. To explore the mechanism by which ACEIs suppress calcineurin activation, we also studied the effects of cyclosporin A (CsA), a calcineurin inhibitor, on the regression of cardiac hypertrophy and calcineurin activation.

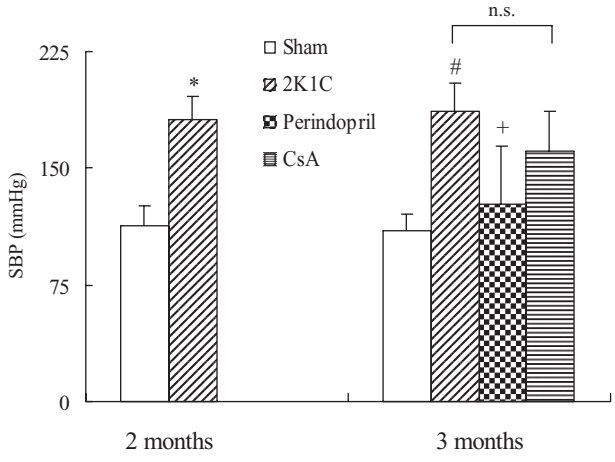

Fig. 1. *Systolic blood pressure measured in aorta. The blood pressure of 2- or 3-month 2K1C rats was higher than that of age-matched sham rats. Perindopril treatment decreased the blood pressure, and there was no significant difference in blood pressure between 3-month 2K1C rats and CsA-treated 2K1C rats. Data are shown as mean±SEM. *$p<0.05$ vs. 2-month sham rats; $^{\#}p<0.05$ vs. 3-month sham rats; $^{+}p<0.05$ vs. 3-month 2K1C rats. n.s. means no significance.*

Methods

Animals

Male Sprague-Dawley (SD) rats weighing 210±25 g were obtained from the animal center of Nanjing Medical University (China). Two kidney-one clip (2K1C) renovascular hypertension was induced by placing a silver clip (internal diameter 0.25 mm) around the left renal artery through a flank incision. The right kidney was left undisturbed. Sham-operated rats underwent the same experimental procedure but no clip was placed. Systolic blood pressure (SBP) was measured weekly by the indirect tail-cuff method using an MRB-IIIA indirect tail-cuff sphygmomanometer (Shanghai Hypertension Institute, Shanghai, China). Rats were maintained on sterile tap water and a regular rodent diet ad libitum. Two months after the clipping, when cardiac hypertrophy had already developed, the rats were randomly divided into three groups: one group was given placebo (0.9% saline) as a control, one group was administered perindopril (1 mg/kg body weight daily; Servier, Neuilly, France), and one group was administered CsA (20 mg/kg body weight daily; Novartis, Basel, Switzerland). All treatments were started at 2 months after clipping, and continued until 3 months.

Echocardiographic Analysis

Transthoracic echocardiography was performed utilizing a Hewlett Packard 5500 instrument with a 12 MHz microprobe. The short-axis dimension was recorded at the level of the tip of the papillary muscle, and the thickness of the interventricular septum (IVS) and left ventricular posterior wall (LVPW) were measured at end-diastole. The LV fractional shortening

Table 2. Perindopril or CsA Administration Induced the Regression of Cardiac Hypertrophy

Parameter	2 months after clipping		3 months after clipping			
	Sham	2K1C	Sham	2K1C	Perindopril	CsA
n	11	15	11	13	8	11
Body weight (g)	392±6	426±8	431±11	492±9	459±6	378±8
Tibial length (mm)	39.8±1.1	39.7±1.5	39.9±1.3	40.3±1.3	40.1±1.7	41.0±1.2
LV weight (mg)	800±8	1,120±11*	900±9	1,250±16$^{\#}$	1,040±12$^{\dagger,\$}$	960±13$^{\dagger,\$}$
LV weight/tibial length (mg/mm)	20.2±1.4	28.2±1.8*	22.8±1.4	30.9±2.3$^{\#}$	23.5±2.1$^{\dagger,\$}$	24.1±2.0$^{\dagger,\$}$
IVS thickness (mm)	2.13±0.04	2.59±0.03*	2.25±0.04	2.94±0.04$^{\#}$	2.25±0.04$^{\dagger,\$}$	2.28±0.04$^{\dagger,\$}$
LVPW thickness (mm)	2.13±0.04	2.58±0.03*	2.25±0.04	2.91±0.04$^{\#}$	2.24±0.04$^{\dagger,\$}$	2.22±0.03$^{\dagger,\$}$
Cross-sectional area of cardiomyocyte (μm^2)	240±9	704±38*	303±13	903±42$^{\#}$	372±99$^{\dagger,\$}$	330±23$^{\dagger,\$}$

Data are presented as mean±SEM. *$p<0.05$ *vs*. 2-month sham rats; $^{\#}p<0.05$ *vs*. 3-month sham rats; $^{\dagger}p<0.05$ *vs*. 3-month 2K1C rats; $^{\$}p<0.05$ *vs*. 2-month 2K1C rats. CsA, cyclosporine A; LV, left ventricular; IVS, interventricular septem; LVPW, left ventricular posterior wall.

(FS) and ejection fraction (EF) were calculated. The tracings were analyzed by one observer who had no knowledge of the study groups.

Histological Analysis

At 2 or 3 months after clipping, the rats were anesthetized by intraperitoneal injection of ketamine hydrochloride (50 mg/kg). A "home-made" catheter was inserted through the right carotid artery into the aorta, and SBP was recorded by a computer data acquisition workstation (BL410 biopsy laboratory system; Chengdu, Sichuan Province, China). Following the homodynamic study, LV weight (LVW) and tibial length (TL) were measured, and LVW was corrected for TL for quantitative analysis. A part of the LV was fixed with 10% formalin for 16 to 24 h and embedded in paraffin. Transverse sections (3 μm thickness) were prepared and stained with hematoxylin-eosin for routine histological examination. The myocyte cross-sectional areas were measured from myocytes that were cut transversely and exhibited both a nucleus and an intact cell membrane. At least 100 cells were assessed per LV with the software package QWin (Leica, Bensheim, Germany), and the average value was used for analysis.

Calcineurin Activity Assay

The activity of calcineurin in lysates of LV samples was determined as described previously (*13*) with a small modification. Tissue was homogenized in a buffer containing 50 mmol/L Tris-HCl (pH 7.5), 0.5 mmol/L dithiothreitol, and protease inhibitors, and cell debris was removed by centrifugation. The reaction solutions A (50 mmol/L Tris-HCl [pH 7.4], 0.5 mmol/L dithiothreitol, 0.2 g/L BSA, 10 mmol/L *p*-nitrophenyl phosphate [PNPP], 2 mmol/L $CaCl_2$, 0.3 μmol/L calmodulin) and B (identical to A but with 3 mmol/L EGTA instead of $CaCl_2$ and calmodulin) were dispensed into separate wells of a 96-well plate, and a test solution prepared as described above was then added into the wells containing solution A or B. After incubating for 30 min at 30°C, the absorbance at 405 nm was read. Calcineurin activity was calculated as phosphate released in the presence of calmodulin and Ca^{2+} (sample with solution A) minus phosphate released in the presence of EGTA instead of calmodulin and Ca^{2+} (sample with solution B), and the activity was normalized for the protein concentration.

Western Blot Analysis

The protein expression of calcineurin in lysates of LV samples was analyzed by Western blotting using an anti-calcineurin (catalytic subunit) antibody (sc 9070; Santa Cruz Biotechnology Inc., Santa Cruz, USA), the resulting images were densitometrically quantified, and the calcineurin protein levels were expressed as the values relative to the control (2-month sham-operated rats)

Semi-Quantitative Reverse Transcription–Polymerase Chain Reaction

Total RNA was extracted from LV samples using the modified single-step method of RNA isolation by acid guanidinium thiocyanate–phenol–chlorlform extraction. Complementary DNA was then synthesized from 2 μg of total RNA with an oligo(dT)12–18 primer and reverse transcriptase. Polymerase chain reaction (PCR) was performed with primers specific for calcineurin and GAPDH mRNAs (Table 1), using GAPDH mRNA as an internal standard. The PCR products of each target gene were verified by sequencing.

Statistical Analysis

All data are expressed as the means±SEM. Two-way ANOVA and post hoc *q* test were carried out for multiple comparisons among groups. A value of $p<0.05$ was considered statistically significant.

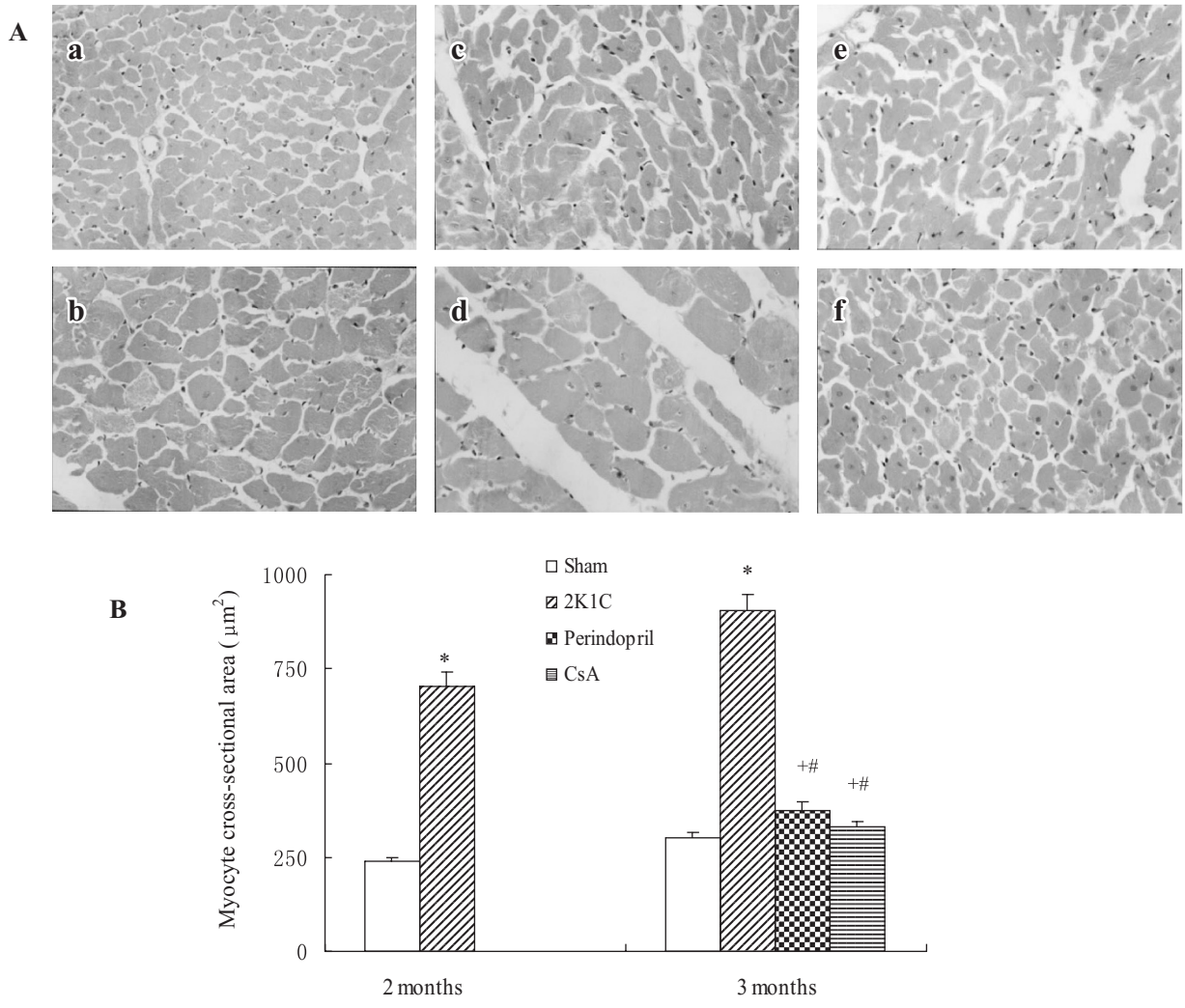

Fig. 2. *Histological analysis. A: Representative high magnification views of LV wall stained by H&E. a and c: cross-sectional area of cardiac myocytes in the hearts of 2- or 3-month sham rats; b and d: increase in cardiomyocyte area in 2- or 3-month 2K1C rats; e: treatment with perindopril; f: treatment with CsA. B: Cross-sectional area of cardiomyocytes. Data are shown as mean±SEM. *$p < 0.05$ vs. age-matched sham rats; $^{+}p < 0.05$ vs. 3-month 2K1C rats; $^{\#}p < 0.05$ vs. 2-month 2K1C rats.*

Results

Effect of Perindopril on the Reversal of Cardiac Hypertrophy

Two months after 2K1C, SBP monitored in the aorta of the rats was increased, and this elevation of blood pressure was also observed in untreated rats at 3 months after operation (untreated 3-month 2K1C rats). Treatment with perindopril significantly reduced blood pressure, and there was no significant difference in blood pressure between the perindopril-treated 2K1C rats and the untreated sham-operated rats. In contrast, blood pressure was not affected by the treatment with CsA (Fig. 1).

The LVW of untreated 2K1C rats at 3 months after operation (untreated 3-month 2K1C rats) was increased by 39% compared with that of age-matched sham-operated rats, and this increase was attenuated by 60% by treatment with perindopril and prevented by CsA treatment (Table 2). The LVW/TL was 35.5% greater in untreated 3-month 2K1C rats than in age-matched sham-operated rats, and treatment with perindopril or CsA significantly reduced the increase in the LVW/TL. Moreover, the LVW and LVW/TL of perindopril- or CsA-treated 3-month 2K1C rats was significantly less than that of 2-month 2K1C rats, suggesting that perindopril or CsA treatment induced regression of cardiac hypertrophy (Table 2).

To further quantify the reversal of hypertrophy in 2K1C rats, we analyzed individual myocyte size. Microscopic anal-

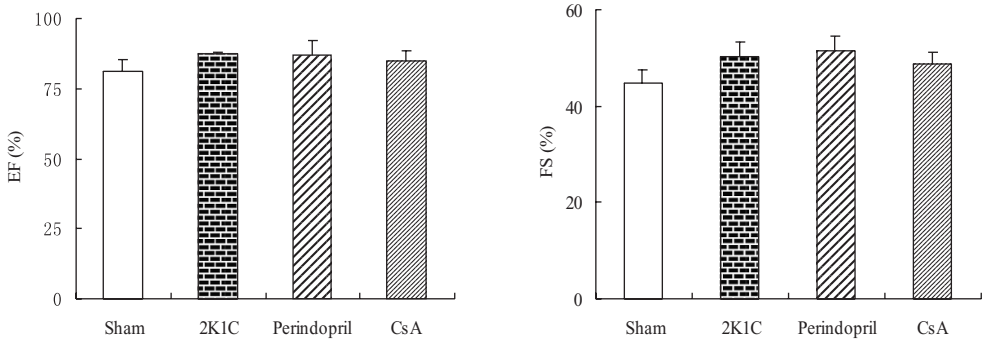

Fig. 3. *Cardiac systolic function detected by echocardiography at 3 months after operation. There was no significant difference in EF or FS among sham, 2K1C, perindopril- and CsA-treated rats.*

ysis of hematoxylin and eosin (H&E)–stained cardiac histological sections revealed a noticeable increase in the cross-sectional area of cardiac myocytes in either 2- or 3-month 2K1C rats, compared with that in age-matched sham-operated rats. However, 1 month of perindopril or CsA treatment resulted in the reversal of myofibre hypertrophy (Table 2). The cross-sectional area of perindopril- or CsA-treated 3-month 2K1C rats was smaller than that of 2-month 2K1C rats (Fig. 2).

Echocardiography revealed that the thicknesses of IVS and LVPW were greater in 2-month 2K1C rats than in age-matched sham rats, and in 3-month 2K1C rats, there was a further increase in the thickness of both IVS and LVPW (Table 2). The thickness of IVS and LVPW was found to decrease in perindopril-treated 3-month 2K1C rats as compared with that of untreated 2-month 2K1C rats, suggesting that perindopril treatment not only attenuated the development but also induced the regression of cardiac hypertrophy. These data were also consistent with the gravimetric measurements of LVW and LVW/TL. CsA treatment showed a similar ability to regress cardiac hypertrophy (Table 2).

Effect of Perindopril on Cardiac Systolic Function *In Vivo*

Three months after clipping, the LV systolic function, measured as EF and FS, of sham-operated rats was similar to that of 2K1C rats. Echocardiograms revealed that the EF and FS of both perindopril- and CsA-treated 2K1C rats were similar to those of untreated 2K1C rats, suggesting that LV systolic function is preserved after the treatment with perindopril or CsA despite the reversal of cardiac hypertrophy (Fig. 3).

Inhibition of Calcineurin by Perindopril

In order to examine whether perindopril treatment inhibits the activation of calcineurin in the hearts of 2K1C rats, we detected calcineurin activity and the calcineurin protein and mRNA expression levels.

Calcineurin activity in the LV samples of 2- or 3-month 2K1C rats was increased by 39% or 24%, respectively, compared with age-matched sham-operated rats, and the increase was suppressed significantly by treatment with perindopril or CsA (Fig. 4).

The calcineurin protein expression level was increased in the LV sample of 2- or 3-month 2K1C rats compared with age-matched sham-operated rats. In 2-month 2K1C rats, the calcineurin protein level was increased to 209±15% ($p<0.01$), ranging from 151% to 267% (the calcineurin protein level in 2-month sham-operated rats was set as 100%). In 3-month 2K1C rats, the calcineurin protein level was increased to 250±14% ($p<0.01$). The calcineurin protein level was decreased to 83% or 127% in the perindopril- or CsA-treated groups, respectively (Fig. 4).

The calcineurin mRNA expression level in the LV of 2- or 3-month 2K1C rats was significantly higher than that of the age-matched sham-operated rats, and this elevation of calcineurin mRNA expression was suppressed completely by the treatment with perindopril or CsA (Fig. 4).

Discussion

In the present study, we showed that the ACEI perindopril induced the regression of cardiac hypertrophy, and prevented the increase in calcineurin activity and in the calcineurin protein and mRNA expression levels in rats with renovascular hypertension. These results suggest that calcineurin may contribute to Ang II signaling *in vivo*, and inhibition of endogenous Ang II–induced activation of calcineurin is important for the attenuation and regression of cardiac hypertrophy by ACEI in renovascular hypertensive rats.

The renin-angiotensin system is crucial for the maintenance of blood pressure, fluid, and sodium homeostasis and also plays a role in the pathogenesis of hypertension. In the 2K1C model of renovascular hypertension, one renal artery is constricted to reduce renal perfusion, and the other kidney remains untouched. In response to low renal arterial pressure, the plasma renin concentration is rapidly increased and then

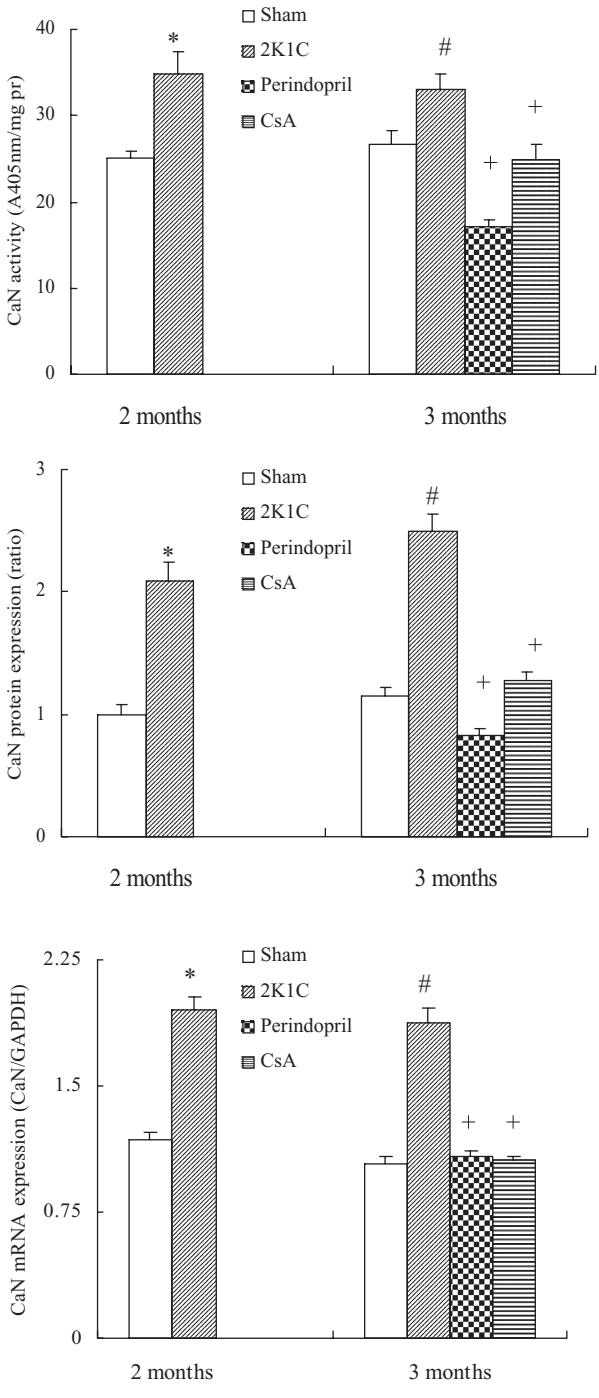

Fig. 4. *The changes of calcineurin activity and its protein and mRNA expression levels in different groups. Two- or 3-months after clipping, calcineurin activity and its protein and mRNA expression levels were increased in 2K1C rats compared with age-matched sham rats. The activation of calcineurin signaling pathway in 2K1C rats was inhibited by the treatment with perindopril or CsA. All data are expressed as mean±SEM. *$p < 0.05$ vs. 2-month sham rats; #$p < 0.05$ vs. 3-month sham rats; +$p < 0.05$ vs. 3-month 2K1C rats.*

the renin-angiotensin-aldosterone system is activated, resulting in chronic elevation of blood pressure and subsequent cardiac hypertrophy.

Calcineurin is a calcium/calmodulin-activated protein phosphatase, and an elevation in the intracellular calcium concentration leads to calmodulin saturation, which results in displacement of the autoinhibitory domain from the catalytic subunit of calcineurin, thus activating the enzyme (*14*). Calcineurin has been reported to play a key role in the development of cardiac hypertrophy (*5–8*). In this study, to determine whether calcineurin is activated in cardiac hypertrophy caused by renovascular hypertension, we used three distinct assays: a calcineurin enzymatic assay, a Western blot assay, and reverse transcription-PCR (RT-PCR). The results showed that calcineurin activity was increased in the hearts of 2K1C rats, accompanied by the development of cardiac hypertrophy, suggesting that activation of the calcineurin signaling pathway is associated with cardiac hypertrophy in renovascular hypertensive rats. Moreover, the levels of calcineurin mRNA and protein expression were also increased in 2K1C rats compared with age-matched sham-operated rats. These data suggest that cardiac calcineurin activity, in addition to being regulated by calcium/calmodulin, might also be regulated at the level of gene or protein expression. The study of Ritter *et al*. (*15*) in patients with myocardial hypertrophy caused by hypertrophic obstructive cardiomyopathy or aortic stenosis found that, in addition to the increment of calcineurin activity in myocardial hypertrophy, there is a proteolysis of the calcineurin A C-terminus containing the autoinhibitory domain. Recently it was also found that Ang II stimulation of cardiomyocytes leads to proteolysis of the autoinhibitory domain of calcineurin (*16*). Moreover, it was recently demonstrated that the phosphatase calcineurin is translocated to the nucleus, and targeted inhibition of the nuclear import of calcineurin could prevent myocardial hypertrophy (*17*). These results suggest that calcineurin activity is regulated on several levels.

The fact that perindopril or CsA induced the regression of cardiac hypertrophy in our model was verified by three independent methods: gross pathology, *in vivo* echocardiography, and examination of the cardiac myocyte area. Cardiac hypertrophy is a fundamental, adaptive response of the heart to multiple stimuli. The activation of the calcineurin signaling pathway in the 2K1C rats observed in the present study was inhibited by the treatment with ACEI perindopril or calcineurin inhibitor CsA. The activation of calcineurin as well as the development of cardiac hypertrophy was inhibited in the hearts of renovascular hypertensive rats treated with perindopril, suggesting that inhibition of the calcineurin activation may be a cause of the perindopril-induced regression of cardiac hypertrophy. Calcineurin represents a potential "sensing" molecule that links alterations in ventricular load (through calcium) with molecular signaling and changes in gene expression. Indeed, inhibition of calcineurin signaling may be beneficial for decoupling an increase in inotropy from

maladaptive decompensation and heart failure. However, the molecular mechanism whereby inhibition of calcineurin leads to regression of hypertrophy is not understood. Presumably, calcineurin activity is necessary to maintain coordinated signaling with other intracellular signaling pathways, resulting in a balanced hypertrophic response. Attenuation of calcineurin activity may simply lead to a profile of unbalanced signaling, which results in diminished efficiency to promote the hypertrophic state (*18*). This interpretation is consistent with a large body of literature that has shown attenuation of cardiac hypertrophy by inhibition of other intracellular pathways, suggesting coordinated intracellular signaling networks. In addition to the calcineurin pathway, there are other signaling cascades involved in the development and maintenance of myocardial hypertrophy, such as mitogen-activated protein kinases (including extracellular signal–regulated kinase [ERK], c-Jun N-terminal kinase [JNK], and p38), protein kinase C (PKC) and protein kinase A as well. There is an integrated model of signal transduction in the heart such that multiple pathways are necessary for timely and effective hypertrophy. Specific inhibition of central regulatory pathways likely diminishes the activation of other interdependent signal transduction pathways (*19*). Calcineurin not only leads to the nuclear factor of activated T cell (NFAT) dephosphorylation and its nuclear translocation, but also promotes the activation of JNK and certain PKC isoforms. When CsA inhibits the calcineurin signaling pathway, the activity of JNK and PKC is also decreased (*20*). It has been reported that the calcineurin and MEK-ERK1/2 pathways constitute a codependent signaling module in cardiomyocytes that coordinately regulates the growth response (*21*), and that carabin is a negative feedback inhibitor of the calcineurin signaling pathway that also mediates crosstalk between calcineurin and Ras (*22*). In our laboratory, we also found that JNK mRNA expression was upregulated in renovascular hypertensive rats, and that perindopril treatment induced the down-regulation of JNK mRNA expression (data not shown). Inhibition of multiple intracellular signaling pathways may be the basis of the reversal of cardiac hypertrophy by perindopril or CsA.

In addition to the inhibition of calcineurin, however, we cannot ignore the blood pressure–lowering effect of perindopril on the regression of cardiac hypertrophy. The antihypertrophic effect of an AT1 receptor blocker has been shown to be greater than that of hydralazine, despite the greater antihypertensive effect of hypralazine, in spontaneously hypertensive rats (*23*). It has also been demonstrated that a non-antihypertensive dose of AT1 receptor blocker attenuates the development of cardiac hypertrophy and fibrosis as well as the activation of calcineurin in rats with salt-sensitive hypertension (*24*). Matsumoto *et al*. found that the ACEI imidapril reduced LV mass in hemodialysis patients by a mechanism that was independent of changes in blood pressure (*25*). It is also reported that blockade of Ang II receptors may attenuate the structural changes in the heart and blood vessels of hypertensive animals independent of a reduction in blood pressure (*26*). In our laboratory, we also found that there was a trend of attenuation of cardiac hypertrophy by a non-antihypertensive dose of perindopril in 2K1C rats (data not shown). These observations suggest that blood pressure reduction alone is not sufficient to prevent cardiac hypertrophy. Accordingly, perindopril reverses the cardiac hypertrophy in renovascular hypertensive rats mainly through the inhibition of calcineurin signaling pathways induced by Ang II, rather than mainly through lowering the blood pressure.

Finally, our study demonstrates that LV systolic performance is preserved despite the reversal of cardiac hypertrophy in renovascular hypertensive rats. This result is consistent with that reported by Hill *et al*. (*12*) in a model of aortic-banded mice. The underlying mechanism may involve a sustained Anrep effect.

In conclusion, this study demonstrated that ACEI perindopril induced a regression of cardiac hypertrophy in rats with renovascular hypertension, possibly through inhibition of the calcineurin signaling pathway. Given the multifactorial nature of hypertrophic signaling, it is likely that many regulatory pathways coordinately participate in the overall hypertrophic response (*21*). Further studies are necessary to elucidate the connections between calcineurin and other intracellular signaling molecules and thereby provide a better understanding of the regulation of cardiac hypertrophy. In addition, further investigations will be needed to elucidate whether a non-antihypertensive dose of perindopril induces regression of cardiac hypertrophy and inhibits calcineurin activity in the heart to the same extent as in this study.

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