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Cardiac Calcineurin during Transition from Hypertrophy to Heart Failure in Rats

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We studied an alteration of calcineurin expression in the heart and its modification by cyclosporin A and an ACE inhibitor, *temocapril*, using Dahl salt-sensitive (DS) rats with hypertensive left ventricular hypertrophy (LVH) and congestive heart failure (CHF). Calcineurin protein expression in the LV myocardium was increased in the LVH stage, but then decreased during CHF transition. Chronic cyclosporin A treatment (10 mg/kg/day), which inhibits calcineurin activity, could not block the increases of LV weight and dimensions and did not improve the LV systolic function during the CHF transition. In contrast, chronic temocapril treatment (20 mg/kg/day) restored the downregulation of calcineurin expression, but progression of the hypertrophic process was inhibited. Therefore, cardiac calcineurin is increased in the hypertensive LVH and may be involved in the development of the adaptive hypertrophic process. However, calcineurin expression is downregulated during CHF transition and may no longer play a major role in the pathogenesis of myocardial hypertrophy in the failing hearts.

Key Words: **calcineurin; Dahl salt-sensitive rats; hypertrophy; heart failure; cyclosporin A; ACE inhibitor.**

Calcineurin is a serine/threonine phosphatase regulated by intracellular Ca^{2+} and calmodulin (1, 2). Recently, this phosphatase has been shown to mediate the evolution of cardiac hypertrophy by activating a transcriptional factor NFAT (nuclear factor of T cells) in cultured myocytes and transgenic mouse models (3). Some investigators reported that calcineurin inhibition by immunosuppressants prevented or attenuated left ventricular hypertrophy (LVH) (4, 5), whereas others claimed that the calcineurin inhibition failed to block the hypertrophic process (6–9). More recent studies showed inconsistent results regarding the changes of calcineurin in failing human myocardium (10, 11). Lim and Molkentin (10) reported an increase in calcineurin activity, but Tsao *et al.* (11) revealed a decrease in calcineurin expression in the failing hearts. Thus, controversies exist regarding the exact roles of calcineurin in cardiac hypertrophy. In particular, it remains unclear whether this pathway is altered in decompensation process to congestive heart failure (CHF), and whether the calcineurin inhibition is effective for the prevention of pathological hypertrophy in the CHF stage. The calcineurin pathway may play different roles in the compensated LVH and in the decompensated CHF.

The present study investigated the cardiac expression of calcineurin in the compensated LVH and decompensated CHF stages, using the Dahl salt-sensitive rat model (12–14). To elucidate functional roles of calcineurin, we assessed whether cyclosporin A (CsA), which inhibits calcineurin activity to activate NFAT (15), could block the process of pathological hypertrophy during CHF transition. Furthermore, we also studied effects on the calcineurin pathway of an angiotensin converting enzyme (ACE) inhibitor as a clinically-relevant therapy of CHF (16, 17).

MATERIALS AND METHODS

Dahl salt-sensitive rat model. The Dahl salt-sensitive (DS) and Dahl salt-resistant (DR) rats (Eisai Co., Tokyo, Japan) were fed a diet containing 8% NaCl after the age of 6 weeks. The DS rats developed systemic hypertension and compensated, concentric LVH at the age of 11 weeks (LVH stage, $n = 5$), and then manifested transition to CHF characterized by LV dilatation and dysfunction at 15–17 weeks (CHF stage, $n = 5$) (13, 14). In contrast, the DR rats remained normotensive, and maintained normal cardiac geometry and function, therefore serving as age-matched controls in the LVH and CHF stages (each $n = 4$). The animals underwent tail-cuff blood pressure monitoring and echocardiography (Model HP77010 with a 7.5-MHz probe, Hewlett-Packard, Palo Alto, CA) in the LVH and CHF stages, and the hemodynamic parameters were obtained as described elsewhere (13, 14). The animals used in the study were treated in accordance with the *Guide for Care and Use of Laboratory*

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TABLE 1

In Vivo Characteristics at the LVH and CHF Stages

Age	Group	*n*	Body weight (g)	LV/body weight (mg/g)	SBP (mmHg)	EDD (mm)	ESD (mm)	FS (%)	PWT (mm)
11 weeks	DR	4	350 ± 19	1.95 ± 0.22	140 ± 6	6.5 ± 0.3	3.3 ± 0.2	55 ± 3	1.51 ± 0.07
	DS-LVH	5	324 ± 22	2.80 ± 0.34*	238 ± 10*	6.2 ± 0.4	2.2 ± 0.2	60 ± 5	2.08 ± 0.18*
17 weeks	DR	4	413 ± 24	1.85 ± 0.18	135 ± 6	7.5 ± 0.2	3.4 ± 0.3	56 ± 4	1.47 ± 0.08
	DS-CHF	5	294 ± 49*	4.73 ± 0.91*	232 ± 11*	8.5 ± 0.7*	6.1 ± 0.9*	31 ± 6*	1.86 ± 0.11*

Note. DR, Dahl salt-resistant rats; DS, Dahl salt-sensitive rats; LVH, left ventricular hypertrophy; CHF, congestive heart failure; SBP, systolic blood pressure; EDD, LV end-diastolic diameter; ESD, LV end-systolic diameter; FS, fractional shortening; PWT, LV posterior wall thickness.

*$P < 0.01$ and **$P < 0.05$ compared with age-matched DR.

Animals by the U.S. National Institutes of Health (NIH Publication No. 8523, revised 1985) and also with the institutional guidelines of Kyoto University Graduate School of Medicine.

Chronic cyclosporin A treatment. Twelve DS rats were treated with either of CsA (Sandimmune, Sandoz, 10 mg/kg/day, $n = 8$) or vehicle alone ($n = 4$) from the LVH stage to the CHF stage. CsA or vehicle was administered subcutaneously once a day. This dose of CsA was shown to yield sufficiently high blood level and eliminate virtually the calcineurin activity in the rat LV myocardium (8). Premature death due to pulmonary infection occurred in two rats treated with CsA. At the time of the examination, body weight tended to be less in the CsA-group than in the vehicle-treated group (272 ± 43 vs. 329 ± 62 g), but the difference was insignificant. The reduced weight gain in the CsA-treated rats was also observed by others (8).

Chronic ACE inhibitor treatment. Ten DS rats and four DR rats were assigned to one of the following three groups: 1) control DR rats ($n = 4$), 2) DS rats treated with vehicle ($n = 5$), 3) DS rats treated with an ACE-inhibitor, temocapril (Sankyo Co., Tokyo, Japan) at 20 mg/kg/day ($n = 5$). The dose of temocapril was determined so that systemic blood pressure was comparable between the vehicle- and temocapril-treated groups. Each vehicle or drug was administered orally by a gastric tube once a day from the LVH stage to the CHF stage.

Preparation of protein extract. The animals were anesthetized with pentobarbital sodium (35 mg/kg, i.p.). The heart was excised and perfused retrogradely with heparinized phosphate-buffered saline (PBS) to wash out the blood. The LV free wall myocardium was excised and homogenized on ice in the homogenization buffer (20 mmol/L Tris pH 7.5, 150 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L EGTA, 1% (v/v) Triton X-100, 2.5 mmol/L sodium pyrophosphate, 1 mmol/L β-glycerophosphate, 1 mmol/L Na3VO4, 1 mg/mL leupeptin and 1 mmol/L phenylmethylsulfonyl fluoride). The tissue homogenate was centrifuged at 12,000*g* for 30 min at 4°C, and the supernatant was collected as protein extract. Protein concentration was measured by the Bradford method (Bio-Rad Protein Assay Kit, Bio-Rad Laboratories, Hercules, CA), and the total yield of protein recovered per gram of the myocardial samples was comparable in each animal.

Western blot analysis. The protein extract from the LV myocardium (50 μg) was mixed with the electrophoresis buffer (62.5 mmol/L Tris, pH 6.8, 2% (w/v) sodium dodecyl sulfate (SDS), 10% (v/v) glycerol, 50 mmol/L dithiothreitol, 0.1% (w/v) bromphenol blue) and boiled for 5 min. The samples were subjected to 10% SDS–polyacrylamide gel electrophoresis (PAGE) and transferred to a nitrocellulose membrane (Hybond ECL, Amersham International, Buckinghamshire, UK), as previously described (18). Immunoblots were performed using an anti-calcineurin mouse monoclonal antibody that recognizes a 61 kDa calmodulin-binding catalytic subunit A (Transduction Laboratories, Lexington, KY, 1:1000 dilution), and then with a horseradish peroxidase (HRP)-conjugated anti-IgG antibody (Amersham, 1:2000 dilution). The blotted membrane was exposed on the film, using the enhanced chemiluminescence method (ECL, Amersham). The images were also analyzed by computer-assisted densitometry (NIH image software).

Statistics. The results are expressed as mean values ± SD. Data comparisons among groups were performed by analysis of variance, and a P value of less than 0.05 was considered significant.

RESULTS AND DISCUSSION

Hemodynamic Characteristics in the LVH and CHF Stages

The DS rats developed systemic hypertension and LVH. Echocardiographic data revealed presence of concentric LVH and preserved fractional shortening in the LVH stage. However, the animals then manifested a transition to CHF, and the LV cavity was dilated and fractional shortening was markedly decreased (Table 1), as reported previously (13, 14). In contrast, the DR rats maintained normal cardiac geometry and function throughout the course of the experiments.

Calcineurin Expression in the LVH and CHF Stages

The calcineurin protein level was increased (a mean of 1.7-fold of the age-matched control DR rats, $P < 0.05$) in the DS rats of the LVH stage (Fig. 1). The present data supports a previous concept that the calcineurin pathway is involved in the development of cardiac hypertrophy (3–6, 19). However, some recent studies reported that calcineurin inhibition could not prevent the development of pressure-overload LVH in spontaneously hypertensive rats and aortic stenosis mice (7–9). The functional effects of the calcineurin pathway in these models may be offset and/or masked by upregulation of other hypertrophy-related signaling pathways. Therefore, these previous results (7–9) do not simply refute our results.

However, cardiac calcineurin expression was decreased to reach a mean of 0.6-fold of the controls ($P < 0.05$) in the CHF stage (Fig. 1). This finding is similar

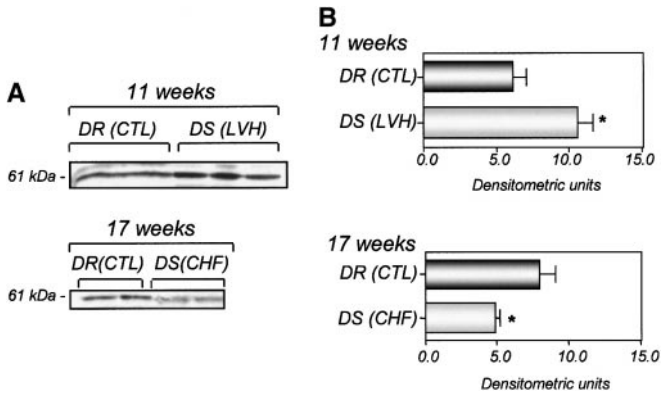

FIG. 1. Calcineurin immunoblot analysis. (A) Calcineurin expression in the LV myocardium of the Dahl salt-sensitive (DS) rats and control (CTL) DR rats at 11 and 17 weeks. LVH, LV hypertrophy; CHF, congestive heart failure. Data presented are representative examples. (B) Bar graphs showing the mean values and ranges of 1 SD for the densitometric analysis in all animals. * = $P < 0.05$ vs. the age-matched DR rats.

to the observation by Tsao *et al.* (11) who revealed a decreased mRNA expression of calcineurin A-β, predominant isoform in myocardium, in the failing human heart samples. The decreased expression of calcineurin is considered to result in an inactivation of a transcriptional NFAT3 (3, 20, 21) and to affect the expression of growth/hypertrophy-related genes. In addition, the intrinsic phosphatase activity of calcineurin may be decreased (9), which also possibly contribute to an inactivation of NFAT3. However, it is notable that despite the decrease in calcineurin expression, the hypertrophic change was still in progress during the CHF transition, as evidenced by a further increase in the LV weight (Table 1). This finding suggests that the hypertrophic process in the failing hearts may not be mediated by the activation of calcineurin pathway.

Effects of Chronic CsA Treatment on LV Remodeling

To further assess a potential role of calcineurin in the failing hearts, we tested whether the chronic calcineurin inhibition by CsA could block the progression of pathological hypertrophy during the CHF transition. As shown in Fig. 2, no substantial differences were observed in the LV/body weight ratio (3.6 ± 0.5 vs. 3.3 ± 0.4 g/kg) as well as in the echocardiographic LV end-diastolic diameter (6.6 ± 0.3 vs. 6.4 ± 0.5 mm) or LV posterior wall thickness (2.0 ± 0.1 vs. 2.0 ± 0.1 mm) between the CsA- and vehicle-treated groups. In addition, the LV fractional shortening was not improved by the CsA treatment. Thus, CsA could not block the LV hypertrophic process during the CHF transition. These data support the concept that the calcineurin pathway no longer involves in the progression of pathologic hypertrophy during cardiac decompensation.

The lack of effects of CsA on the LV hypertrophy may be explained by a possibility that the calcineurin inhibitory effects is offset by other pharmacological effects of CsA. It was shown that CsA increased angiotensin II receptors independently from calcineurin inhibition (22), which causes vasoconstriction and systemic hypertension (23) and can promote cardiac hypertrophy (24). However, in the present results, systolic blood pressure was not significantly different in the CsA-treated group (232 ± 19 mmHg) and in the vehicle-treated group (239 ± 16 mmHg), suggesting a lack of substantial vasoconstrictive effects through angiotensin II receptors. In addition, we previously observed that the angiotensin II-induced activation of growth-promoting signaling such as a JNK cascade was actually downregulated in the failing myocardium (25).

Effects of Chronic ACE Inhibition

We also examined effects of chronic ACE inhibition with temocapril, more conventional treatment of CHF. As shown in Fig. 3, chronic temocapril treatment restored the calcineurin expression in the DS rats to a level comparable to that in the control DR rats. Chronic ACE inhibition is well known to inhibit cardiac hypertrophy, independently from a hemodynamic load level (16, 17). In the present results, systemic blood pressure was comparable between the vehicle-

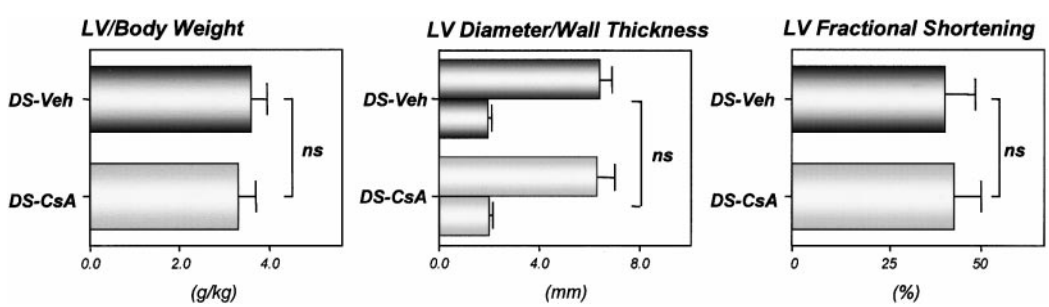

FIG. 2. Effects of chronic cyclosporin A treatment. Bar graphs showing the LV/body weight ratio (left), the echocardiographic LV end-diastolic diameter and wall thickness (*middle*) and the LV fractional shortening (right) in the vehicle-treated DS rats (DS-Veh) and the CsA-treated DS rats (DS-CsA). No substantial difference was observed in these parameters between the two groups.

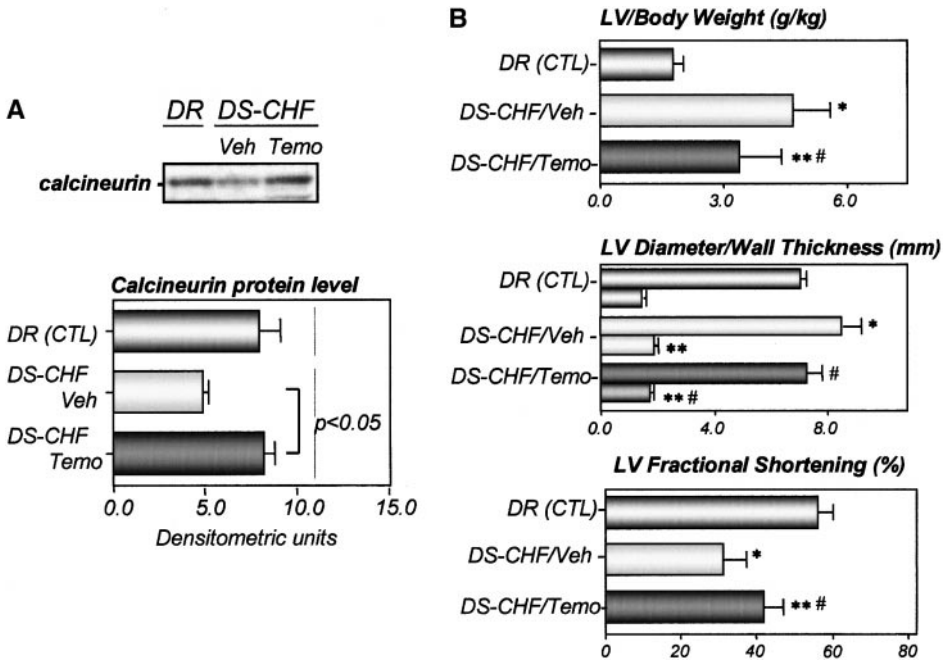

FIG. 3. Effects of chronic temocapril treatment. (A) Representative calcineurin immunoblot and the result of densitometric analysis in all animals. Abbreviations are the same as in former figures. Temo = temocapril. (B) Bar graphs showing the LV/body weight ratio (top), the echocardiographic LV end-diastolic diameter and wall thickness (middle), and the LV fractional shortening (bottom) in the control (CTL) DR rats, the DS rats treated with vehicle, and the DS rats treated with temocapril. * = $P < 0.01$ and ** = $P < 0.05$ vs. DR(CTL), # = $P < 0.05$ vs. DS(CHF)/Veh.

and the temocapril-treated DS rats (237 ± 11 and 235 ± 12 mmHg, P = NS). Despite the non-hypotensive dose of temocapril, the LV weight was reduced (3.4 ± 1.0 vs. 4.7 ± 0.9 g/kg, $P < 0.01$). The echocardiographic LV diameter and wall thickness were decreased (7.5 ± 0.6 vs. 8.5 ± 0.7 mm, 1.7 ± 0.1 vs. 1.9 ± 0.1 mm, respectively, both $P < 0.01$) and the LV fractional shortening was also improved in the temocapril-treated DS rats. Thus, the regression of hypertrophy occurred even when the calcineurin expression was restored during ACE inhibition. This finding also refute a hypothesis that the calcineurin activity is still crucial for the pathologic hypertrophy of failing myocardium.

The decreased calcineurin expression may occur as a result of secondary negative-feedback for the augmented calcineurin activation by the cardiac renin-angiotensin system in the failing hearts (26). Cardiac ACE inhibition suppresses the effect of excessive tissue *Ang*II, thereby leading to the restoration of calcineurin expression. However, the pathological hypertrophic process is regulated by crosstalk of a number of signaling pathways, and in particular relation to the renin-angiotensin system, other hypertrophy-related pathways probably play more important roles than the calcineurin pathway does. Future studies are needed to elucidate this important issue, but our data at least indicate that the calcineurin pathway cannot be a primary target for the treatment of heart failure. In addition, the present data also suggest that caution should be taken when assessing the expression and activity of calcineurin in the human failing hearts treated with ACE inhibitors (10).

In conclusion, cardiac calcineurin may be involved in the development of adaptive hypertrophy in the compensated, pressure-overload LVH. However, the calcineurin expression is progressively downregulated during the CHF transition and is considered not to play a predominant role in the pathological hypertrophy of failing myocardium.

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