Calcineurin Inhibitor Attenuates the Development and Induces the Regression of Cardiac Hypertrophy in Rats With Salt-Sensitive Hypertension

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Background—It remains unclear how hemodynamic overload induces cardiac hypertrophy. Recently, activation of calcium-dependent phosphatase, calcineurin, has been elucidated to induce cardiac hypertrophy. In the present study, we examined the role of calcineurin in load-induced cardiac hypertrophy by using Dahl salt-sensitive (DS) rats, which develop both pressure and volume overload when fed a high salt diet.

Methods and Results—In the DS rat heart, the activity of calcineurin was increased and cardiac hypertrophy was induced by high salt diet. Treatment of DS rats with the calcineurin inhibitor FK506 (0.1 or 0.01 mg/kg twice daily) from the age of 6 weeks to 12 weeks inhibited the activation of calcineurin in the heart in a dose-dependent manner and attenuated the development of load-induced cardiac hypertrophy and fibrosis without change of hemodynamic parameters. Additionally, treatment with 0.1 mg/kg twice daily but not with 0.01 mg/kg twice daily of FK506 from the age of 12 weeks to 16 weeks induced regression of cardiac hypertrophy in DS rats. Load-induced reprogramming of gene expression was also suppressed by the FK506 treatment.

Conclusions—These results suggest that calcineurin is involved in the development of cardiac hypertrophy in rats with salt-sensitive hypertension and that inhibition of calcineurin could induce regression of cardiac hypertrophy. (Circulation. 2000;102:1996-2004.)

Key Words: hypertrophy ■ hemodynamics ■ hypertension

Although cardiac hypertrophy is an adaptive response to various stimuli, prolonged hypertrophy is associated with ischemia, arrhythmia, sudden death, and heart failure. Therefore, it is very important to understand the underlying mechanism of cardiac hypertrophy and prevent or treat it. Many reports have indicated that intracellular Ca\(^{2+}\) plays an important role in gene expression and growth in a variety of cell types including cardiac myocytes. However, it is unknown how Ca\(^{2+}\) regulates these events in cardiomyocytes. Recently, Molkentin et al. have demonstrated the importance of the Ca\(^{2+}\)-dependent phosphatase calcineurin and its downstream transcription factor NF-AT3 in the development of cardiac hypertrophy. Transgenic mice expressing activated calcineurin or NF-AT3 in the heart showed marked cardiac hypertrophy, and some of them progressed to dilated cardiomyopathy with interstitial fibrosis and then congestive heart failure. The calcineurin inhibitors cyclosporine A (CsA) and FK506 prevented cardiac hypertrophy in activated calcineurin transgenic mice and humoral factor–induced hypertrophy of cultured cardiomyocytes of neonatal rats. In addition, Sussman et al. reported that several transgenic mouse models that show hypertrophic cardiomyopathy could be treated with CsA. These results suggest that calcineurin plays a pivotal role in the development of cardiac hypertrophy induced by various causes. Although cardiac hypertrophy is induced by a variety of factors such as hemodynamic overload (mechanical stress), genetic factors, and humoral factors, mechanical stress is clinically the most important cause for cardiac hypertrophy. Sussman et al. reported that CsA prevented pressure overload–induced cardiac hypertrophy produced by constriction of the abdominal aorta, and Meguro et al. reported that CsA attenuated cardiac hypertrophy induced by constriction of the transverse aorta. Additionally, we have quite recently reported that FK506 inhibited the pressure overload–induced activation of calcineurin and prevented the pressure overload–induced cardiac hypertrophy and fibrosis produced by constriction of the abdominal aorta. In contrast, other groups reported that calcineurin inhibitors did not prevent overload-induced cardiac hypertrophy in spontaneously hypertensive rats (SHR) or produced by constriction of the abdominal or transverse aorta. Therefore, it is controversial whether the calcineurin signaling...
pathway plays a critical role in the development of cardiac hypertrophy induced by hemodynamic overload. Furthermore, it is unknown whether suppression of calcineurin induces the regression of cardiac hypertrophy once cardiac hypertrophy develops.

In this study, we examined the following: (1) whether the calcineurin activity was actually elevated in the hypertrophic heart, (2) how large a dose of the calcineurin inhibitor FK506 was required to suppress the overload-induced elevation of calcineurin activity in the heart, and (3) whether suppression of calcineurin activation in the heart prevented the development or induced the regression of overload-induced cardiac hypertrophy. In the present study, we used Dahl salt-sensitive (DS) rats as the overload-induced cardiac hypertrophy model. The DS rat has unique features as an animal model of cardiac hypertrophy. This model shows the rapid onset of hypertension and develops both pressure overload– and volume overload–induced cardiac hypertrophy only with a high salt diet. In contrast, the Dahl salt-resistant (DR) rat, which is overfed–induced cardiac hypertrophy only with a high salt diet, develops. This model shows the rapid onset of hypertension and develops both pressure overload– and volume overload–induced cardiac hypertrophy as well as the heart.

### Methods

#### Animals

Male inbred DS and DR rats were obtained from Nihon Seibutsu Zairyo Inc (Tokyo, Japan). Rats were handled in accordance with the Tokyo University Guide for the Care and Use of Laboratory Animals. Rats were fed a diet containing 0.3% NaCl until the age of 6 weeks, then they were fed an 8% NaCl diet. Systolic blood pressure was measured with an indirect tail-cuff method (BP-98A, Softron). FK506 was injected intramuscularly in DS rats from the age of 6 weeks to 12 weeks or from the age of 12 weeks to 16 weeks. Rats were divided into 4 groups: (1) DR rats, (2) DS rats, (3) DS rats administered a high dose of FK506 (0.1 mg/kg body wt twice daily), and (4) DS rats administered a low dose of FK506 (0.01 mg/kg body wt twice daily) (n=12, each group) (Table 1). After all rats were deeply anesthetized by intraperitoneal injection of sodium pentobarbital (50 mg/kg body wt), rats were killed and the hearts were subjected to further analysis.

#### Calcineurin Enzymatic Assay

The activity of calcineurin in the left ventricle (LV) was determined as described previously. Fresh tissue was homogenized in hypotonic lysis buffer (50 mmol/L Tris-HCl, pH 7.5, 0.05% Tween-20, 1 mmol/L EDTA, 1 mmol/L CaCl₂, 1 mmol/L PMSF, 1 μg/mL pepstatin, 1 μg/mL leupeptin, 1 mmol/L DTT), and phosphatase buffer (100 mmol/L MOPS pH 7.0, 0.4 mmol/L CaCl₂, 2 mmol/L MnCl₂, 10 μg/mL BSA, 100 mmol/L calmodulin, 500 mmol/L okadaic acid) was added to the supernatant. The calcineurin substrate GST-RII peptide, which was phosphorylated by protein kinase A in the presence of [γ-32P]ATP, was incubated with 2 μg of tissue lysate for 30 minutes at 30°C. Liberated 32P was determined by the Cherenkov method.

#### Echocardiographic Analysis

At the age of 6 weeks, 12 weeks, and 16 weeks, transthoracic echocardiographic analysis was performed repeatedly in the same animal with the HP Sonos 100 (Hewlett-Packard Co) with a 10-MHz imaging transducer, and the progress of overload-induced LV hypertrophy was evaluated as described previously.

#### Histological Analysis

For histological analysis, hearts were fixed by perfusion with 10% formalin. Fixed hearts were embedded in paraffin, sectioned at 4-μm thickness, and stained with hematoxylin and eosin or by the van Gieson method. The myocyte cross-sectional diameter was measured in sections stained with hematoxylin and eosin, and suitable cross sections were defined as having nearly circular capillary profiles and nuclei (n=100, each group). To determine the degree of collagen fiber accumulation, we selected 5 fields at random and calculated the ratio of van Gieson–stained fibrosis area to total myocardium area as described previously with the software NIH IMAGE (NIH, Research Service Branch) for image analysis.

#### RNA Analysis

Twenty micrograms of total RNA, extracted from the fresh LV samples with RNAzol (Tel-text), was separated on a 1.0% agarose/formaldehyde gel and blotted onto a Hybond-N membrane (Amer sham Co). cDNA was labeled by a random priming method with [α-32P]dCTP. Hybridized bands were quantified with NIH IMAGE.

#### Statistical Analysis

All data are expressed as the mean±SEM. Two-way ANOVA and Fisher’s exact test for post hoc analysis were carried out for multiple comparisons among 3 or more groups. A value of P<0.05 was considered statistically significant.

### Results

FK506 Suppresses Pressure Overload–Induced Activation of Calcineurin Without Affecting Hemodynamic Parameters

In DS rats fed the high salt diet from the age of 6 weeks, blood pressure gradually increased and reached the level of ≈200 mm Hg by the age of 12 weeks (Figure 1A). With the high salt diet for 4 more weeks, the blood pressure of DS rats increased >220 mm Hg by the age of 16 weeks (Figure 1B).
Treatment with FK506 did not affect blood pressure in DS rats (Figure 1, A and B). The blood pressure of DR rats did not significantly change during this period with the same high salt diet (Figure 1, A and B). Relative calcineurin activity in the hearts of 12- and 16-week-old DS rats was significantly higher than that of DR rats of the same age (Table 1). These results indicate that hemodynamic overload but not high salt diet itself induces calcineurin activation in the hearts of DS rats. The work overload–induced elevation of calcineurin activity was suppressed completely by the high dose of FK506 and partly by the low dose of FK506 in both 12- and 16-week-old rats (Table 1).

FK506 Attenuates Development of Cardiac Hypertrophy

Approximately half of DS rats died during the FK506 treatment, and there was no difference between the 0.1 and 0.01 mg/kg FK506 groups (Table 1). We dissected all of the dead DS rats and found that all of the dead animals showed severe pulmonary infection with empyema. Although the reason is not clear at present, Dahl rats may be more susceptible to infection than Wistar rats, which were not infected even with 1 mg/kg FK506.9 The rats that survived (n=6 to 7) were apparently healthy and used for further analysis (Table 1). The LV weight (LVW) of 12-week-old DS rats was significantly higher than that of 12-week-old DR rats (Figure 2A). Both at high and low doses, FK506 strongly attenuated the increase in LVW of DS rats (Figure 2A). However, since treatment with FK506 decreased the body weight (BW) (Figure 2B), there was no significant difference in the LVW-to-BW ratio (LVW/BW) between DS rats treated with FK506 and those that were not treated with FK506 (Figure 2C). Because there was no significant difference in tibial length (TL) among DR rats, DS rats, and DS rats treated with FK506, we used TL as the internal control instead of BW.18,19 The LVW-to-TL ratio (LVW/TL) was significantly higher in DS rats than in DR rats (Figure 2D). Treatment with low and high doses of FK506 significantly attenuated the overload-induced increase in LVW/TL (Figure 2D).

Echocardiographic analysis revealed that the thickness of both the interventricular septum (IVS) and left ventricular posterior wall (PW) was larger in DS rats than in DR rats (Table 2). Chronic treatment with either dose of FK506
attenuated the increment of wall thickness without affecting cardiac function (Table 2).

Microscopic analysis revealed that hemodynamic overload for 6 weeks markedly increased the cross-sectional diameter of cardiac myocytes (Figure 3A). Treatment with both high and low doses of FK506 from 6 weeks to 12 weeks attenuated the increase of the cross-sectional diameter of cardiac myocytes (Figure 3A). Moreover, hemodynamic overload-induced marked perivascular fibrosis in the heart (Figure 3B). The hemodynamic overload-induced fibrosis was completely inhibited by treatment with the high dose of FK506 and partly by treatment with the low dose of FK506 (Figure 3B). There was no significant difference in the number of inflammatory cells in hearts between treated and untreated rats.

**Treatment with High Dose of FK506 Induces Regression of Cardiac Hypertrophy**

LVW was significantly larger in DS rats than in DR rats at the age of 16 weeks (Figure 4A). Treatment with the high dose of FK506 for 4 weeks significantly attenuated the increase in LVW in DS rats (Figure 4A). DS rats lost BW from the age of 12 weeks to 16 weeks irrespective of the treatment as reported previously, and the BW of 16-week-old DS rats was significantly smaller than that of DR rats (Figure 4B). Both the LVW/BW and LVW/TL were significantly larger in DS rats than in DR rats, and treatment with the high dose of FK506 but not with the low dose of FK506 significantly attenuated the increase in the LVW/BW as well as the LVW/TL (Figure 4, C and D). Moreover, the LVW/TL of 16-week-old DS rats that were treated with the high dose of FK506 was significantly less than that of untreated 12-week-old DS rats (Figure 4D), suggesting that the FK506 treatment-induced regression of cardiac hypertrophy.

Echocardiographic analysis revealed that hemodynamic overload for an additional 4 weeks (weeks 12 to 16) was associated with further increase of the thickness of both IVS and PW (Table 3). At the high dose but not low dose FK506 treatment not only attenuated the development but also induced the regression of cardiac hypertrophy without any impairment of cardiac function (Table 3). The thickness of IVS and PW was decreased in 16-week-old DS rats by treatment with the FK506 as compared with that of untreated 12-week-old DS rats (Table 3).

Microscopic analysis revealed that the cross-sectional diameter of cardiac myocytes was significantly larger in DS rats than in DR rats at the age of 16 weeks (Figure 5A). At the high dose but not at the low dose, FK506 induced regression of hypertrophy of cardiac myocytes in DS rats (Figure 5A). The cardiomyocyte diameter of treated 16-week-old DS rats was smaller than that of nontreated 12-week-old DS rats. Additionally, hemodynamic overload for a further 4 weeks markedly induced progression of the perivascular fibrosis in the hearts of 16-week-old DS rats (Figure 5B). Treatment with FK506 from the age of 12 weeks to 16 weeks attenuated the development of the overload-induced fibrosis in a dose-dependent manner (Figure 5B).

**Calcineurin Is Involved in Load-Induced Reprogramming of Gene Expression**

Induction of fetal-type genes is a genetic response to hemodynamic overload. We thus examined whether calcineurin was involved in gene expression during overload-induced...
cardiac hypertrophy. Hemodynamic overload for 6 weeks upregulated fetal genes such as atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP), and skeletal α-actin genes in the heart (Figure 6, A and B). In contrast, the sarcoplasmic reticulum Ca\(^{2+}\)-ATPase (SERCA2) gene was downregulated in the hearts of DS rats, as previously reported\(^{20}\) (Figure 6, A and B). Treatment with FK506 from 6 weeks to 12 weeks dose-dependently inhibited the upregulation of the BNP gene and the downregulation of the SERCA2 gene (Figure 6, A and B). The upregulation of ANP and skeletal α-actin genes was not affected by treatment with FK506 (Figure 6, A and B).

**Discussion**

In this study, treatment with the high dose of FK506 for 6 weeks abolished the overload-induced activation of calcineurin and attenuated the development of cardiac hypertrophy in 12-week-old DS rats without change of hemodynamic parameters (Figures 1, 2, and 3 and Table 1). In addition, treatment with the high dose of FK506 from the age of 12 weeks to 16 weeks induced significant regression of cardiac hypertrophy (Figures 4 and 5). Furthermore, we demonstrated that treatment with the high dose of FK506 inhibited cardiac hypertrophy.
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but also hypertriglyceridemia and hyperinsulinemia, 21,22

diet.14 –16 DS rats show not only salt-sensitive hypertension

onset of hypertension without variation among animals and

of hypertensive cardiac hypertrophy. They show rapid

phy model. DS rats have several unique features as a model

we used DS rats as the overload-induced cardiac hypertro-

weeks (Figures 4 and 5).

induce the regression of cardiac hypertrophy at the age of 16

(Figures 2 and 3). At the low dose, however, FK506 did not

of cardiac hypertrophy and fibrosis in 12-week-old DS rats

sition levels of interleukin-1

human hypertension. It has also been reported that expres-

suggesting that DS rats have some features similar to

clinical setting, suppressed the overload-induced elevation of

FK506, which was almost the same as the dose used in a

clinical setting, suppressed the overload-induced elevation of
calcineurin activity by \( \approx 40\% \) and attenuated the development

cardiac hypertrophy and fibrosis in 12-week-old DS rats

(Figures 2 and 3). At the low dose, however, FK506 did not

induce the regression of cardiac hypertrophy at the age of 16

weeks (Figures 4 and 5).

There are controversial reports on the role of calcineurin

in overload-induced cardiac hypertrophy.5,8–13 Although

the reason of the discrepancy is not clear at present, it may

come from the model of hemodynamic overload. It has

been reported that CsA does not prevent overload-induced

cardiac hypertrophy, as in the case of constriction of the

ascending or transverse aorta12,13 or of SHR.8 In this study,

have independently modified LV mass,28,29 all these results

suggest that activation of calcineurin is involved in load-

induced cardiac hypertrophy.

FK506 inhibited overload-induced upregulation of the

BNP gene and downregulation of the SERCA2 gene but did

not alter the expression of ANP or skeletal \( \alpha \)-actin genes

(Figure 6). In our previous study with an abdominal aortic

banding model, however, an increase in ANP expression

was also inhibited by FK506 treatment.9 In our previous

study, we treated the rats of abdominal aortic constriction

with 1 mg/kg per day of FK506, and this treatment

completely prevented pressure overload–induced calcineurin

activation and cardiac hypertrophy.9 Because DS rats

were susceptible to infection with unknown reason, we

treated DS rats with 0.1 mg or 0.01 mg/kg twice daily of

FK506 in the present study, and this treatment inhibited
load-induced calcineurin activation in a dose-dependent manner and attenuated load-induced cardiac hypertrophy. These observations suggest that complete inhibition of load-induced calcineurin activation is required to inhibit load-induced upregulation of the ANP gene. Quite recently, Taigen et al. demonstrated that target inhibition of calcineurin by expressing noncompetitive peptide inhibitors cain and A-kinase anchoring protein, by using
adenoviral-mediated gene transfer, attenuated agonist-induced calcineurin activity, cardiomyocyte hypertrophy, and upregulation of the ANP gene. These noncompetitive peptide inhibitors may be useful to elucidate the role of calcineurin in load-induced cardiac hypertrophy while avoiding severe side effects of calcineurin inhibitors.

It has been reported that NF-AT3 activates transcription of some cardiac genes in concert with cardiac-enriched transcription factor GATA4. Additionally, it was reported that activation of calcineurin selectively upregulates slow-fiber-specific genes in skeletal myocytes through NF-AT3-MEF2 association and that calcineurin is involved in skeletal muscle hypertrophy by activating GATA2 and NF-ATc. It has been reported that MEF2 plays an important role in gene regulation also in the heart and that NF-ATc plays a vital role in the morphogenesis of the cardiac valves and septum during heart development. Furthermore, it has recently been reported that intracellular Ca\(^{2+}\) homeostasis regulates calcineurin activity and plays a vital role in cardiac development or development of dilated cardiomyopathy. Meseall et al demonstrated that calreticulin, which is a ubiquitous Ca\(^{2+}\)-binding protein and located in the endoplasmic reticulum lumen, plays a role in cardiac development as a component of the calcineurin-NF-AT3–GATA4 transcription pathway. Sussman et al reported that alteration of intracellular calcium handling induced calcineurin activation, altered sarcomeric structure, and induced dilated cardiomyopathy in tropomodulin-overexpressing transgenic mice. From these results and observations, calcineurin may be involved in the regulation of many cardiac genes through activation of the NF-AT family including NF-AT3 and NF-ATc and may be involved in cardiac hypertrophy and development.

In the DS rat heart, the activity of calcineurin was increased and cardiac hypertrophy was induced by high salt diet. Treatment of DS rats with the calcineurin inhibitor FK506 (0.01 or 0.1 mg/kg twice daily) from the age of 6 weeks to 12 weeks inhibited the activation of calcineurin in the heart and attenuated the development of load-induced cardiac hypertrophy and fibrosis without change of hemodynamic parameters. Treatment with 0.1 mg/kg twice daily of FK506 from the age of 12 weeks to 16 weeks induced regression of cardiac hypertrophy in DS rats. Load-induced reprogramming of gene expression was also suppressed by FK506.

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References


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