Calcineurin Inhibitor Attenuates Left Ventricular Hypertrophy, Leading to Prevention of Heart Failure in Hypertensive Rats

Yasushi Sakata, MD; Tohru Masuyama, MD, PhD; Kazuhiro Yamamoto, MD, PhD; Nagahiro Nishikawa, MD; Hideyuki Yamamoto, MD, PhD; Hiroya Kondo, MD, PhD; Keiko Ono, MD; Kinya Otsu, MD, PhD; Tsunehiko Kuzuya, MD, PhD; Takeshi Miwa, PhD; Hiroshi Takeda, MD, PhD; Eishichi Miyamoto, MD, PhD; Masatsugu Hori, MD, PhD

Background—There is controversy regarding the contribution of calcineurin activation to the development of pressure-overload left ventricular (LV) hypertrophy and heart failure. The aim of this study was to explore whether the inhibition of calcineurin may prevent the transition to heart failure in hypertensive rats and, if so, to clarify in which developmental stage of LV hypertrophy calcineurin plays a key role.

Methods and Results—Dahl salt-sensitive rats placed on an 8% NaCl diet from the age of 7 weeks (hypertensive rats) were randomized to no treatment (n=6) or treatment with the calcineurin inhibitor FK506 (1 mg·kg⁻¹·d⁻¹) from 8 weeks (FKE, n=7) or from 17 weeks (FKL, n=7). Rats placed on a 0.3% NaCl diet were defined as control rats (n=6). The administration of FK506 from 8 weeks attenuated, although it did not block, LV hypertrophy observed in the untreated rats and prevented the transition to heart failure. The development of LV fibrosis, however, was not attenuated by the administration of FK506 from 8 weeks. The administration of FK506 from 17 weeks brought no benefit for cardiac remodeling or LV function and failed to prevent heart failure.

Conclusions—Calcineurin inhibition, if started from the initial stage of pressure overload, attenuated the development of LV hypertrophy without any effect on LV fibrosis and prevented the transition to heart failure. The activation of calcineurin is involved in the development of LV hypertrophy but not of LV fibrosis, and this involvement may be crucial at the initial stage. (Circulation. 2000;102:2269-2275.)

Key Words: hypertrophy ■ heart failure ■ hypertension ■ calcineurin
Preclinical studies have used age-matched control rats (n=3 per group). FK506 was administered from 8 weeks as in FKE rats. The LV was excised and immediately stored at −80°C. The frozen rat heart ventricles were weighed and homogenized with 10 volumes of a homogenization buffer containing 20 mmol/L Tris-HCl (pH 7.5), 2 mmol/L EDTA, 2 mmol/L EGTA, 0.1% Triton X-100, 0.5 mmol/L DTT, and protease inhibitors (10 μg/mL leupeptin, 1 μg/mL pepstatin A, 1 mmol/L PMSF) with an Ultra Turrax (IKA-Werk). The homogenate was centrifuged at 14 000 rpm for 20 minutes at 4°C to obtain the supernatant as the tissue extract. The protein concentration in the tissue extract was measured with a BioRad protein assay reagent. Calcineurin activity was measured using 32P-labeled casein as a substrate. Casein was phosphorylated by cAMP-dependent protein kinase (cAMP-kinase) with 0.2 mmol/L [γ-32P]ATP (3000 to 5000 cpm/pmol) overnight. Phosphorylated casein was heat-treated at 65°C for 5 minutes to remove cAMP-kinase activity and collected by ammonium sulfate fractionation (0% to 80%) in the presence of 1 mg/mL BSA. The protein was washed 3 times with 80% ammonium sulfate and dialyzed against a buffer containing 10 mmol/L Tris-HCl (pH 7.5), 10 mmol/L 2-mercaptoethanol, and 10% (vol/vol) glycerol for 18 hours. The reaction mixture contained, in 25 μL 50 mmol/L HEPES (pH 7.5), 1 mmol/L DTT, 0.1 mmol/L MnCl2, 1 mmol/L CaCl2, 1.5 μmol/L calmodulin, 0.2 μmol/L calyculin A to inhibit protein phosphatases 1 and 2A, and 100 μg/mL 32P-labeled casein. In addition, the phosphatase activity was measured without CaCl2 and calmodulin in the presence of 200 μmol/L trifluoperazine. Calcineurin activity was determined as the activity in the presence of trifluoperazine without CaCl2 and calmodulin subtracted from the activity in the presence of CaCl2 and calmodulin. The reaction was initiated by the addition of 3 μL of tissue extract. After incubation at 30°C for 10 minutes, the reaction was terminated by the addition of 100 μL of 20% TCA and 25 μL of 6 mg/mL BSA. The samples were centrifuged at 14 000 rpm for 10 minutes at 4°C, and 100 μL of the supernatant was used for counting the amount of 32P-labeled inorganic phosphate with a liquid scintillation counter. All assays were performed in duplicate, and the activity was corrected for the protein concentration. The calcineurin activity was expressed as a percentage of the mean value of the age-matched control rats. The calcineurin activity at 13 weeks was elevated in the untreated rats compared with age-matched normal controls (182±31% versus 100±25%, respectively), and the elevation was depressed by the chronic administration of FK506 (101±14%).

Statistical Analysis

Results are expressed as mean±SEM. Data were assessed with commercially available statistical software (STATVIEW version 4.5, Abacus Concepts). Differences at specific stages between groups were assessed by 1-factor ANOVA and Fisher’s test. Two-factor ANOVA for repeated measures was followed by Fisher’s test for testing serial changes. A value of P<0.05 was considered statistically significant.

Results

Hemodynamics and Heart Failure

None of the control rats developed hypertension or heart failure. Aortic pressure was higher in the FK(−), in FKE, and in FKL rats than in the control rats. (Figure 1). Aortic pressure was slightly decreased at 17 and 20 weeks in FKE rats and at 20 weeks in FKL rats compared with the FK(−) rats. The lung/body wt increased in the FK(−), rats reflecting congestive heart failure at 20 weeks (Table 2). It was reduced in FKE rats but not in FKL rats. LV end-diastolic pressure was elevated and τ was prolonged in the FK(−) and FKL rats, but these abnormalities were not evident in FKE rats. LV systolic function assessed from endocardial and midwall...
fractional shortening was not deteriorated in FK(−), FKE, or FKL rats throughout the experiment (Table 1 and Figures 2 and 3). The +dP/dt max was elevated in FKE rats but reduced in FKL rats at 20 weeks (Table 2). End-systolic stress was equivalent among FK(−), FKE, and control rats but was increased in FKL rats at 20 weeks (Table 2).

There were no remarkable changes in the Doppler transmirtal flow velocity pattern throughout the experiment in the control group (Table 1). In contrast, E velocity decreased and A velocity increased in FK(−), FKE, and FKL rats at 13 to 17 weeks (Table 1 and Figures 2 and 3). The FK(−) rats showed increases in E velocity and in the ratio of E velocity to A velocity (E/A ratio) (restrictive pattern) at 20 weeks. The administration of FK506 from 8 weeks prevented the transition to the restrictive pattern at 20 weeks; however, its administration from 17 weeks was not effective.

**LV Geometrical and Histological Changes**

Posterior wall thickness at end diastole and LVMI increased at 13, 17, and 20 weeks in the FK(−) rats compared with the control rats (Tables 1 and 2). The increases were attenuated, however, by the administration of FK506 from 8 weeks. LV end-diastolic dimension was smaller in the FK(−) rats than in the control rats at 13, 17, and 20 weeks. FK506 administration from 8 weeks did not affect LV end-diastolic dimension (Table 1). Changes in LV geometry were not suppressed by FK506 administration after 17 weeks (Tables 1 and 2 and Figures 3 and 4).

Perivascular and interstitial fibrosis were observed in FK(−) rats at 20 weeks, particularly in the subendocardial portion (Figure 5), and did not regress by the chronic administration of FK506. This observation was confirmed by the quantitative data of hydroxyproline content (Table 2).

**Gene Expression**

ANP mRNA level was much higher in FK(−) rats than in the control rats. FK506 administration from 8 weeks reduced the expression of ANP mRNA by 41% compared with FK(−) rats. The expression was not reduced, however, by the treatment starting at 17 weeks (Figure 6).

**Discussion**

The administration of FK506 from the age of 8 weeks attenuated pressure-overload LV hypertrophy and prevented the transition to the restrictive pattern at 20 weeks. FK506 administration after 17 weeks was not effective.

**TABLE 1. Changes in Echocardiographic Parameters in the Control, FK(−), FKE, and FKL Rats**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control group (n=6)</th>
<th>FK(−) group (n=6)</th>
<th>FKE group (n=7)</th>
<th>FKL group (n=7)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PWd, mm</td>
<td>1.2±0.1</td>
<td>1.3±0.1</td>
<td>1.3±0.1</td>
<td>1.2±0.1</td>
</tr>
<tr>
<td>LVDd, mm</td>
<td>6.8±0.1</td>
<td>8.9±0.1</td>
<td>8.8±0.2</td>
<td>7.1±0.2</td>
</tr>
<tr>
<td>FS, %</td>
<td>36±2</td>
<td>29±2</td>
<td>30±1</td>
<td>33±3</td>
</tr>
<tr>
<td>MFS, %</td>
<td>20±1</td>
<td>15±2</td>
<td>14±1</td>
<td>14±1</td>
</tr>
<tr>
<td>E/A</td>
<td>1.6±0.1</td>
<td>2.4±0.4</td>
<td>3.0±0.4</td>
<td>3.4±0.8</td>
</tr>
<tr>
<td>LVMI, mg/g</td>
<td>2.7±0.1</td>
<td>2.2±0.1</td>
<td>3.5±1.1</td>
<td>4.3±0.3</td>
</tr>
</tbody>
</table>

PWd indicates LV posterior wall thickness at end diastole; LVDd, LV end-diastolic dimension; FS, fractional shortening of LV inner diameter; and MFS, midwall fractional shortening of LV inner diameter. Values are mean±SEM.

*P<0.05 vs 7 weeks, †P<0.05 vs 13 weeks, ‡P<0.05 vs 17 weeks, §P<0.05 vs control group, ¶P<0.05 vs untreated group,
#P<0.05 vs FKE group.
to heart failure in Dahl salt-sensitive rats. If FK506 was administered from the age of 17 weeks, however, the reductive or preventive effect on the development of LV hypertrophy or heart failure was no longer evident. These data indicate that the activation of calcineurin is involved in the development of LV hypertrophy and eventual transition to heart failure in our model and that the involvement may be limited to the initial developmental stage of pressure-overload hypertrophy.

Figure 2. M-mode LV echocardiograms (left) and Doppler mitral flow velocity patterns (right) at 13 and 20 weeks in FK(−) (top) and FKE (bottom) rats. In FKE rat, LV wall thickness is reduced compared with FK(−) rat at 13 and 20 weeks. Restrictive pattern seen in FK(−) rat is not observed in FKE rat at 20 weeks.

Figure 3. M-mode LV echogram (left) and Doppler mitral flow velocity pattern (right) at 17 and 20 weeks in FK(−) rat (top) and FKL rat (bottom). In both rats, LV hypertrophy is observed at 20 weeks. At 20 weeks, restrictive pattern was seen in FK(−) and FKL rats.
Calcineurin Inhibition From the Initial Stage and LV Geometry

The administration of FK506 from the age of 8 weeks reduced, although it did not block, the increase in LVMI. The expression of ANP mRNA was also reduced in FKE, supporting the idea that calcineurin inhibition is effective in attenuating LV hypertrophy. End-systolic stress remained normal in FKE, suggesting that LV hypertrophy was attenuated to such a degree that it did not interfere with adaptation to pressure overload. Interestingly, histological LV fibrosis or LV hydroxyproline content had not regressed at 20 weeks in FKE rats. Thus, calcineurin activation is likely to contribute to the development of LV hypertrophy but little to the progression of LV fibrosis. The effect on LV hypertrophy was evident from the initial developmental stage of pressure-overload hypertrophy.

The importance of calcium-dependent phosphatase calcineurin and NF-ATc, was first described by Molkentin et al.\(^1\) They studied the effect of pharmacological inhibition of calcineurin activity in transgenic mice to find that the calcineurin transcriptional pathway plays a key role in the development of pressure-induced hypertrophy. Meguro et al.\(^2\) observed the attenuation of LV hypertrophy by pharmacological inhibition of calcineurin activity. These reports, however, were followed by in vivo studies\(^3,4,9\) showing that the role of calcineurin activation in LV hypertrophy was not as large in conventional models of pressure-overloaded hypertrophy as in the studies by Molkentin et al and Meguro et al. In these studies, administration of cyclosporin A or FK506 did not prevent or attenuate pressure-overload hypertrophy when assessed at 14 to 28 days after banding of the aorta.\(^3,4,9\)

The discrepancy between our study and these in vivo studies may be explained by the difference in the characteristics of the models of pressure-induced LV hypertrophy. We may speculate that the most important difference is the pattern of increasing blood pressure. In our model, systolic blood pressure gradually rose from 8 to 13 weeks. In contrast, blood pressure rises immediately after the operation in the aortic banding model. We have already demonstrated that the pattern of blood pressure elevation regulates the phenotype of heart failure in rats with genetically identical backgrounds.\(^5\) In addition, the sustained change in aortic input impedance affects the geometry of LV hypertrophy in rats with aortic banding.\(^10\) These data suggest that the duration and/or type of pressure overload may influence the phenotype of heart failure and geometry of LV hypertrophy. Differences in the duration or type of pressure overload may well account for the difference in the activated neurohumoral factors and/or signal transcriptional pathway; thus, it is not surprising if calcineurin may be related to only a certain phenotype of LV hypertrophy. Further studies are necessary to clarify the difference in the importance of signal transcriptional pathways among various types of pressure-overloaded heart.

Administration of FK506 from 8 weeks did not affect LV hydroxyproline content at 20 weeks, suggesting that the activation of calcineurin is not involved in the development of fibrosis in our model. Because the activation of calcineurin is targeted to myocytes but not to fibroblasts, collagen synthesis in fibroblasts may well be facilitated by other factors, such as angiotensin II, that are independent of the calcineurin transcriptional pathway.\(^11\)

Calcineurin Inhibition From the Initial Stage and LV Function

In this study, administration of FK506 from 8 weeks prevented the increases in lung/body wt ratio and LV end-diastolic pressure (LVEDP), and diastolic blood pressure (\(\Delta P/dt_{max}\)). These results are consistent with previous reports\(^3,4,9\) that calcineurin inhibition reduces ANP expression, LV mass, and LV diastolic function. The administration of FK506 from 8 weeks did not affect LV mass at 20 weeks in FKE rats, suggesting that the effect of calcineurin inhibition was not large at this time point. However, the effect of calcineurin inhibition on end-systolic stress and myocardial stiffness constant was evident from the initial stage of pressure overload.

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stolic pressure and the transition to a restrictive pattern in the transmitral flow velocity pattern. To the best of our knowledge, this is the first report that calcineurin inhibition prevents the transition to heart failure in a conventional heart failure model induced by pressure overload.

We have already demonstrated that heart failure develops mainly as a result of diastolic dysfunction in our model. In terms of LV diastolic function, the myocardial stiffness constant increased in FK(−) rats compared with the normal controls but was not significantly reduced by the administration of FK506 from 8 weeks. This result is consistent with histological data showing that the degree of LV fibrosis was not affected by FK506 administration. Structural remodeling of extracellular matrix has been implicated in the alteration of myocardial stiffness. For example, Thai et al showed that reduction of fibrosis by administration of angiotensin II type 1 receptor antagonist was associated with the improvement of myocardial stiffness constant. Thus, the beneficial effects of the administration of FK506 cannot be considered to occur through changes in myocardial stiffness constant or in the degree of fibrosis. Then, the question arises why heart failure did not develop in FKE regardless of the unchanged myocardial stiffness constant. The development of LV hypertrophy was restrained in FKE, and this should account for the improvement in LV chamber compliance and in the prophylaxis of LV diastolic failure, because attenuation of LV hypertrophy decreases LV chamber stiffness constant even with an unchanged myocardial stiffness constant. In fact, the increase in LV chamber stiffness in FK(−) was reduced in FKE, as evidenced by the result that LV end-diastolic pressure was lower in FKE than in FK(−) with similar LV end-diastolic dimension. Furthermore, τ was shortened in FKE compared with FK(−), indicating that LV relaxation was not deteriorated in FKE despite mild LV hypertrophy. It is still unknown why FK506 improved LV relaxation. This issue may be solved in future by studies of the effect of the administration of FK506 on calcium handling in hypertrophied myocytes.

Calcineurin Inhibition in the Advanced Stage
FK506 and cyclosporin A are not suitable for prophylactic administration because of their critical side effects. It would be clinically beneficial, however, if short-term calcineurin inhibition from the decompensated stage improved LV function and prevented the transition to heart failure. In our study, treatment with FK506 from 17 weeks did not shorten the time constant, decrease the myocardial stiffness constant, decrease lung/body wt and LV end-diastolic pressure, or decrease LVMI and LV hydroxyproline content. Thus, the inhibition of calcineurin starting just before the decompensated stage did not prevent the development of cardiac remodeling, the deterioration of LV function, or the transition to heart failure in our model. The activation of calcineurin may not participate in the transition process to heart failure in our model. +dP/dt max was smaller in FKL rats than in FKE rats. Because +dP/dt max is a load-dependent index, the decrease in +dP/dt max may be partly explained by increased wall stress in FKL rats. However, the possible detrimental effect of calcineurin inhibition on decompensated heart has to be studied in future.

Conclusions
Calcineurin inhibition from the initial developmental stage of pressure overload attenuated the development of LV

Figure 5. Photomicrographs of Azan Mallory staining of LV in FK(−) rat (left), FKE rat (center), and FKL rat (right) (×100). Interstitial and perivascular fibrosis is evident in FK(−) rat. These fibrous changes are also evident in FKE and FKL rats.

Figure 6. ANP mRNA levels at 20 weeks. Each mRNA level was corrected for an mRNA level of GAPDH and then normalized to mean value of age-matched control group, respectively.

*P < 0.05 vs control group, †P < 0.05 vs FK(−) group, ‡P < 0.05 vs FKE group.
hypertrophy to such a degree that it did not interfere with adaptation and prevented the transition to heart failure without an attenuation of LV fibrosis. Calcineurin inhibition from the advanced developmental stage of pressure overload, however, did not prevent cardiac remodeling, deterioration of LV function, or the transition to heart failure in our model. These data suggest that calcineurin activation is involved in the development of LV hypertrophy but not in the development of LV fibrosis and that the involvement may be crucial at the initial stage but negligible just before the decompensated stage of pressure overload.

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References
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