Calcineurin Plays a Critical Role in Pressure Overload–Induced Cardiac Hypertrophy

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Background—Cardiac hypertrophy is a fundamental adaptive response to hemodynamic overload; how mechanical load induces cardiac hypertrophy, however, remains elusive. It was recently reported that activation of a calcium-dependent phosphatase, calcineurin, induces cardiac hypertrophy. In the present study, we examined whether calcineurin plays a critical role in pressure overload–induced cardiac hypertrophy.

Methods and Results—Pressure overload produced by constriction of the abdominal aorta increased the activity of calcineurin in the rat heart and induced cardiac hypertrophy, including reprogramming of gene expression. Treatment of rats with a calcineurin inhibitor, FK506, inhibited the activation of calcineurin and prevented the pressure overload–induced cardiac hypertrophy and fibrosis without change of hemodynamic parameters. Load-induced expression of immediate-early-response genes and fetal genes was also suppressed by the FK506 treatment.

Conclusions—The present results suggest that the calcineurin signaling pathway plays a pivotal role in load-induced cardiac hypertrophy and may pave the way for a novel pharmacological approach to prevent cardiac hypertrophy. (Circulation. 1999;100:2449-2454.)

Key Words: hypertrophy ■ pressure ■ genes ■ calcineurin

Although cardiac hypertrophy is a beneficial adaptive response of the heart to an increased workload, hypertrophic heart often leads to dilated cardiomyopathy and eventually causes congestive heart failure after sustained overload. Moreover, recent epidemiological studies revealed that cardiac hypertrophy is an independent risk factor for ischemic heart disease, arrhythmia, and sudden death. Therefore, it is even more important to determine the mechanism of the development of cardiac hypertrophy.

Many lines of evidence have suggested that although cardiac hypertrophy can be induced by a variety of factors, such as hemodynamic overload (mechanical stress), genetic factors, and humoral factors, mechanical stress is the most important cause of cardiac hypertrophy. Mechanical stress on cardiac myocytes induces various hypertrophic responses, including activation of protein kinases, reprogramming of gene expression, and an increase in protein synthesis. Among intracellular signaling molecules, protein kinases such as protein kinase C, tyrosine kinases, and the mitogen-activated protein kinase family have been reported to play an important role in the development of cardiac hypertrophy.

Although many reports have suggested that intracellular Ca$^{2+}$ plays an important role in gene expression and growth in a variety of cell types, including cardiac myocytes, it is unknown how Ca$^{2+}$ regulates these events in cardiomyocytes. Recently, Molkentin et al reported that the Ca$^{2+}$-dependent phosphatase calcineurin plays a vital role in the development of cardiac hypertrophy through activation of NF-AT3, a member of a transcription factor family initially discovered in immune T cells. Both transgenic mice expressing activated calcineurin and NF-AT3 showed marked cardiac hypertrophy, and some of them progressed to dilated cardiomyopathy with interstitial fibrosis and showed congestive heart failure. The calcineurin inhibitors cyclosporin A (CsA) and FK506 prevented cardiac hypertrophy of activated calcineurin and NF-AT3 showed marked cardiac hypertrophy, and some of them progressed to dilated cardiomyopathy with interstitial fibrosis and showed congestive heart failure. The calcineurin inhibitors cyclosporin A (CsA) and FK506 prevented cardiac hypertrophy of activated calcineurin transgenic mice and humoral factor–induced hypertrophy of cultured cardiomyocytes of neonatal rats. In addition, Sussman et al also reported that several transgenic mice models that show hypertrophic cardiomyopathy can be treated with CsA. These results suggest that calcineurin plays a vital role in the development of cardiac hypertrophy induced by various causes. In the present study, using a pressure-overload model of abdominal aortic constriction, we examined whether calcineurin is also involved in load-induced cardiac hypertrophy.
Methods

Rat Preparation
Male Wistar rats (8 weeks old; 230 to 250 g) obtained from Nihon Seibutsu Zairyo Inc (Tokyo, Japan) were divided into 3 groups: (1) sham-operated rats, (2) pressure-overloaded rats without FK506, and (3) pressure-overloaded rats with FK506 (n=14, each group). Pressure overload was produced by constriction of the abdominal aorta as described previously.\(^{14}\) Briefly, the rats were anesthetized with an injection of a cocktail of ketamine HCl 100 mg/kg and xylazine 5 mg/kg IP. The abdominal aorta was constricted above the renal arteries by a 4-0 silk suture tied around both the aorta and a blunted 22-gauge needle, which was then pulled out. FK506, 1 mg \(\times\) kg body wt \(^{-1}\) \(\times\) d \(^{-1}\) IM, was administered from 3 days before operation through 3 weeks after. After rats were killed, hearts were removed, rinsed in PBS buffer, and separated into the right and left ventricles (LV). Total RNA (20 \(\mu\)g), extracted from the LV samples with RNAzol (Tel-text), was separated on a 1.0% agarose/formaldehyde gel and blotted onto a Hybond-N membrane (Amersham Co). cDNA was labeled by the random priming method with \([\alpha-^32\text{P}]\text{dCTP}\). Hybridized bands were quantified with NIH IMAGE software (NIH, Research Service Branch).

Hemodynamic Measurements In Vivo
To measure short- and long-term hemodynamic effects of aortic constriction, the right carotid artery was cannulated with a 24-gauge polyethylene catheter. The transducer (Baxter, model MP 5100) was connected to a Mac Laboratory system (model 400/s, AD Instruments), and the blood pressure and heart rate were measured.

Calcineurin Enzymatic Assay
The activity of calcineurin in lysates of LV samples was determined as described previously.\(^{15}\) Tissue was homogenized in 50 \(\mu\)L of hypotonic lysis buffer (50 mmol/L Tris-HCl [pH 7.5], 0.05% Tween-20, 1 mmol/L EDTA, 1 mmol/L CaCl\(_2\), 1 mmol/L PMSF, 1 \(\mu\)g/mL pepstatin, 1 \(\mu\)g/mL leupeptin, and 1 mmol/L DTT) and subjected to 1 round of freeze and thaw. After cell debris had been removed by centrifugation, 50 \(\mu\)L of phosphatase buffer (100 mmol/L MOPS [pH 7.0], 0.4 mmol/L CaCl\(_2\), 2 mmol/L MnCl\(_2\), 10 \(\mu\)g/mL BSA, 100 mmol/L calmodulin, and 500 mmol/L okad acid) was added to the supernatant. A calcineurin substrate, GST-RII peptide, which was fixed to glutathione-cellulose beads, was first phosphorylated by protein kinase A in the presence of \([\gamma-^32\text{P}]\text{ATP}\). Phosphorylated RII peptide was incubated with 2 \(\mu\)g of tissue lysate for 30 minutes at 30°C. Reactions were stopped, and the liberated \(^32\text{P}\) was determined by the Cherenkov method.

Echocardiographic Analysis
At 20 days after operation, transthoracic echocardiographic analysis was performed with HP sonos 100 (Hewlett-Packard Co) with a 10-MHz imaging transducer as described previously.\(^{16}\) Rats were weakly anesthetized with an injection of a cocktail of ketamine HCl 50 mg/kg and xylazine 2.5 mg/kg IP. When the rats partially recovered from anesthesia, M-mode images of the LV were recorded.

Histological Analysis
For histological analysis, hearts were fixed with 10% formamide by perfusion fixation. Fixed hearts were embedded in paraffin, sectioned at 4-\(\mu\)m thickness, and stained by the van Gieson method for collagen. To determine the degree of collagen fiber accumulation, we selected 5 fields randomly and calculated the ratio of van Gieson–stained fibrosis area to total myocardial area as described previously with the image analysis software NIH IMAGE.\(^{16}\)

Statistical Analysis
All data are expressed as the mean\(\pm\)SEM. Multiple comparisons among \(\geq 3\) groups were carried out by 2-way ANOVA and Fisher’s exact test for post hoc analysis. A value of \(P<0.05\) was considered statistically significant.

Results
Calcineurin Inhibitor FK506 Suppresses Pressure Overload–Induced Activation of Calcineurin Without Affecting Hemodynamic Parameters
Blood pressure monitored at the right carotid arteries was increased from 74.6 \(\pm\) 5.7 to 145.5 \(\pm\) 6.5 mm Hg soon after aortic constriction, and the elevation of blood pressure was also observed at 3 weeks after the operation (144.8 \(\pm\) 2.7 mm Hg, Figure 1A). FK506, 1 mg/kg body wt IM, was injected every day from 3 days before constriction of the abdominal aorta up to 3 weeks after. All rats survived throughout the treatment protocol, and hemodynamic parameters, such as the blood pressure and heart rate, were not affected by this treatment (Figure 1A). To examine whether pressure overload changes the calcineurin activity, we performed calcineurin enzymatic assays.\(^{16}\) Relative calcineurin activity in hearts from control rats (no operation nor injection of FK506) was 684 \(\pm\) 54 cpm/\(\mu\)g protein (Figure 1B). Al-
though sham operation did not change the activity of calcineurin (674±14 cpn/µg), pressure overload for 2 hours significantly increased the relative calcineurin activity to 910±22 cpn/µg in the heart (Figure 1B). Treatment with FK506 completely suppressed the elevation of the calcineurin activity (500±31 cpn/µg) (Figure 1B). Pressure overload–induced elevation of the calcineurin activity was still observed at 3 weeks after operation (975±37 cpn/µg), and chronic treatment with FK506 also completely inhibited the elevation (631±48 cpn/µg) (Figure 1B).

**FK506 Prevents Pressure Overload–Induced Cardiac Hypertrophy**

Pressure overload for 3 weeks induced marked cardiac hypertrophy (Figure 2A). Chronic treatment with FK506 completely prevented the development of pressure overload–induced cardiac hypertrophy (Figure 2A). There was no significant difference in body weight among sham-operated rats, banded rats, and banded rats treated with FK506. Pressure overload for 3 weeks increased LV weight by 38% and ratio of LV weight to body weight by 40%, and treatment with FK506 completely prevented increase. Data are mean±SEM (n=7 each group). *P<0.05 vs sham-operated rats.

**Calcineurin Is Involved in Pressure Overload–Induced Reprogramming of Gene Expression**

Induction of immediate-early-response genes and fetal genes is a genetic response to hemodynamic overload. The activation of calcineurin has been reported to upregulate the brain...
genes was not affected by the treatment (Figure 5).

FK506 treatment, whereas upregulation of c-fos, c-jun, and BNP genes (Figure 5A and SC), and fetal genes, such as skeletal α-actin and atrial natriuretic peptide (ANP) genes (Figure 5B and 5D), in the heart as previously reported.13 Treatment with FK506 inhibited the pressure overload–induced regulation of some of these genes. Upregulation of c-fos, BNP, and ANP genes and downregulation of the SERCA2A gene were fully inhibited by the FK506 treatment, whereas upregulation of c-jun and skeletal α-actin genes was not affected by the treatment (Figure 5).

Discussion

This study demonstrated that calcineurin is activated by pressure overload in the heart and that the activated calcineurin plays a critical role in the development of load-induced cardiac hypertrophy. While we were preparing the manuscript, several groups reported controversial results.13,19–21 Sussman et al13 reported that 20 mg/kg CsA treatment prevents acute pressure overload–induced cardiac hypertrophy. Meguro et al19 reported that CsA could attenuate but not prevent LV hypertrophy, and Luo et al20 and Zhang et al21 reported that calcineurin inhibitors had no effects on load-induced cardiac hypertrophy in the same pressure-overload model as ours. Although the reason for the discrepancy is not known at present, it may come from the dose and the route of administration of the calcineurin inhibitors. Luo et al injected 20 to 40 mg/kg CsA SC or 2 to 4 mg/kg FK506 IV, and quite a high percentage of banded rats (30% to 90%) died with these treatments.20 The treatment by Zhang et al21 was similar to that by Luo et al, and mortality was also very high (33% to 67%). Sussman et al13 also reported that all rats of the banded group were dead within 1 week after 20 mg/kg CsA treatment. Systemic administration of CsA or FK506 dose-dependently induces many side effects, such as infection, renal failure, diabetes mellitus, and hypertension.22 Because our preliminary experiments revealed that injection of FK506 10 mg/kg body wt IP or IM killed many rats and caused serious side effects, such as cachexia and infection, and that injection of 1 mg/kg FK506 IM was enough to inhibit load-induced activation of calcineurin in the heart without any side effects, we injected 1 mg/kg FK506 every day from 3 days before operation up to 3 weeks after in the present study. In this dose, no rats were dead until at least 3 weeks after operation, and no decrease in body weight or infection was observed in our treatment (n = 7 each) (Figure 3). From this point of view, the dose and the route of administration of calcineurin inhibitors might be quite critical to evaluate their role in the development of load-induced cardiac hypertrophy.

Treatment with FK506 fully inhibited pressure overload–induced cardiac hypertrophy and fibrosis (Figures 2, 3, and 4). FK506, a macrolide compound isolated from Streptomyces tsukubaensis, has potent immunosuppressive properties.23 FK506 is 10 to 100 times more potent than CsA.24 FK506 binds to FK506 binding protein (FKBP), and the FK506/FKBP complex inhibits calcineurin.24 In cardiac myocytes, it has been reported that FK506 binds to FKBP and opens the ryanodine receptor, resulting in an increase in intracellular calcium concentration.24–26 Therefore, it is possible that the FK506 modulated the ryanodine receptor in this study. However, the following observations suggest that treatment with FK506 suppressed the development of cardiac hypertrophy through inhibition of the calcineurin activity. The FK506 treatment suppressed the load-induced elevation of calcineurin activity, and our preliminary results show a good correlation between the calcineurin activity and the degree of cardiac hypertrophy (Figure 1B, unpublished observations). In addition, CsA, which binds to another protein, cyclophilin, showed similar inhibitory effects on the development of load-induced cardiac hypertrophy (unpublished observations).

Many lines of evidence have suggested that vasoactive peptides, including angiotensin II (Ang II) and endothelin–1, play an important role in mechanical stress–induced cardiac hypertrophy.2,27–29 It has also been reported that Ang II plays a critical role in this pressure-overload rat model of abdominal aortic constriction3 and that Ang II–induced hypertrophy of cultured cardiomyocytes is inhibited by CsA.11 Therefore, it is conceivable that FK506 effectively prevented pressure overload–induced cardiac hypertrophy in this model by inhibiting Ang II–induced calcineurin activation in the heart. It has been reported that CsA is not effective in preventing load-induced cardiac hypertrophy, which is induced by constriction of the ascending or transverse aorta.29,30 Because Ang II is not involved in the pressure-overload model of ascending or transverse aortic constriction,31 it is possible that CsA was not effective in preventing load-induced hypertrophy in that model.29,30 Whether calcineurin is involved in the development of load-induced cardiac hypertrophy may depend on the model of hemodynamic overload. Although it remains unclear how activated calcineurin induces cardiac hypertrophy, activation of NF-AT3 may play an important role.11 In analogy with activated T cells,12 NF-AT3 may upregulate cytokines of the interleukin-6 family, such as 

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<th>sham</th>
<th>banding</th>
<th>banding + FK506</th>
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<tbody>
<tr>
<td>IVS (mm)</td>
<td>1.32±0.04</td>
<td>1.94±0.17∗</td>
<td>1.23±0.07</td>
</tr>
<tr>
<td>PW (mm)</td>
<td>1.28±0.05</td>
<td>1.90±0.16∗</td>
<td>1.13±0.02</td>
</tr>
<tr>
<td>EF (%)</td>
<td>76.8±1.0</td>
<td>79.8±3.3</td>
<td>79.2±1.7</td>
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Figure 4. Representative chart of transthoracic M-mode echo-cardiograms. Pressure overload increased thickness of both IVS and PW, whereas treatment with FK506 completely inhibited increment of wall thickness without affecting cardiac function. EF indicates LV ejection fraction. *P<0.05 vs sham-operated rats (n=7 each).
cardiotrophin-1 and leukemia inhibitory factor, which are strong inducers of cardiomyocyte hypertrophy.\textsuperscript{32} The mechanism of induction of cardiac hypertrophy and fibrosis by activated calcineurin awaits further investigation.

FK506 inhibited load-induced expression of some of the immediate-early-response genes and fetal genes, but upregulation of \textit{c-jun} and skeletal \textit{\alpha-actin} genes was not affected by FK506 (Figure 5). These results suggest that calcineurin is involved in pressure overload–induced expression of some specific genes but not of all genes and that FK506 inhibited load-induced gene expression by suppressing calcineurin activation in the heart without affecting hemodynamic load.

Molkentin et al\textsuperscript{11} reported that NF-AT3 activates transcription of some cardiac genes in concert with the cardiac enriched transcription factor GATA4. Recently, it was reported that activation of calcineurin selectively upregulates slow-fiber–specific genes in skeletal myocytes through NF-AT3-MEF2 association.\textsuperscript{33} Because MEF2 plays an important
role in gene regulation in the heart as well. Calcineurin may be involved in the regulation of many cardiac genes through activation of NF-AT3. Molkentin et al13 using the transgenic mouse strategy, first demonstrated that activated calcineurin could induce cardiac hypertrophy. Sussman et al13 next showed that calcineurin may be involved in the generation of hypertrophic cardiomyopathy. The present study suggests that calcineurin plays a critical role in the development of the load-induced cardiac hypertrophy, which is most often observed clinically. In addition, Lim et al15 recently demonstrated that calcineurin is activated in the heart in human heart failure. Because of severe side effects, it may not be realistic to use calcineurin inhibitors in the present form to prevent the development of cardiac hypertrophy. However, because the possibility exists that inhibition of calcineurin may prevent not only the development of cardiac hypertrophy but also that of heart failure, it should be worthwhile to develop novel calcineurin inhibitors.

Acknowledgments
This work was supported by a Grant-in-Aid for Scientific Research on Priority Areas from the Ministry of Education, Science, Sports, and Culture of Japan and by the Program for Promotion of Fundamental Studies in Health Science of the Organization for Drug ADR Relief, R&D Promotion, and Product Review of Japan.

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Circulation. 1999;100:2449-2454
doi: 10.1161/01.CIR.100.24.2449

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