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

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Background—Although activation of the Ca\(^{2+}\)-dependent phosphatase calcineurin has been reported to induce cardiomyocyte hypertrophy, whether calcineurin is involved in pressure overload–induced cardiac hypertrophy remains controversial.

Methods and Results—We examined in the present study the role of calcineurin in pressure overload–induced cardiac hypertrophy using transgenic mice that overexpress the dominant negative mutant of calcineurin specifically in the heart. There were no significant differences in body weight, blood pressure, heart rate, heart weight, and the cardiac calcineurin activity between the transgenic mice and their littermate wild-type mice at basal state. The activity of calcineurin was markedly increased by pressure overload produced by constriction of the abdominal aorta in the heart of wild-type mice but less increased in the heart of the transgenic mice. Pressure overload induced increases in heart weight, wall thickness of the left ventricle, and diameter of cardiomyocytes; reprogramming of expressions of immediate early response genes and fetal-type genes; activation of extracellular signal–regulated protein kinases; and fibrosis. All these hypertrophic responses were more prominent in the wild-type mice than in the transgenic mice.

Conclusions—These results suggest that calcineurin plays a critical role in the development of pressure overload–induced cardiac hypertrophy. 

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Key Words: calcineurin n hypertrophy n genes n pressure

Cardiac hypertrophy is recognized in many cardiovascular diseases, such as hypertension, valvular diseases, and myocardial infarction, and is an independent risk factor for cardiac morbidity and mortality. Cardiac hypertrophy is induced by a variety of factors, such as vasoactive peptides, catecholamines, cytokines, and growth factors; however, mechanical stress is most important as an initial stimulus. Hypertrophic stimuli induce an increase in protein synthesis with reprogramming of gene expression by activation of various signaling molecules, such as protein kinase C, tyrosine kinases, Ras, the mitogen-activated protein (MAP) kinase family, and the Janus kinase (JAK)/signal transducer and activator of transcription (STAT) family.

See p 9

Ca\(^{2+}\) is an important second messenger in various cellular processes, including cell growth and survival. In response to growth stimuli, many types of cells increase their cytosolic Ca\(^{2+}\) levels, and the elevated Ca\(^{2+}\) activates many effectors, including calcineurin, a Ca\(^{2+}\)/calmodulin–dependent protein phosphatase that is highly conserved in evolution and widely distributed in many tissues. Calcineurin plays pivotal roles in neuronal functions and immune responses. Calcineurin has attracted great attention as a critical mediator for cardiac hypertrophy. Transgenic mice that overexpress constitutively active forms of calcineurin and of its downstream transcription factor nuclear factor of activated T cells (NFAT) showed marked cardiac hypertrophy, whereas calcineurin inhibitors, such as cyclosporin A and FK506, significantly suppressed phenylephrine- and angiotensin II–induced cardiomyocyte hypertrophy in vitro. The role of calcineurin in the development of pressure overload–induced cardiac hypertrophy, however, is unclear. Five research groups, including ours, have reported that calcineurin plays a critical role in the development of pressure overload–induced cardiac hypertrophy, and several other groups reported an opposite conclusion. Because in those studies, many animals lost body weight (BW) and died, possibly of severe side effects of calcineurin inhibitors, it might be difficult to reach a conclusion. To elucidate the precise role of cal-

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cineurin in the development of pressure overload–induced cardiac hypertrophy while avoiding drug toxicity, we created transgenic mice that overexpress the catalytically inactive cardiac hypertrophy while avoiding drug toxicity, we created transgenic mice that overexpress the catalytically inactive form of calcineurin only in the heart. We show here that aortic constriction–induced cardiac hypertrophy was attenuated in the transgenic mice compared with wild-type mice.

Methods

Mice and Transgene

The dominant negative (DN) mutants of human calcineurin A subunit (CnA) were previously described.20 These mutants were generated by deleting the autoinhibitory domain in the calcineurin A subunit (CnA). The dominant negative (DN) mutants of human calcineurin A subunit (CnA) were constructed by deleting the autoinhibitory domain in the calcineurin A subunit (CnA).

Pressure-Overload Model

The abdominal aorta above the left renal artery was constricted as previously described.21 The abdominal aorta above the left renal artery was constricted as previously described.21 To monitor the hemodynamic effects of aortic constriction–induced cardiac hypertrophy, we generated transgenic mice that expressed the DNCnA with HA tag specifically in the heart under the control of \( \alpha \)-myosin heavy chain promoter (Figure 1A).20 DNCnA can bind to the calcineurin B subunit, but it is catalytically inactive and interferes with NFAT4 translocation.17

Echocardiographic Analysis

Transthoracic echocardiographic analysis was performed with an HP Sonos 100 (Hewlett-Packard Co) with a 10-MHz imaging transducer as described previously.18

Calcineurin Activity

The activity of calcineurin was determined by use of phosphorylated GST-RII peptide as a substrate as previously described.22 with some modifications. We separated calmodulin-bound calcineurin (active calcineurin, >100 kDa) from free calcineurin (inactive calcineurin, <100 kDa) by centrifugation of cell lysates at 1500g for 10 minutes with Ultrafree-MC Centrifugal Filter Units (Millipore). The phosphorylated GST-RII peptide was incubated with the samples in the Ca\(^{2+}\)-free condition.

Western Blot Analysis of Calcineurin Expression

The expressions of DNCnA and endogenous CnA were analyzed by Western blotting using an anti-HA antibody (Mitsubishi Biochemical Laboratories) or an anti-CnA antibody (Santa Cruz Biotechnology, Inc). Immunoreactivity was detected by an enhanced chemiluminescence reaction system (Amersham) according to the manufacturer’s directions.

Northern Blot Analysis

Total RNA (10 \( \mu \)g) was size-fractionated in 1.2% formaldehyde agarose gels and transferred to nylon membranes. The blots were hybridized with the [\( \alpha \)-\( ^{32} \)P]dCTP-labeled (Du Pont-New England Nuclear Co) cDNA fragment as previously described.19

Assay of Extracellular Signal–Regulated Protein Kinase Activity

Extracellular signal–regulated protein kinase (ERK) activities were measured with myelin basic protein (MBP)–containing gel as previously described.19 In brief, cell lysates from LV tissue were electrophoresed on SDS-polyacrylamide gel containing 0.5 mg/mL MBP. Phosphorylation of MBP was detected by incubation of the gel with [\( \gamma \)-\( ^{32} \)P]ATP.

Histological Analysis

The transverse diameter of cardiomyocytes stained by hematoxylin and eosin was measured by micrometers (\( \mu \)m) in 20 different randomly chosen points from a cross section of LV free wall. The extent of LV fibrosis was measured in 8 fields randomly selected from a section by calculating the ratio of azan-stained fibrosis area divided by total myocardium area.18 Five sections of each heart were measured.

Statistics

All values are expressed as mean±SEM of 6 experiments in each instance. Comparisons among 3 groups were made by 1-way ANOVA followed by Dunnett’s modified t test. Values of \( P < 0.05 \) were considered statistically significant.

Results

Expression of DNCnA in Mice

To determine the role of calcineurin in the development of pressure overload–induced cardiac hypertrophy, we generated transgenic mice that expressed the DNCnA with HA tag specifically in the heart under the control of \( \alpha \)-myosin heavy chain promoter (Figure 1A).20 DNCnA can bind to the calcineurin B subunit, but it is catalytically inactive and interferes with NFAT4 translocation.17 Northern blot analysis using 10 \( \mu \)g of total RNA and cDNA probe corresponding to N1 to 407 of DNCnA revealed that although endogenous CnA gene was not detected, the DNCnA gene was abundantly expressed in the heart of both lines of the transgenic mice.

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(Figure 1B). The DNCnA gene was not expressed in other tissues examined, such as brain, liver, skeletal muscle, lungs, and intestine of the transgenic mice (data not shown). We also examined the expression of DNCnA and the endogenous CnA at the protein level by Western blot analysis using an anti-HA antibody and an anti-CnAβ antibody that was raised against a carboxy terminus peptide of the CnAβ. The DNCnA protein was abundantly expressed in the heart of both transgenic lines (Figure 1C, lane 1 and 2). There was no significant difference in the expression levels of endogenous CnA between the transgenic and wild-type mice (Figure 1C, lane 5 to 8). Expression levels of DNCnA were >10-fold higher than those of endogenous CnA in the transgenic heart.

**Inhibition of Calcineurin Activity in the Heart of Transgenic Mice**

To determine whether DNCnA effectively functions as a dominant negative mutant in the transgenic heart, we examined the activity of calcineurin in the heart using an enzymatic phosphatase assay after separation of activated calcineurin. There was no difference in the basal activity of calcineurin between the wild-type heart (15 520 ± 1500 cpm/mg, n = 5) and the transgenic heart (14 850 ± 1050 cpm/mg, n = 5). After 30 minutes under pressure overload, the activity of calcineurin was significantly increased in the heart of wild-type mice, as reported before in rats

\[ \text{ICP} = 10.5 ± 0.6 \, \text{mmHg} \]

\[ \text{BW} = 190 ± 12^* \, \text{g} \]

\[ \text{HW/BW} = 4.1 ± 0.2 \, \text{mg/g} \]

while in the heart of transgenic mice, the increase of calcineurin activity was attenuated (21 450 ± 1520 cpm/mg, n = 5, \( P < 0.05 \) versus versus wild-type mice). These results suggest that the cardiac-specific overexpression of DNCnA effectively suppresses the activation of calcineurin in the heart.

**Suppression of Cardiac Hypertrophy in Transgenic Mice**

A 2D long-axis image was used to evaluate the wall thickness of the left ventricle (LV). M-mode images taken during 10 successive beats revealed that the interventricular septum and the posterior wall of the LV were significantly thicker in wild-type mice compared with the transgenic mice at 3 weeks after aortic constriction (Figure 2).

After 3 weeks of aortic banding, the heart weight (HW) and the ratio of HW to BW (HW/BW) were significantly suppressed in the transgenic mice, whereas the increases in HW and HW/BW were significantly in wild-type mice, as reported before in rats

\[ \text{HW} = 1750 ± 0.2 \, \text{g} \]

\[ \text{HW/BW} = 5.1 ± 0.3 \, \text{mg/g} \]

\[ \text{HW/BW} = 1.2 ± 0.5 \% \]

These results clearly indicate that calcineurin plays a critical role in pressure overload–induced cardiac hypertrophy.

Induction of specific gene expression is one of hypertrophic responses to hemodynamic overload.\(^2\)\(^3\) We thus examined the expressions of immediate early response genes and fetal genes in the hearts at 2 hours and 3 weeks after aortic constriction, respectively (Figure 4). Pressure overload upregulated mRNA levels of c-fos, c-jun, and brain natriuretic peptide (BNP) genes at 2 hours and atrial natriuretic peptide (ANP) and skeletal α-actin (sk. α-actin) genes at 3 weeks in wild-type mice. Expression of sarcomplastic reticulum Cα\(^+\) ATPase (SERCA2) gene was downregulated by pressure overload in the wild-type heart. Although the expression of c-jun and sk. α-actin genes was upregulated by pressure overload in the transgenic mice as well, mRNA levels of c-fos, ANP, BNP, and SERCA2 genes remained unchanged in the hearts of transgenic mice. Thus, calcineurin might play an important role in pressure overload–induced reprogramming of some specific genes.

**Pressure Overload–Induced Cardiac Hypertrophy**

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<td>HW/BW, mg/g</td>
<td>4.1 ± 0.2</td>
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WT and TG mice were sham-operated (Sham) or subjected to abdominal aortic constriction for 3 weeks as described in Methods. BW, HW, and HW/BW ratio were determined. Results are shown as mean ± SEM.

*\( P < 0.05 \) vs sham-operated WT mice; †\( P < 0.05 \) vs WT mice subjected to aortic constriction.

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**Histological analysis showed that after 3 weeks, aortic constriction induced marked cardiac hypertrophy in wild-type mice, whereas the transgenic mice developed less cardiac hypertrophy in response to pressure overload (Figure 3).** The transverse diameter of cardiomyocytes was increased significantly, from 10.5 ± 1.3 to 19.1 ± 0.8 μm (\( P < 0.05 \)), in wild-type mice, whereas there was no significant difference in transverse diameter of cardiomyocytes between sham-treated and banding-treated transgenic mice (sham, 10.0 ± 1.2 μm versus banding, 13.0 ± 1.3 μm). The extent of LV fibrosis was significantly reduced in the transgenic mice compared with that in wild-type mice (transgenic mice, 1.3 ± 0.2% versus wild-type mice, 2.1 ± 0.5% of a whole section, n = 5, \( P < 0.05 \)).

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**Figure 2.** M-mode echocardiograms. a and b, Sham operation; c and d, 3 weeks after aortic constriction. Data are expressed as mean ± SEM. *\( P < 0.05 \) vs sham-operated wild-type (WT) mice. †\( P < 0.05 \) vs WT mice with aortic constriction. IVS indicates interventricular septum; LVPW, left ventricular posterior wall.
Suppression of Protein Kinases in Transgenic Mice

ERKs play an important role in the development of cardiac hypertrophy. Calcineurin has been reported to be involved in Ca\(^{2+}\)-mobilizing agent–induced activation of ERKs in mouse M1 myeloid leukemic cells, and the activity of ERKs was increased in the heart of the transgenic mice, which express a constitutively active form of calcineurin. We also observed that calcineurin plays a critical role in isoproterenol-induced activation of ERKs in cultured cardiomyocytes of neonatal rats (unpublished observation). We therefore examined whether calcineurin is involved in pressure overload–induced activation of ERKs in the heart in vivo. Constriction of the abdominal aorta for 30 minutes markedly activated ERKs in the heart of wild-type mice (Figure 5). The pressure overload–induced activation of ERKs was attenuated in the heart of transgenic mice (Figure 5), suggesting that calcineurin is critically involved in pressure overload–induced activation of ERKs in the heart.

### Discussion

Ca\(^{2+}\) plays an important role in various cellular processes, such as cell growth and survival, including cardiac hypertrophy. The mechanism of how Ca\(^{2+}\) is involved in the development of cardiac hypertrophy, however, was unclear. Recently, calcineurin has attracted great attention as a mediator of Ca\(^{2+}\)-induced cardiac hypertrophy. Overexpression of constitutively active mutants of calcineurin and of its downstream transcription factor NFAT3 induced marked cardiac hypertrophy in transgenic mice. The calcineurin inhibitors cyclosporin A and FK506 suppressed phenylephrine- and angiotensin II–induced cardiomyocyte hypertrophy in vitro. Although 5 research groups, including ours, have demonstrated that calcineurin plays a critical role in pressure overload–induced cardiac hypertrophy, several other groups have reported that cyclosporin A and FK506 had no suppressive effect on pressure overload–induced cardiac hypertrophy. The reason for the discrepancy is not clear at present, but there are several possibilities. First, because calcineurin is expressed abundantly in the heart, it is possible that the dose of calcineurin inhibitors was not enough to completely inhibit the activity of calcineurin in the heart. Many studies did not examine the activity of calcineurin before and after administration of calcineurin inhibitors. Although some of them measured the activity of calcineurin, it is difficult to determine the precise activity of calcineurin. In the present study, we measured the activity of calcineurin after separating activated calcineurin from nonactivated calcineurin, which enabled us to accurately determine the activity of endogenous calcineurin. Second, the role of calcineurin may differ with animal species and the model of pressure overload–induced cardiac hypertrophy. The third and most likely possibility is the severe cytotoxicity of calcineurin inhibitors. Both cyclosporin A and FK506 induce severe adverse reactions, such as

### Figure 3

Cardiac hypertrophy induced by pressure overload. Hearts were excised from transgenic (TG) and wild-type (WT) mice that were subjected to pressure overload for 3 weeks or sham operation. a to c, Hearts were sectioned longitudinally and stained with hematoxylin and eosin. Cardiac hypertrophy was observed in WT mice subjected to aortic banding (b) but not in sham-operated WT mice (a) or in TG mice (c). d to f, High-magnification views of hematoxylin and eosin–stained LV wall. Pressure overload induced an increase in cardiomyocyte size in WT mice (e) but not in sham-operated WT mice (d) or in TG mice (f). g to i, Azan staining. g, Sham-operated WT mice. An increase in perivascular and interstitial fibrosis was observed in WT (h) but not in TG mice subjected to aortic banding (i).

### Figure 4

Specific gene expression. Wild-type (WT) and transgenic (TG) mice were subjected to sham operation or to constriction of abdominal aorta. Northern blot analysis was performed as described in Methods. A, Induction of immediate early response genes by acute pressure overload (2 hours). B, Expression of fetal-type and SERCA2 genes by pressure overload for 3 weeks. Ethidium bromide staining of 18S ribosomal RNA is presented to show integrity of RNA. Representative autoradiograms from 3 independent experiments are shown.

### Figure 5

ERK activity. Wild-type (WT) and transgenic (TG) mice were subjected to either sham operation or aortic constriction for 30 minutes, and activity of ERKs in heart was measured by "in gel assay" as described in Methods. Representative autoradiogram showing MBP phosphorylation.
infection, renal failure, and cachexia, which could cause death. Indeed, most animals showed marked loss of BW, and many animals died after the operation under treatment with calcineurin inhibitors. It should be difficult to reach a conclusion when >50% of animals died during the experiments. To evaluate the role of calcineurin in pressure overload–induced cardiac hypertrophy while avoiding the severe side effects of calcineurin inhibition, we used transgenic mice that overexpress DNCnA specifically in the heart.

In the basal state, there was no significant difference in the activity of calcineurin and HW between transgenic mice and their littermate wild-type mice, suggesting that the basal calcineurin activity is low in the heart. In the transgenic mice, pressure overload induced less marked hypertrophic responses, including attenuated increases in HW, LV wall thickness, cardiomyocyte size, and myocardial fibrosis and reprogramming of some specific genes and activation of ERKs, compared with wild-type mice. Because there was no difference between transgenic mice and wild-type mice in BW and hemodynamic parameters, such as cardiac function, HR, and BP, these results clearly indicate that calcineurin is critically involved in pressure overload–induced cardiac hypertrophy. Although it is suggested that calcineurin induces cardiac hypertrophy by activating its downstream transcription factor NFAT3, there may be other pathways, including ERKs, through which calcineurin induces cardiac hypertrophy. Further studies are necessary to elucidate the downstream mechanism of how calcineurin induces cardiac hypertrophy.

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