Calcineurin Expression, Activation, and Function in Cardiac Pressure-Overload Hypertrophy

Hae W. Lim, PhD; Leon J. De Windt, PhD; Leonard Steinberg, MD; Tyler Taigen, BS; Sandra A. Witt, BS; Thomas R. Kimball, MD; Jeffery D. Molkentin, PhD

Background—Vascular hypertension resulting in increased cardiac load is associated with left ventricular hypertrophy and is a leading predicator for progressive heart disease. The molecular signaling pathways that respond to increases in cardiac load are poorly understood. One potential regulator of the hypertrophic response is the calcium-sensitive phosphatase calcineurin.

Methods and Results—We showed that calcineurin enzymatic activity is increased 3.2-fold in the heart in response to pressure-overload hypertrophy induced by abdominal aortic banding in the rat. Western blot analysis further demonstrates that calcineurin A (catalytic subunit) protein content and association with calmodulin are increased in response to pressure-overload hypertrophy. This increase in calcineurin protein content was prevented by administration of the calcineurin inhibitor cyclosporine A (CsA). CsA administration attenuated load-induced cardiac hypertrophy in a dose-dependent manner over a 14-day treatment protocol. CsA administration also partially reversed pressure-overload hypertrophy in aortic-banded rats after 14 days. CsA also attenuated the histological and molecular indexes of pressure-overload hypertrophy.

Conclusions—These data suggest that calcineurin is an important upstream regulator of load-induced hypertrophy. (Circulation. 2000;101:2431-2437.)

Key Words: aorta ■ hypertrophy ■ pressure

Cardiac left ventricular hypertrophy (LVH) is an adaptive response to numerous forms of cardiovascular stress that temporarily augments cardiac performance by reducing wall tension.1 Although this response is initially beneficial, it often progresses to decompensation and heart failure if the initiating stimulus is not alleviated. LVH in humans is thought to be the single greatest predisposing risk factor for cardiac morbidity and death.2 However, the molecular controllers that sense cardiovascular disease states and initiate cardiac hypertrophy are poorly understood.

Cardiac hypertrophy can be induced by hemodynamic overload, ischemic disease, neurohumoral factors, or intrinsic defects in cardiac structural protein genes.3,4 Studies in cultured cardiomyocytes and in animal models of heart disease have implicated the mitogen-activated protein kinase cascade in cardiac hypertrophy. Hypertrophic agonists such as angiotensin II, endothelin-1, cardiotrophin-1, and catecholamines were shown to activate the mitogen-activated protein kinase cascade in cultured cardiomyocytes.5–11 Recent investigation suggests that both a JNK-dependent and a p38-dependent pathway are capable of initiating hypertrophy of cultured neonatal cardiomyocytes.12–14

Another intracellular regulatory pathway implicated in cardiac hypertrophy involves the calcium-regulated phosphatase calcineurin and the transcription factor NF-AT3.15 This calcineurin-dependent pathway was first identified in T cells as an early response pathway to T-cell receptor activation mediated through increases in intracellular calcium.16 Activated calcineurin directly dephosphorylates cytosolic NF-AT transcription factors, resulting in their nuclear translocation and activation of immune response genes. The immunosuppressive drugs cyclosporine A (CsA) and FK506 prevent T-cell–mediated responses through inhibition of calcineurin activity.17

Cardiac overexpression by transgenesis of either activated calcineurin or a constitutively nuclear NF-AT3 mutant produced substantial hypertrophy that rapidly progressed to heart failure. These studies were extended to demonstrate prevention of phenotypic hypertrophy with cyclosporine or FK506 in genetically altered mouse models of cardiomyopathy and in a pathophysiological model of pressure-overload hypertrophy in the rat.18 In contrast, a number of recent studies have reported that calcineurin inhibitors are ineffective in preventing pressure-overload hypertrophy in aortic-banded rodent models.19–23

We showed that calcineurin is activated early in the time course of load-induced hypertrophy and that activation is maintained long term. Treatment of aortic-banded rats with

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Results
Calcineurin Is Dynamically Regulated During Progression of Load-Induced Hypertrophy

It has been previously shown that load-induced hypertrophy results in altered intracellular calcium handling. 27 To determine whether calcineurin is activated by pressure-overload hypertrophy, we used 3 distinct assays: (1) a Western blot, (2) a calmodulin coimmunoprecipitation assay, and (3) a calcineurin-specific enzymatic assay.

Rats were subjected to abdominal aortic banding for 6 hours, 1, 3, 7, 14, 21, or 42 days, at which time their hearts were collected for analysis. Left ventricular and atrial protein extracts were subjected to calmodulin immunoprecipitation followed by a calcineurin-specific Western blot (Figure 1A). Levels of immunoprecipitated calcineurin were normalized to levels of calmodulin coprecipitated and are represented graphically below each Western blot. The data demonstrate calcineurin association with calmodulin as early as 1 day after aortic banding in the left ventricle. Association then reached a maximum within 3 days and was maintained through day 42, whereas calcineurin was not activated in the atria (Figure 1A). Atria were included for control purposes, which did not demonstrate increased calcineurin-calmodulin association. These results were consistent across 2 animals each at the indicated time points, although only 1 is shown for simplicity.

Unprecipitated protein was also subjected to Western blotting to quantify absolute levels of calcineurin and calmodulin in each sample. The data demonstrate invariant calmodulin levels but an increase in total calcineurin protein content at days 7 and 14 in the left ventricle and to a lesser extent in the atria. This increase in total calcineurin protein was seen in 4 of 4 samples at day 14 and 2 of 2 at day 7.

Total calcineurin enzymatic activity after 14 days of banding was also increased 3.2-fold (P < 0.05) in the left ventricle as assayed by dephosphorylation of a 32P-labeled RII peptide (Figure 1B). Because the increase in calcineurin activity at day 14 is also associated with increased calcineurin protein content, it is difficult to infer an increase in specific activity. However, the calmodulin-calcineurin immunoprecipitation assay shows increased association at time points when calcineurin protein content is invariant, suggesting an increase in specific activity of calcineurin.

Methods
Generation and Treatment of Aortic-Banded Rats
Sprague-Dawley (Harlan, Indianapolis, Ind) rats (weight 100 to 225 g, all female) were anesthetized with isoflurane, and their abdominal aortas were exposed. The aorta was constricted below the celiac trunk and above the superior mesentric artery using 1-0 silk and 21-gauge wire, which is removed to generate a defined constriction. CsA (Sandimmune, Novartis) was administered subcutaneously into the nape of the neck 1 day before aortic banding and continued for 14 additional days. For the prevention study, 19 and 13 rats were initially banded and placed on 2 dosages of CsA (10 mg/kg twice daily or 4 mg/kg twice daily), of which 14 and 11 survived the treatment protocol, respectively. In addition, 13 untreated, banded rats were generated, of which 10 survived the 14-day protocol. Six sham animals were analyzed in 2 separate groups (with or without CsA) without deaths.

For Western Blots and Assay for Calcineurin
To demonstrate activated calcineurin, protein extracts were made from either the left ventricle or atria in immunoprecipitation buffer (20 mmol/L NaPO4, 150 mmol/L NaCl, 2 mmol/L MgCl2, 0.1% NP40, 10% glycerol, 10 mmol/L NaF, 0.1 mmol/L sodium orthovanadate, 10 mmol/L sodium pyrophosphate, 1 mmol/L DTT, 10 μg/mL leupeptin, 10 μg/mL aprotinin, 10 μg/mL pepstatin, 10 μg/mL TPCK, and 10 μg/mL TLCK). For immunoprecipitation, 400 μg of protein extract (~30 to 50 μL) was incubated with 5 μg of calmodulin rabbit polyclonal antibody (Zymed) in 100 μL at 4°C with gentle rocking for 1 hour followed by the addition of 50 μL of protein A/G agarose (Santa Cruz) and another hour of incubation at 4°C. The samples were washed 3 times with 200 μL of immunoprecipitation buffer and subjected to SDS-PAGE. Immunodetection was then performed with a calcineurin antibody (Transduction Laboratories) followed by a calmodulin-specific antibody (Zymed). Blots were quantified for fluorescence with a Storm 860 PhosphorImager (Molecular Dynamics).

Calcineurin Phosphatase Assay
Left ventricles were homogenized in equal volumes of cell lysis buffer (50 mmol/L Tris-Cl pH 7.5, 0.1 mmol/L NaCl, 5 mmol/L DTT, 1 mmol/L EDTA, pH 8.0, 1 mmol/L PMSF, 5 μg/mL pepstatin, 5 μg/mL leupeptin, and 5 μg/mL aprotinin) and sonicated at 4°C. The resulting supernatants (25 μg of protein) were assayed in buffer consisting of 20 mmol/L Tris-Cl pH 7.5, 0.1 mmol/L NaCl, 6 mmol/L MgCl2, 0.5 mmol/L CaCl2, 1 mmol/L DTT, and 50 μg/mL BSA. Phosphatase activity was measured as the dephosphorylation rate of a synthetic [32P]-ATP–labeled phosphopeptide substrate (R-II peptide; Peninsula Labs) in the presence of 0.5 mmol/L CaCl2, 1.0 μmol/L calmodulin, and 1.0 μmol/L okadaic acid as described previously. 28 Activity was blocked with the addition of 500 μmol/L calcineurin autoinhibitory peptide (Calbiochem), and total activity was determined as the difference between the blocked and unblocked states.

Quantitative mRNA Analysis
Determination of mRNA levels of a subset of hypertrophic markers was performed as described previously in detail, including the sequence of the rat-specific oligonucleotides that were used. 29 Blots were quantified on a PhosphorImager (Molecular Dynamics). The signal intensity of each RNA dot was normalized to the corresponding GAPDH signal to account for equal loading.

Statistics
All data are presented as mean±SEM and were analyzed by a 1-way ANOVA between the indicated groups by the use of a Bonferroni multiple comparison test when appropriate or an unpaired Student’s t test, and significance was assigned a value of P < 0.05. Tests were performed with the Instat software package (Graphpad).

CsA at 2 different doses significantly attenuated calcineurin activation and load-induced hypertrophy after 14 days. CsA administration also partially reversed load-induced hypertrophy once established.
ratios. For examination of hypertrophy reversal, 14-day banded rats were treated with CsA for an additional 14 days. Weight loss did not affect heart–to–body weight ratios calculated for each group (Figure 2).

Table. To control for weight loss, we restricted the diets of either sham rats or aortic-banded rats to 16.7% of the weight-adjusted daily caloric requirements (very low caloric diet, VLCD).25 Sham rats on a caloric-restricted diet lost 28% of their body weight yet maintained a nearly identical heart–to–body weight ratio as ad libitum feed shams (Table). More importantly, caloric-restricted, aortic-banded rats had identical increases in heart–to–body weight ratios compared with CsA (Sandimmune) for 1 day before abdominal aortic banding and maintained on CsA for 14 additional days (Table). Control groups included aortic-banded rats without CsA, sham rats, and sham rats treated with CsA. Heart–to–body weight ratios were calculated for each group (Figure 2). The data demonstrate that aortic banding induced a significant increase in heart–to–body weight ratio after 14 days (34%) (P<0.05), which was prevented in a dose-dependent manner by CsA treatment (P<0.05 for each group). At the highest dosage of CsA, a large attenuation was observed, whereas at a ×0.4 dosage a modest attenuation was still noted (P<0.05). CsA treatment did not influence the heart–to–body weight ratios of sham animals, nor did it substantially increase the mortality rates of either the sham or banded groups.

Figure 2. CsA significantly prevents hypertrophy in aortic-banded rats. Aortic-banded rats demonstrated 34% increase in heart–to–body weight ratio after 14 days. CsA treatment at 10 mg/kg or 4 mg/kg twice daily attenuated this increase in heart–to–body weight ratio. Also shown are sham and banded rats subjected to VLCD to induce significant weight loss to control for weight loss in CsA-banded group. VLCD banded rats maintained hypertrophic heart–to–body weight ratio. #P<0.05 compared with sham group. §P<0.05 compared with with CsA (Sandimmune) for 1 day before abdominal aortic banding and maintained on CsA for 14 additional days (Table). Control groups included aortic-banded rats without CsA, sham rats, and sham rats treated with CsA. Heart–to–body weight ratios were calculated for each group (Figure 2). The data demonstrate that aortic banding induced a significant increase in heart–to–body weight ratio after 14 days (34%) (P<0.05), which was prevented in a dose-dependent manner by CsA treatment (P<0.05 for each group). At the highest dosage of CsA, a large attenuation was observed, whereas at a ×0.4 dosage a modest attenuation was still noted (P<0.05). CsA treatment did not influence the heart–to–body weight ratios of sham animals, nor did it substantially increase the mortality rates of either the sham or banded groups.

Aortic-banded animals treated with CsA lost a small percentage of their body weight during the treatment protocol (Table). To control for weight loss, we restricted the diets of either sham rats or aortic-banded rats to 16.7% of the weight-adjusted daily caloric requirements (very low caloric diet, VLCD).25 Sham rats on a caloric-restricted diet lost 28% of their body weight yet maintained a nearly identical heart–to–body weight ratio as ad libitum feed shams (Table). More importantly, caloric-restricted, aortic-banded rats had identical increases in heart–to–body weight ratios compared

CsA Prevents Pressure-Overload Hypertrophy in Aortic-Banded Rats

<table>
<thead>
<tr>
<th></th>
<th>Sham, 0 d (n=6)</th>
<th>Sham CsA, 0 d (n=6)</th>
<th>Banded, 14 d (n=10)</th>
<th>Banded CsA, 14 d (n=14)</th>
<th>Banded ×0.4 CsA, 14 d (n=11)</th>
<th>Sham (VLCD), 14 d (n=7)</th>
<th>Banded (VLCD), 14 d (n=10)</th>
<th>Banded, 28 d (n=10)</th>
<th>Banded CsA, 14–28 d (reversal), (n=12)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart wt, g</td>
<td>0.850±0.019</td>
<td>0.770±0.031</td>
<td>1.025±0.015</td>
<td>0.725±0.025</td>
<td>0.966±0.024</td>
<td>0.551±0.019</td>
<td>0.583±0.027</td>
<td>1.060±0.039</td>
<td>0.863±0.037</td>
</tr>
<tr>
<td>Pretreatment body wt, g</td>
<td>228±3</td>
<td>213±3</td>
<td>216±7</td>
<td>223±6</td>
<td>210±6</td>
<td>203±3</td>
<td>236±13</td>
<td>229±8</td>
<td></td>
</tr>
<tr>
<td>Posttreatment body wt, g</td>
<td>246±4</td>
<td>222±3</td>
<td>225±5</td>
<td>200±6</td>
<td>228±10</td>
<td>151±5</td>
<td>128±2</td>
<td>248±8</td>
<td>229±10</td>
</tr>
<tr>
<td>Heart/body wt, g/mg</td>
<td>3.41±0.10</td>
<td>3.46±0.12</td>
<td>4.56±0.09*</td>
<td>3.61±0.14†</td>
<td>4.23±0.12†</td>
<td>3.64±0.06</td>
<td>4.58±0.22*</td>
<td>4.28±0.17†</td>
<td>3.79±0.08††</td>
</tr>
<tr>
<td>CsA-blood, ng/mL</td>
<td>...</td>
<td>1956±409</td>
<td>...</td>
<td>3104±948</td>
<td>842±477</td>
<td>...</td>
<td>...</td>
<td>...</td>
<td>...</td>
</tr>
</tbody>
</table>

Rats were pretreated with CsA 1 day before abdominal aortic banding and continued on drug or vehicle for 14 additional days. CsA was administered twice daily at a dosage of 10 mg/kg (20 mg/kg per day) or 4 mg/kg (8 mg/kg per day) in the ×0.4 CsA treatment group. After 14 days, the rats were killed and weighed; hearts were removed and weighed and dissected for molecular analysis. Because the banded CsA-treated animals lost weight, we included a study of aortic-banded rats maintained on a VLCD for 14 days, resulting in a significant loss of weight in both the sham and banded groups. Weight loss did not affect heart–to–body weight ratios. For examination of hypertrophy reversal, 14-day banded rats were treated with CsA for an additional 14 days.

Values shown are mean±SEM.

*P<0.05 compared with sham group, †P<0.05 compared with 14-day banded only group, ‡P<0.05 compared with 28-day banded only group.
with ad libitum feed, banded animals despite a significant weight loss (Figure 2).

Calcineurin-specific Western blots were performed on protein extracts derived from nonbanded hearts, 14-day banded hearts, or 14-day banded hearts treated with CsA. The data show that CsA administration effectively prevented the increase in total calcineurin protein content, whereas calmodulin levels did not vary (Figure 3). It is likely that the total increase in calcineurin protein content by day 14 is part of a secondary mechanism that is associated with hypertrophy itself, so that CsA treatment that attenuates the hypertrophy response also blocks this increase in calcineurin protein content.

Effects of Calcineurin Inhibition on Reversal of Pressure-Overload Hypertrophy

To determine whether calcineurin inhibition could reverse pressure-overload hypertrophy, rats were banded for 14 days and treated with CsA at 10 mg/kg twice daily for an additional 14 days (Table). An additional control group included untreated rats that were subject to aortic banding for 28 days. Analysis of heart-to-body-weight ratios demonstrated a partial reversal of hypertrophy in the CsA-treated rats compared with either the 14- or 28-day banded, untreated controls (P<0.05) (Figure 4A and Table). These data indicate that CsA significantly but only partially reverses pressure-overload hypertrophy caused by abdominal aortic banding.

We extended this analysis to include a longitudinal assessment of left ventricular wall thickness by echocardiography within the same animals before and after CsA treatment. Fourteen-day aortic-banded rats had an average left ventricular wall thickness of 0.262±0.0099 cm, whereas these same animals after an additional 14 days of CsA treatment had an average wall thickness of 0.229±0.0088 (P<0.05) (Figure 4B). These data demonstrate a longitudinal reversal of hypertrophy within the same animals after 14 days of CsA treatment.

Histological and Molecular Analysis of CsA-Treated Rats

We performed histological analysis of left ventricular cardiac tissue to examine myocyte fiber sizes, organization, and fibrosis. Hematoxylin and eosin–stained sections on gross inspection appeared normal between sham, banded, and banded with CsA groups, although the banded group appeared to have larger fibers (Figure 5, A through C). Trichrome staining of sections between the 3 treatment groups did not reveal a qualitative difference in fibrosis after

Discussion

We previously reported that calcineurin-inhibitory drugs (CsA and FK506) could prevent hypertrophy/ cardiomyopathy in 3 genetic models of heart disease and in aortic-banded rats over a period of 6 days. More recently, 4 studies have reported that CsA and FK506 are ineffective in preventing pressure-overload hypertrophy in aortic-banded rodents. Analysis of the details between our study and the 4 negative studies suggests a number of technical differences that may account for the disparate results. The studies by Müller et al and Ding et al were performed in aortic-banded mice at the
level of the transverse aorta and ascending aorta, respectively. However, in a very similar study that used transverse aortic–
banded mice, a statistically significant attenuation of hyper-
rophy was observed with CsA.23 The reasons for these
differing accounts in the mouse are uncertain.

In abdominal aortic–banded rats, 2 separate studies did not
report a significant attenuation of pressure-overload hyper-
trophy with CsA or FK506.19,21 Although these studies failed
to identify attenuation with CsA, careful inspection of the
data suggests a trend. Luo et al19 reported a 42% increase in
heart–to–body weight ratio in the banded group and only a
27% increase in the banded, CsA-treated group (highest
dose). Zhang et al21 reported a 47% increase in left ventricle–
to–body weight ratio in the banded group but only a 21% and
27% increase in banded animals at 2 different dosages of CsA
(4-week study), yet these differences were not interpreted as
significant, given the manner in which the data were normal-
ized to blood pressure gradient between the groups.

A potential criticism of our data is that CsA has a
nonspecific effect that diminishes the “health” of the heart or
animal. However, sham animals treated with CsA did not
have a decrease in heart weight, nor did CsA prevent

Figure 5. Histological analysis of hearts from banded and CsA-treated animals. A through C, Hematoxylin and eosin histological stain-
ing (×200) reveals similar morphology between sham, banded, and banded with CsA groups (n=4, each). D through F, Trichrome
staining (×200) reveals comparable staining for fibrosis after 14 days in each group (n=4 each). G through I, Myocyte membranes were
stained with wheat germ agglutinin (WGA)-TRITC conjugate at 50 μg/mL to accurately identify myofiber surface area. J, NIH software
image analysis of WGA-TRITC-stained confocal images (×400) demonstrates increased myofiber surface area in banded hearts com-
pared with sham hearts (P<0.05), whereas CsA blocked this increase (P<0.05) (n=50 to 80 outlines from 4 separate hearts in each of
the 3 groups).
hypertrophy in RAR transgenic mice\textsuperscript{18} or NF-AT3 transgenic mice (data not shown). Another potential variable relates to the observation that banded CsA groups either lost body weight or failed to gain weight over 14 days of treatment (Table). However, the VLDL control group demonstrated the same degree of relative hypertrophy despite a $>30\%$ loss in body weight (Table).

Blood CsA levels (3104 ng/mL) in the banded group were 6 to 10 times higher than is achieved clinically in humans.\textsuperscript{29} However, it is difficult to interpret the relevance of this dosage between rat and humans, given the known differences in physiology and drug metabolism. Indeed, effective immunosuppression in the mouse was reported to require substantially higher CsA blood levels than is required in humans.\textsuperscript{30} Alternatively, calcineurin protein content is thought to be higher in the heart compared with T cells, necessitating higher doses to achieve inhibition.\textsuperscript{31}

This report also raises the question as to other potential mechanisms (calcineurin independent) whereby CsA might affect cardiac hypertrophy. We previously demonstrated that both CsA and FK506 were capable of preventing cardiac myopathy in tropomodulin transgenic mice.\textsuperscript{18} The significant biological effects in common to both CsA and FK506, which each bind different immunophilin proteins, have been attributed to their ability to inhibit calcineurin.\textsuperscript{32} These data suggest that the observed attenuation and partial reversal of cardiac hypertrophy by CsA is primarily due to calcineurin inhibition, although we cannot formally rule out other mechanisms of action.

The calcineurin-inhibitory drugs CsA and FK506 are currently prescribed to prevent allograft tissue rejection after organ transplantation. Anecdotal reports have suggested that calcineurin inhibitors have a negative effect on the heart. Long-term CsA therapy is reported to cause LVH in heart transplantation recipients.\textsuperscript{32} In addition, 2 separate case report studies found hypertrophic cardiomyopathy in pediatric liver transplantation recipients receiving FK506.\textsuperscript{33,34}

In contrast to these case reports, our analysis of intrinsic and extrinsic animal models of cardiac hypertrophy would suggest that calcineurin inhibitors antagonize cardiac hypertrophy. The reason for these disparate results either reflects a temporally regulated phenomenon or is related to the higher doses required to inhibit cardiac calcineurin activity. The clinical reports represent long-term effects associated with CsA and FK506, whereas our animal studies were performed in the short term and at high doses. The side effects associated with CsA and FK506 probably exclude these drugs as long-term therapeutics for human heart disease, but it also suggests new avenues for drug discovery with cardiac specificity.

Acknowledgments
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