Cyclosporine Attenuates Pressure-Overload Hypertrophy in Mice While Enhancing Susceptibility to Decompensation and Heart Failure

Tomomi Meguro, Chull Hong, Kuniya Asai, Gen Takagi, Timothy A. McKinsey, Eric N. Olson, Stephen F. Vatner

Abstract—Left ventricular hypertrophy (LVH) is a compensatory mechanism to cope with pressure overload. Recently, a calcineurin pathway mediating LVH and its prevention by cyclosporine was reported. We examined whether calcineurin mediates LVH due to pressure overload in mice. Pressure overload was induced by aortic banding in 53 mice (32 treated with cyclosporine [25 mg · kg⁻¹ · d⁻¹], 21 treated with vehicle). There were 17 sham-operated mice (9 treated with vehicle, 8 treated with cyclosporine). At 3 weeks after surgery, LV weight to body weight was greater in the nontreatment banded group (4.39±0.16 mg/g) than in the cyclosporine-treated banded group (3.95±0.14 mg/g, P<0.05), with both groups being greater compared with the entire group of sham-operated mice (3.02±0.04 mg/g). The pressure gradient between the ascending and abdominal aorta was not different between the cyclosporine-treated (49.6±6.1 mm Hg) and nontreatment groups (48.7±4.6 mm Hg). Although LV systolic pressure was lower in the cyclosporine-treated banded animals, LV systolic wall stress was similar in the nontreatment banded group and in the cyclosporine-treated group. However, LV dP/dt was lower (P=0.05) in the cyclosporine-treated banded group (4774±656 mm Hg/s) than in the nontreatment banded group (6604±516 mm Hg/s). During the protocol, 23 of 32 mice in the cyclosporine-treated group and 9 of 21 mice in the nontreatment group died. All deaths occurred within 10 days after surgery. Deaths caused by heart failure were 7.2-fold higher (P<0.05) in the cyclosporine-treated group, whereas deaths due to other causes were not different between the 2 groups. In addition, LV function of mice was assessed at 48 hours after banding; LV ejection fraction measured with echocardiography was lower (P<0.05) in the cyclosporine-treated banded group (66±3.0%) than in the nontreatment banded group (79±1.5%), whereas LV systolic wall stresses were similar. Calcineurin phosphatase activity was depressed similarly in both cyclosporine-treated groups compared with both nontreatment groups. Thus, cyclosporine could attenuate, but not prevent, LVH at the expense of inhibiting an important compensatory mechanism in response to pressure overload, resulting in reduced LV wall stress and function and increased susceptibility to decompensation and heart failure. (Circ Res. 1999;84:735-740.)

Key Words: calcineurin ■ Ca²⁺ ■ left ventricular hypertrophy ■ aortic banding

Left ventricular hypertrophy (LVH) is an important compensatory mechanism in response to volume or pressure overload.¹² Many factors such as stretch,³ angiotensin II,⁴ endothelin,⁵,⁶ sympathomimetic stimulation,⁷,⁸ and growth factors⁹ are mechanisms that mediate LVH. These factors also increase the concentration of [Ca²⁺]. Calcineurin, a Ca²⁺-regulated phosphatase, is activated by high concentrations of [Ca²⁺], and activates the genes for hypertrophy by dephosphorylating NF-AT.¹⁰–¹² Recently, Molkentin et al¹⁰ reported that cyclosporine blocks this pathway in vitro and prevents hypertrophy in transgenic mice, in which the calcineurin pathway is enhanced. It is not clear whether this pathway is universal, ie, whether it mediates pressure-overload LVH as well as hypertrophy in transgenic models. An equally important question is whether LVH is salutary or deleterious, ie, if LVH is a beneficial compensatory mechanism, then blocking this mechanism may be deleterious and lead to decompensation and development of heart failure.

Accordingly, the first goal of the present study was to determine whether cyclosporine prevents LVH due to aortic banding–induced pressure overload in mice. The second goal was to determine whether the incidence of decompensation to heart failure increased, pari passu, with the inhibitory action on the development of LVH. The third goal was to assess LV function to determine whether this was impaired by cyclosporine in the mice with aortic banding.
Materials and Methods

CD-1 mice (12-week-old male, 33 to 38 g; Charles River, Wilmington, Mass) were used for this study and were maintained in accordance with Guide for the Care and Use of Laboratory Animals (NIH 85-23, revised 1985). Animals were individually housed in microisolator cages in a specific pathogen-free room and exposed to a cycle of 12 hours light/12 hours dark. An acclimation period of at least 1 week was provided before initiating the experimental protocol. The mice were anesthetized with a mixture of ketamine (0.065 mg/g), xylazine (0.013 mg/g), and acepromazine (0.002 mg/g). The chest and neck were shaved, and mice were placed in a supine position, and a midline cervical incision was made to intubate the trachea. The adapter was connected to a rodent ventilator (Harvard Apparatus). Mice were ventilated with a tidal volume of 0.2 mL and a respiratory rate of 110 breaths per minute. The chest was opened at the second intercostal space. Aortic constriction was performed by ligating the transverse thoracic aorta between the innominate artery and left common carotid artery with a 27-gauge needle using a 7-0 nylon suture with the aid of a dissecting microscope. The chest was closed, and the pneumothorax was reduced. The remaining animals underwent thoracotomy without constricting the aorta.

The animals were divided into 6 groups at autopsy: group 1, sham-operated mice with cyclosporine treatment (n=8); group 2, sham-operated mice treated with vehicle (n=9); group 3, banded mice with cyclosporine treatment (n=32); and group 4, banded mice treated with vehicle (n=21). Cyclosporine (25 mg·kg⁻¹·d⁻¹, subcutaneously) or vehicle was initiated 2 days before banding and continued for 22 days. Blood was sampled from the inferior vena cava for measurement of cyclosporine concentration and renal function (blood urea nitrogen [BUN], creatinine, and potassium levels). Two groups of mice were studied for 2 days after banding: group 5, with cyclosporine (n=14) and group 6, without cyclosporine (n=17). Calcineurin phosphatase activity was measured in 11 mice, which were banded, but not treated, 9 mice, which were banded and treated, 6 sham nontreated, and 7 sham-treated mice. Echocardiography was performed at 2 days or 3 weeks after banding using methods previously used in our laboratory. Briefly, mice were anesthetized with a mixture of ketamine (0.065 mg/g), xylazine (0.013 mg/g), and acepromazine (0.002 mg/g) injected intraperitoneally. After the chest was shaved, the mice were positioned prone on a warmed saline pad for support. ECG leads were attached to each limb using needle electrodes (Grass Instruments). Echocardiography was performed using an Interspec Apogee X-200 ultrasonograph (Interspec ATL). A dynamically focused 9-MHz annular array transducer was applied from below, using the saline bag as a standoff. The heart was scanned using M-mode guided by a short-axis view of the 2-dimensional mode. Frozen frames and ECG were printed on a Sony color printer (UP-5200, Sony Corp). The images were scanned into a Power Macintosh 7200 and digitized at 300 pixels per inch. Gray-scale equalization was made using the Adobe Photoshop program (Adobe Systems Corp), and the images were imported into the NIH Image program (National Institutes of Health) for measurement. LV diameters, anterior wall thickness, and posterior wall thickness were measured using leading edge-to-leading edge convention, and LV ejection fraction was calculated. Stroke volume was calculated as (LV end-diastolic diameter)²−(LV end-systolic diameter)². Cardiac output was calculated as the product of stroke volume and heart rate. Total peripheral resistance was calculated as the quotient of mean abdominal aortic pressure and cardiac output. LV systolic wall stress was calculated as follows: LV systolic wall stress=1.36×(aortic systolic pressure×LV end-systolic diameter)/(2×systolic wall thickness).

To measure arterial pressure, 2 high-fidelity catheter tip transducers (1.4F, Millar) were used; one was inserted into the right carotid artery and the other into the left femoral artery and carefully advanced to the ascending aorta and abdominal aorta, respectively, at either 2 days or 3 weeks after banding under the same anesthesia as described above. The pressures in the ascending aorta and abdominal aorta were measured simultaneously. The pressure gradients between the systolic pressure in the ascending and abdominal aorta were calculated. All pressure signals were recorded on a multichannel tape recorder (PC200Ax, Sony Corp) and played back on a multichannel oscillograph (Gould-Brush). The pressure in one mouse in the banded treatment group could not be measured because of death during anesthesia. After measurement of aortic pressure, the catheter in the ascending aorta was advanced to the left ventricle for measurement of LV pressure and LV dP/dt.

In subgroups of mice, arterial pressure and heart rate were measured in the conscious state after recovery from insertion of an arterial catheter. Animals surviving the 22 days of treatment were anesthetized deeply, and the heart, lungs, liver, and kidney were removed and their weights were measured. Organ weights were normalized to body weight. In animals that died spontaneously, organs were also removed at autopsy. In these animals, the deaths were attributed to rupture of the thoracic aorta, if this was observed. The deaths were ascribed to heart failure, if pleural effusion had occurred, and then increased lung weight to body weight was confirmed. Deaths were attributed to unknown causes, if neither of the above was observed.

Calcineurin phosphatase activity in heart extracts was determined as described previously with slight modifications. LVs were excised from animals and immediately frozen in liquid nitrogen.

### TABLE 1. Hemodynamics at 2 Days in Banded Mice

<table>
<thead>
<tr>
<th></th>
<th>Nontreatment</th>
<th>Cyclosporine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Conscious</td>
<td>n=6</td>
<td>n=7</td>
</tr>
<tr>
<td>Systolic ascending aortic pressure, mm Hg</td>
<td>155±7.1</td>
<td>154±6.5</td>
</tr>
<tr>
<td>Mean aortic pressure, mm Hg</td>
<td>120±5.3</td>
<td>118±5.5</td>
</tr>
<tr>
<td>Heart rate, bpm</td>
<td>565±44</td>
<td>527±43</td>
</tr>
<tr>
<td>Anesthetized</td>
<td>n=11</td>
<td>n=11</td>
</tr>
<tr>
<td>Systolic ascending aortic pressure, mm Hg</td>
<td>159±4.7</td>
<td>142±3.5*</td>
</tr>
<tr>
<td>Mean aortic pressure, mm Hg</td>
<td>102±3.2</td>
<td>94±2.3</td>
</tr>
<tr>
<td>Heart rate, bpm</td>
<td>247±12</td>
<td>261±15</td>
</tr>
<tr>
<td>LV systolic wall stress, g/cm²</td>
<td>244.4±10.8</td>
<td>246.4±15</td>
</tr>
<tr>
<td>LV/BW, mg/g</td>
<td>3.53±0.1</td>
<td>3.31±0.1</td>
</tr>
</tbody>
</table>

*P<0.05, cyclosporine-treated group vs nontreatment banded group.
Frozen LVs were pulverized using a mortar and pestle and transferred to a buffer solution containing 0.1 mol/L MOPS (pH 7.0), 2 mmol/L EDTA, 1 mmol/L PMSF, and a protease inhibitor cocktail (Complete, Boehringer-Mannheim). The resuspended tissue was subjected to 15 strokes with a polypropylene pestle, and cell debris was pelleted by centrifugation at 4°C for 10 minutes at 12,000g. The protein concentration in the clarified supernatant was measured using BioRad protein assay reagent. Calcineurin activity in LV extracts was determined by measuring the rate of dephosphorylation of a 32P-labeled R-II peptide (Biomol) in the presence of 20 μg of heart protein. Reaction mixtures contained 675 pmol of radiolabeled R-II substrate, 20 mmol/L Tri-S (pH 8.0), 100 mmol/L KCl, 6 mmol/L MgCl₂, 100 μmol/L CaCl₂, 500 μmol/L DTT, 100 mmol/L calmodulin (Calbiochem), and 500 mmol/L okadaic acid (Calbiochem) to inhibit protein phosphatases 1 and 2A. After a 20-minute incubation at 30°C, free 32P was separated from the R-II substrate using Dowex AG 50W-X7 cation exchange resin (BioRad) and quantitated by scintillation counting. To distinguish calcineurin activity from background phosphatase activity, reactions were conducted in the absence or presence of 20 nmol/L each of cyclosporin A (Sandoz, Novartis) and recombinant human cyclophilin (Sigma), which forms a complex that specifically binds to and inhibits calcineurin.

Results

Hemodynamics

At 3 weeks after banding, the pressure gradient between the ascending aorta and abdominal aorta was not different in the nontreatment banded group (48.7±4.6 mm Hg) versus the cyclosporine-treated banded group (49.6±6.1 mm Hg), whereas systolic ascending aortic pressure in the cyclosporine-treated banded group (129±5.4 mm Hg) was lower (P<0.05) than in the nontreatment banded group (149±5.4 mm Hg). However, LV systolic wall stresses were similar in the 2 groups (Figure 1). LV dP/dt was lower (P=0.05) in the cyclosporine-treated banded group (4774±656 mm Hg/s) than in the nontreatment banded group (6604±516 mm Hg/s).

At 48 hours after banding, values for arterial pressure were similar to those observed 3 weeks after banding in anesthetized mice (Table 1). However, arterial pressure measurements were no longer different when measured in the conscious state (Table 1). At this time, calculated total peripheral resistance was similar in the 2 groups (6.2±0.4 versus 6.8±0.6 mm Hg·mL⁻¹·min⁻¹), indicating that cyclosporine did not induce peripheral vasodilation. Rather, cyclosporine reduced arterial pressure by impairing LV function in the cyclosporine-treated banded group. LV ejection fraction was depressed, but total peripheral resistance and the pressure gradients were not different in the 2 groups.

Morphology

Three weeks after surgery, LV weight to body weight (LV/BW) ratio increased by 44% in the nontreatment banded group. In the cyclosporine-treated banded group, the LV/BW ratio rose by 32%, which was significantly lower (P<0.05) than in the nontreatment banded group (78.5±1.5%), whereas pressure gradients were similar in the 2 groups (Figure 2). LV systolic wall stresses were similar in the 2 banded groups at 2 days after banding (Figure 1).

<table>
<thead>
<tr>
<th>TABLE 2. Hemodynamics at 3 Weeks</th>
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<tr>
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<tr>
<td></td>
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<tr>
<td>Systolic ascending aortic pressure, mm Hg</td>
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<tr>
<td>Mean ascending aortic pressure, mm Hg</td>
</tr>
<tr>
<td>Systolic abdominal aortic pressure, mm Hg</td>
</tr>
<tr>
<td>Mean abdominal aortic pressure, mm Hg</td>
</tr>
<tr>
<td>Pressure gradient, mm Hg</td>
</tr>
<tr>
<td>Heart rate, bpm</td>
</tr>
<tr>
<td>Body weight, g</td>
</tr>
<tr>
<td>LV/BW, mg/g</td>
</tr>
</tbody>
</table>

*P<0.05, cyclosporine-treated group vs nontreatment banded group.
systolic wall stress, less LVH developed in the cyclosporine-treated group. The RV/BW ratio was not different among the 4 groups.

The extent of LVH was also compared in the 2 groups with matched systolic aortic pressures, by eliminating all animals with systolic aortic pressure >150 mm Hg. Under these conditions, by design, systolic aortic pressure was similar in the nontreatment banded (136±7 mm Hg, n=6) and cyclosporine-treated banded groups (129±5 mm Hg, n=8), but the LV/BW ratio was still higher (P<0.05) in the nontreatment banded animals (4.32±0.19 mg/g) versus the cyclosporine-treated banded animals (3.83±0.09 mg/g).

Blood Chemistries

The serum concentration of circulating cyclosporine in treated mice was >1000 ng/mL (Table 3). The serum creatinine and BUN levels were not different among the 4 groups, but the potassium level was elevated in the cyclosporine-treated group (Table 3).

Premature Deaths

Nine mice of 21 in the nontreatment banded group and 23 mice of 32 in the cyclosporine-treated banded group died prematurely. All deaths were observed within 10 days after banding (Figure 4). We classified the causes of death as congestive heart failure (CHF; 1 nontreatment, 11 cyclosporine-treated), rupture of aorta (5 nontreatment, 9 cyclosporine-treated), or unknown cause (3 nontreatment, 3 cyclosporine-treated). Pleural effusion was observed in 12 mice, which were considered to have died from heart failure. Eleven of these mice were in the cyclosporine-treated group. The lung weight/BW ratio of the group that died from CHF was higher (10.4±0.7 mg/g, P<0.05) than those dying from aortic rupture (7.1±0.3 mg/g) or unknown causes (6.9±0.5 mg/g). The liver weight/BW ratio was also elevated in the CHF group (65.1±4.4 mg/g) versus the other animals that died prematurely (54.6±2.2, P<0.05). The risk of death by CHF was 7.2-fold higher (P<0.05) in the cyclosporine-treated group than in the nontreatment group (Figure 4). Interestingly, even in the animals that died prematurely within the first 10 days, the LV/BW ratio of the mice that died was higher in the nontreatment banded group (3.79±0.14 mg/g) compared with the cyclosporine-treated banded group (3.38±0.08 mg/g, P<0.05).

Calcineurin Activity (Figure 5): Calcineurin phosphatase activity was similar in the nontreatment aortic-banded and sham groups (25.0±2.1 versus 25.2±3.6 pmol·min⁻¹·mg⁻¹). Calcineurin phosphatase activity was significantly (P<0.05) depressed in both aortic-banded treated and sham-treated groups (6.8±1.8 versus 6.7±3.6 pmol·min⁻¹·mg⁻¹).

**Discussion**

LVH is induced by several mechanical and hormonal mechanisms, involving several signal transduction pathways. Recently, Molkentin et al demonstrated that hypertrophy emanating from signals induced by phenyleph-

### TABLE 3. Blood Chemistries

<table>
<thead>
<tr>
<th></th>
<th>Sham</th>
<th>Banded</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Nontreatment (n=9)</td>
<td>Cyclosporine (n=8)</td>
</tr>
<tr>
<td>BUN, mg/dL</td>
<td>21.2±1.3</td>
<td>28.2±1.5</td>
</tr>
<tr>
<td>Creatinine, mg/dL</td>
<td>0.13±0.02</td>
<td>0.17±0.02</td>
</tr>
<tr>
<td>Potassium, mEq/mL</td>
<td>4.3±0.3</td>
<td>4.1±0.2</td>
</tr>
<tr>
<td>Cyclosporine, ng/mL</td>
<td>26.2±1.0</td>
<td>1875±425†</td>
</tr>
</tbody>
</table>

*P<0.05 vs difference between the cyclosporine-treated group and nontreatment banded group.
†P<0.05 vs difference between the cyclosporine-treated group and nontreatment sham group.
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Cyclosporine-treated group could not sustain the same pressure overload–induced LVH in the treated animals. This further supports the notion that calcineurin is a keystone in the signaling pathways of pressure overload hypertrophy. These findings are consistent with previous reports that calcineurin mediates pressure-overload hypertrophy, implying that calcineurin activity is increased in response to increased LV systolic wall stress. Moreover, the regression relationship between LV/BW ratio and LV systolic wall stress was also significantly decreased in the cyclosporine-treated group. Although the pressure gradient between the ascending aorta and abdominal aorta was not different between the cyclosporine-treated banded group and nontreatment banded group, peak systolic aortic pressures were lower in the treated group. However, even when systolic aortic pressures were matched, there was less hypertrophy in the cyclosporine-treated animals. Furthermore, the levels of LV systolic wall stress were similar in nontreatment and cyclosporine-treated banded animals. This suggests the intriguing possibility that the hearts in the cyclosporine-treated group could not sustain the same pressures because of inadequate degree of compensatory LVH, and, consequently, the animals died prematurely from decompensation and CHF. An alternative explanation is that the stimulus for LVH was reduced in the treated group, because systolic aortic pressure was lower in the treated, banded group under anesthesia. However, as noted above, neither LV systolic wall stress nor the pressure gradient was lower in the treated group treated with cyclosporine. Moreover, at 2 days after banding, systolic arterial pressure was not depressed in the treated, banded group in the conscious state.

Nonetheless, to address this possibility, we found that the decrease in peak aortic systolic pressure in the present study was not due to a peripheral vasodilating action of cyclosporine, because calculated total peripheral resistance was similar in the 2 groups. Rather, the decrease in aortic pressure appears to be due to impaired LV function. As noted above, systolic arterial pressure was not reduced in the awake state 2 days after banding in the cyclosporine-treated banded group. These data taken together suggest that the combination of impaired LV function (as assessed by LV ejection fraction and LV dP/dt) and anesthesia, which also impairs cardiac function, caused the reduction in systolic arterial pressure. The LV dysfunction could be due in part to subendocardial hypoperfusion, which is known to occur in LVH. However, without a direct measurement of myocardial blood flow, it is difficult to calculate whether depressed subendocardial perfusion contributes to the mechanism of impaired LV function in the cyclosporine-treated banded mice.

The results from 3 recent preliminary studies on the effects of cyclosporine to inhibit the development of LVH after pressure overload are conflicting: Sussman et al observed a marked positive effect with aortic banding in rats, whereas negative results were noted by others in rats and mice. Interestingly, none of these studies found that heart failure ensued with aortic banding after cyclosporine treatment, although the mortality was unexpectedly high in the cyclosporine-treated group in one of these studies in rats, consistent with what was observed in the present investigation.

There has been a controversy for considerable time whether LVH is salutary or deleterious. The results of the present study should help reconcile that controversy. There was a significant increase in premature mortality due to CHF in the cyclosporine-treated banded mice. Our interpretation of these data is that cyclosporine attenuated the compensatory LVH, which in turn was deleterious, because it blocked a pathway that can compensate and protect the heart against the elevated afterload. In the absence of this protective compensatory action, LV function was impaired, and cardiac failure and death ensued. An alternative interpretation is that those animals that died from CHF would have developed more severe LVH than the animals that survived. If so, there would have been no difference in the LVH observed 3 weeks later between the 2 groups. In that scenario, it is conceivable that cyclosporine exerts no effect in reducing LVH, given that animals destined to exhibit severe LVH die prematurely because of decompression and cardiac failure. However, as noted above, the LV/BW ratio was still higher in nontreated animals that died prematurely compared with cyclosporine-

**Figure 5.** Calcineurin phosphatase activity was similar in the nontreatment aortic-banded and sham groups (25.0±2.1 vs 25.2±3.6 pmol · min⁻¹ · mg⁻¹). Calcineurin phosphatase activity was depressed similarly in both the aortic-banded group treated with cyclosporine (CSA) and the nonbanded group (6.8±5.3 vs 6.7±3.6 pmol · min⁻¹ · mg⁻¹).
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treated banded animals, indicating that even the initial development of LVH was inhibited by cyclosporine. Therefore, the current results support the concept that hypertrophy is a beneficial compensatory mechanism, which protects the heart in the face of pressure overload. Furthermore, it is also possible to conclude that a signaling pathway, other than calcineurin, is required to mediate pressure-overload LVH in the mouse.

Acknowledgment

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References


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