Pressure Overload Induces Severe Hypertrophy in Mice Treated With Cyclosporine, an Inhibitor of Calcineurin

Bo Ding, Robert L. Price, Thomas K. Borg, Ellen O. Weinberg, Philip F. Halloran, Beverly H. Lorell

Abstract—Cardiac hypertrophy is the fundamental adaptation of the adult heart to mechanical load. Recent work has shown that inhibition of calcineurin activity with cyclosporine suppresses the development of hypertrophy in calcineurin transgenic mice and in in vitro systems of neonatal rat cardiocytes stimulated with peptide growth factors. To test the hypothesis that the calcineurin signaling pathway is critical for load-induced hypertrophy in vivo, we examined the effects of cyclosporine treatment on left ventricular hypertrophy induced by experimental ascending aortic stenosis for 4 weeks in mice. Left ventricular systolic pressure was elevated to a similar level in aortic stenosis mice that were treated with cyclosporine versus no drug. Left ventricular mass and myocyte size were similar in treated and untreated aortic stenosis animals and significantly greater than control animals, showing that cyclosporine treatment does not suppress hypertrophic growth. Both treated and untreated animals showed increased left ventricular expression of the load-sensitive gene atrial natriuretic factor. Calcineurin activity was measured in the left ventricle and the spleen from control mice and aortic stenosis mice treated with cyclosporine versus no drug. Levels of calcineurin activity were similar in the spleens of control and untreated aortic stenosis mice. However, calcineurin activity was severely depressed in left ventricular tissue of untreated aortic stenosis mice compared with control mice and was further reduced by cyclosporine treatment. Thus, pathological hypertrophy and cardiac-restricted gene expression induced by pressure overload in vivo are not suppressed by treatment with cyclosporine and do not appear to depend on the elevation of left ventricular calcineurin activity. (Circ Res. 1999;84:729-734.)

Key Words: heart hypertrophy • calcineurin • cyclosporine • atrial natriuretic factor • mice

Hypertrophic growth is crucial for adaptation of the heart to mechanical load. In the absence of the capability for substantive cell division, postnatal cardiac myocytes adapt to load by myocyte enlargement as well as the reinduction of a fetal pattern of cardiac gene expression. It is controversial whether the external stimulus of load initiates myocyte growth via multiple intracellular signaling pathways or by a critical transcriptional pathway on which other signals converge. Calcineurin is a calcium-calmodulin–dependent serine-threonine phosphatase that activates transcription factors of the NFAT family causing their translocation to the nucleus to initiate transcription of cytokines involved in the immune response. The calcineurin system is highly conserved across species and widely distributed in tissues in addition to the immune system. Molkentin et al recently reported that cardiac hypertrophy can be induced in transgenic mice that overexpress calcineurin or its intracellular transcription factor NF-AT3. Pharmacological inhibition of calcineurin with cyclosporine blocked hypertrophy in calcineurin transgenic models; in addition, cyclosporine suppressed hypertrophy and reactivation of the fetal pattern of atrial natriuretic factor gene expression in transgenic mice and in neonatal rat myocytes exposed to the growth peptides angiotensin II and phenylephrine.

These observations have led to the speculations that the elevation of calcineurin activity may regulate hypertrophy in vivo and that cyclosporine may be effective as a clinical therapy in the treatment of pathological hypertrophy and heart failure. However, in clinical heart disease, the predominant stimulus that elicits pathological hypertrophy is pressure overload due to hypertension or valvular heart disease, rather than primary perturbations in neurohormone levels. It is not known if cyclosporine is effective in suppressing hypertrophic growth in humans or normal animals in response to the stimulus of excess load. In the present study, we tested the hypothesis that cyclosporine treatment suppresses left ventricular hypertrophy in mice with experimental pressure overload caused by ascending aortic stenosis.
Materials and Methods

Animal Model of Pressure Overload

Ascending aortic constriction was performed in male FVB/n mice (Charles River, Stoneridge, NY; weight 12 to 15 g). Animals were anesthetized with intraperitoneal ketamine 50 mg/kg and xylazine 2.5 mg/kg, and aortic constriction was created via a left thoracotomy by placing a ligature securely around the ascending aorta and a 26-gauge needle and then removing the needle. Animals with ascending aortic stenosis were then randomized to treatment with cyclosporine (Sandoz, NJ; 25 mg/kg body weight injected subcutaneously twice daily) or no drug for 4 weeks, and age-matched animals served as controls (n=4 to 5 per group). The daily dose of cyclosporine was identical to that reported by Molkentin et al. which was sufficient to block left ventricular hypertrophy in calcineurin transgenic mice. Before euthanasia, in vivo left ventricular hemodynamics were recorded by left ventricular catheterization via direct left ventricular puncture as previously described. The presence of the ascending aortic constriction precludes left ventricular catheterization by a carotid approach in this model. Animal care was in accordance with institutional guidelines.

Assessment of Left Ventricular Hypertrophy

To compare myocyte size, hearts were removed and rinsed for 1 minute in 0.1 mol/L PBS with 50 mmol/L KCl (pH 7.2) and subsequently fixed overnight at 4°C in 4% paraformaldehyde prepared in PBS. Vibratome sections (100 μm) from similar areas of the left ventricles were stained in a 1:20 dilution of rhodamine phalloidin (Molecular Probes) and imaged with a BioRad MRC1000 confocal scanning laser microscope. A minimum of 5 optical sections was prepared in PBS. Vibratome sections from the free wall of the left ventricle of each animal with a Nikon 60X NA 1.4 lens. All images used for myocyte measurements were collected with identical laser, iris, gain, and black level operating parameters. Myocyte widths were measured parallel to the direction of the sarcomeres from unbranched regions of the myocytes near an intercalated disk with the length/profile function in the BioRad MRC1000 COMOS program. Data sets were then compared using Student t tests.

Northern Blot Analyses

Total left ventricular RNA was extracted and purified with TriReagent (Sigma). Twenty micrograms of RNA from aortic stenosis mice treated with cyclosporine, aortic stenosis mice treated with no drug, and age-matched controls was subjected to Northern blot hybridization as previously described, using a 60- bp oligonucleotide probe complementary to the coding region of mouse atrial natriuretic factor (ANF) and a 1.4-kb mouse GAPDH cDNA probe (Ambion). Signals were captured by autoradiography and analyzed by ImageQuant software (Molecular Dynamics). Densitometric values of mRNA levels were quantified by comparison with levels of GAPDH.

Tissue Calcineurin Activity

Calcineurin activity was measured in left ventricular tissue from additional age-matched control aortic stenosis mice 4 weeks after banding, as well as aortic stenosis mice treated with cyclosporine 25 mg/kg injected subcutaneously twice daily. Cohorts of cyclosporine-treated aortic stenosis mice were killed 1 hour after injection and at nadir 12 hours after injection before the second daily dose (n=5 per group). Calcineurin activity was also measured in the spleen from all animals. This assay has been previously described in detail and reports the ability of calcineurin to dephosphorylate a 32P-serine-labeled amino acid substrate. Data are reported as a percentage of peptide hydrolyzed per minute per milligram of protein where 100% peptide hydrolyzed equals 900 pmol. Cyclosporine blood levels were also measured in the treated aortic stenosis mice 1 hour after dosing and at nadir 12 hours after dosing before the second daily dose (n=5 per group).

Results

Parameters of in vivo left ventricular mass and hemodynamics are provided in Table 1. There was no difference in body weight among the groups. In comparison with age-matched control animals, ascending aortic stenosis of 4 weeks’ duration resulted in severe left ventricular hypertrophy characterized by a 52% increase in left ventricular mass and 51% increase in left ventricular mass normalized to body weight (P<0.001 for both). However, left ventricular mass and the left ventricular mass normalized to body weight were similar in aortic stenosis animals treated with cyclosporine or no drug. Thus, treatment with cyclosporine failed to modify the pathological increase in left ventricular mass in vivo. To determine that the magnitude of systolic load was comparable in both aortic stenosis groups, we compared body weight twice daily and begun immediately after aortic banding. Values are mean±SEM. Statistical comparison was done by ANOVA and Student t test for the post hoc analyses of continuous variables. Statistical significance was accepted at P<0.05.

*Values of LV weight, LV/body weight ratio, and LV developed pressure differed between control animals and AS groups at a level of P<0.001. There was no difference in values between the AS group treated with no drug and the AS group treated with cyclosporine.

We also examined cardiac and myocyte morphology using confocal microscopy. Ascending aortic stenosis caused a significant increase in both left ventricular wall thickness and left ventricular myocyte width in comparison with age-matched control animals (Figure 1). Myocyte widths were significantly larger (P<0.01) in both aortic stenosis and aortic stenosis plus cyclosporine animals (19.15±0.28 and 19.5±0.32 μm, respectively) compared with control mice (13.11±0.21 μm). Thus, cyclosporine treatment failed to affect left ventricular remodeling or myocyte hypertrophy in aortic stenosis animals.

Cyclosporine treatment is sufficient to inhibit reactivation of the fetal gene program in calcineurin transgenic animals, including the upregulation of ANF. Thus, we tested whether cyclosporine treatment modifies left ventricular expression of ANF in mice with ascending aortic stenosis. As illustrated in Figure 2, ANF was upregulated in mice with ascending aortic stenosis compared with control animals and did not differ in aortic

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<th>n</th>
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<th>AS</th>
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<td>91.5±7.3*</td>
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<td>LV diastolic pressure, mm Hg</td>
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Left ventricular (LV) hemodynamics (LV systolic developed pressure and LV diastolic pressure) were measured before euthanasia in age-matched control mice and in mice with ascending aortic stenosis (AS) in the presence (AS+cyclosporine) and absence (AS) of 4-week treatment with cyclosporine, an inhibitor of calcineurin, administered by subcutaneous injection 25 mg/kg body weight twice daily and begun immediately after aortic banding. Values are mean±SEM. Statistical comparison was done by ANOVA and Student t test for the post hoc analyses of continuous variables. Statistical significance was accepted at P<0.05.
stenosis mice in the presence and absence of treatment with cyclosporine.

Calcineurin activity\textsuperscript{14–17} was measured in left ventricular tissue and the spleen from age-matched control aortic stenosis mice 4 weeks after banding, as well as aortic stenosis mice treated with cyclosporine 25 mg/kg body weight injected twice daily. Cohorts of cyclosporine-treated aortic stenosis mice were killed 1 hour after injection and at nadir 12 hours after injection before the second daily dose (n=5 per group). The extent of left ventricular hypertrophy in these groups is shown in Table 2. The cyclosporine dosing protocol was sufficient to achieve high blood cyclosporine levels of 15 198 μg/L (range 3360 to 35 280 μg/L) 1 hour after dosing and levels of 4668 μg/L (range 2850 to 6780 μg/L) at nadir 12 hours after dosing before the second daily dose. As shown in Table 2, calcineurin activity was similar in spleens from control and untreated aortic stenosis animals. However, left ventricular calcineurin activity was severely depressed in aortic stenosis mice compared with control mice. In the aortic stenosis mice, cyclosporine treatment caused a further depression in calcineurin activity. Cyclosporine therapy also caused a reduction in calcineurin activity in the spleen.

**Discussion**

The goal of the present study was to determine whether treatment with cyclosporine is effective in modifying left ventricular hypertrophy in aortic stenosis mice. We observed that cyclosporine treatment significantly reduced calcineurin activity in the spleen and left ventricle, suggesting a potential therapeutic benefit in this model of pressure-overload hypertrophy.

**Figure 1.** Confocal micrographs of single optical sections collected from similar regions of the ventricles of representative age-matched control (A and B), aortic stenosis (AS) (C and D), and AS plus cyclosporine (25 mg/kg body weight injected twice daily) (E and F) mice. In both the AS (C) and AS plus cyclosporine-treated animals (E), the left ventricular walls were much thicker and left ventricular chambers more occluded than those of control animals (A). To determine myocyte widths, vibratome sections (100 μm thick) were stained with rhodamine phalloidin, and left ventricular myocyte widths (n=60 to 120 myocytes per heart) were detected using the length/ profile function of the BioRad MRC1000 COMOS software in unbranched regions of myocytes (white arrows) near intercalated disks (black arrowheads). Myocyte widths were significantly larger (P<0.01) in both AS (D) and AS plus cyclosporine animals (F) (19.15±0.28 μm and 19.50±0.32 μm, respectively) compared with control mice (B) (13.11±0.21 μm). However, there was no difference in myocyte width between the AS and the AS plus cyclosporine animals. Scale bar for panels A, C, and E represents 1 mm. Scale bar for panels B, D, and F represents 25 μm.

**Figure 2.** Northern blot analysis of left ventricular mRNA levels of ANF in age-matched control, AS, and AS plus cyclosporine (AS+cyclosporine) mice. Densitometric levels were normalized to GAPDH. Compared with controls, pathological left ventricular expression of ANF is present in AS mice treated with cyclosporine or with no drug compared with control mice. Cyclosporine does not suppress the induction of ANF in AS mice with pressure-overload hypertrophy.
ventricular hypertrophic growth and expression of the cardiac gene ANF in mice with aortic stenosis. Several recent studies suggest a role for the calcineurin transcriptional pathway in the development of cardiac hypertrophy. Recent work has shown that the zinc finger transcriptional protein GATA4 appears to be required for activation of multiple genes that are upregulated in the heart during hypertrophy. The calcium-dependent phosphatase calcineurin dephosphorylates the intracellular transcription factors of the NFAT family, which results in NFAT translocation to the nucleus and interaction with GATA4 to initiate transcription, whereas cyclosporine inhibits its activity. Recent studies suggest that calcineurin inhibition is critical for cardiocyte hypertrophy and expression of ANF induced by phenylephrine and angiotensin II in neonatal rat myocytes maintained in vitro.

Molkentin et al recently reported that cardiac hypertrophy can be induced in transgenic mice that overexpress the calcium-dependent phosphatase calcineurin or the nuclear transcription factor NF-AT3. Pharmacological inhibition of calcineurin-mediated activation of NFAT transcription factors with cyclosporine blocked hypertrophy and suppressed ANF gene expression in calcineurin transgenic models. Sussman et al subsequently reported that administration of the calcineurin inhibitors cyclosporine and FK506 suppressed the development of hypertrophy in transgenic mice, which simulate the rare inherited hypertrophic cardiomyopathies, including models with overexpression of tropomodulin, myosin light chain-2, or β-tropomyosin, but not mice with overexpression of the retinoic acid receptor. These data indicate that cyclosporine inhibits hypertrophy in these transgenic models of cardiomyopathies but do not demonstrate that this strategy is effective in modifying pathological hypertrophy in animal models that more closely simulate the pressure overload of clinical heart disease.

There are only a few reports of the effects of cyclosporine in experimental pressure-overload hypertrophy, and the results are contradictory. Sussman et al briefly cited that cyclosporine treatment prevented the increase in hypertrophy in Sprague-Dawley rats subjected to abdominal aortic banding. However, this study was terminated at only 6 days of treatment because of “lethality” in the cyclosporine-treated banded rats, suggesting either inadvertent toxic levels of cyclosporine causing renal failure and systemic illness or excessively tight acute banding causing acute heart failure. In letters to the editor, Luo et al and Muller et al reported that calcineurin inhibitors had no effect on heart to body weight ratio in rodents subjected to short-term abdominal aortic banding. Each of these brief reports of short-term effects of calcineurin inhibition had limitations, including absence of measurements of myocyte morphology and cardiac calcineurin activity, as well as lack of hemodynamic estimates of left ventricular load.

Our studies in ascending aortic stenosis mice show that treatment with cyclosporine fails to modify severe pathological hypertrophy induced by load at either the heart or myocyte levels and does not suppress load-induced pathological expression of left ventricular ANF. Cyclosporine treatment results in partial, not complete, suppression of measured calcineurin activity and downstream events of dephosphorylation of NFAT transcription factors and nuclear DNA binding. The team of Halloran has previously shown that higher concentrations of cyclosporine are required to partially inhibit tissue calcineurin activity in vivo than in vitro, and higher blood concentrations are required to achieve similar levels of suppression of tissue calcineurin activity levels in mice than in humans. In the present study, the measured blood cyclosporine levels at both peak and nadir exceed the IC50 for inhibition of tissue calcineurin activity in mice. Our data show that the dosing protocol was sufficient to achieve ~80% reduction in measured calcineurin activity levels in the spleen.

In the present study, we made the unexpected and novel observation that left ventricular tissue levels of calcineurin activity are depressed in 4-week aortic stenosis mice. These data strikingly differ from the report of Sussman et al who...
measured cardiac tissue calcineurin activity in tropomodulin-overexpressing transgenic mice and observed a 2-fold increase compared with levels in wild-type hearts. In the present study, we observed that levels of calcineurin activity were similar in the spleens from age-matched control mice and untreated aortic stenosis mice and comparable to values previously reported in mice.15 These data indicate that left ventricular calcineurin activity is suppressed in response to pressure overload in aortic stenosis mice and that this is not secondary to systemic disease or circulating factors affecting other organs.

These observations suggest that the elevation of cardiac calcineurin activity is not required for pathological hypertrophy in response to mechanical load in vivo. Furthermore, our finding that left ventricular calcineurin activity is depressed in the aortic stenosis mouse raises the hypothesis that depression of tissue calcineurin activity is critical or permissive for the development of pathological pressure-overload hypertrophy. This report has several limitations. We do not know whether cyclosporine therapy is efficacious in preventing late decompensation and transition to overt heart failure or whether activation of calcineurin differs during early hypertrophy, which was studied in these experiments, versus late decompensation. Extensive studies will be needed to examine the mechanisms of the downregulation of calcineurin activity in early pressure overload and to determine whether this change in the calcineurin signaling pathway occurs in other species and models of hypertrophy.

Our findings provide insight into the issue of whether there are multiple redundant outside-in signaling pathways that are sufficient to transduce the stimulus of load versus a single master intracellular effector pathway.4 Excess stimulation of multiple neurohormonal signaling pathways, including angiotensin II and norepinephrine, is sufficient to induce the hypertrophic phenotype in cultured myocytes and transgenic animals with forced overexpression of their receptors or effector proteins.24–26 However, the role of these peptide growth factor pathways, which appear to depend on calcineurin activation in vitro, as critical mediators of load-induced hypertrophy is controversial. For example, stretch of cultured neonatal cardiocytes results in local release of angiotensin II and activation of the AT1 receptor, which appears to be required for the hypertrophic response in this in vitro system.27 However, our laboratory9 has shown that AT1 receptor antagonism does not suppress hypertrophic growth in animals with aortic stenosis, and Harada et al28 have demonstrated that hearts of mice with AT1 receptor knockout exhibit a similar degree of hypertrophy as wild-type mice in response to aortic constriction. Kudoh et al29 have also shown that mechanical stretch evokes hypertrophic responses in cardiocytes from AT1 knockout mice. These findings, and our observation that cyclosporine fails to suppress pressure-overload hypertrophy, suggest that activation of the calcineurin transcriptional pathway is not mandatory for load-induced hypertrophy and that mechanotransduction is mediated by multiple signaling pathways.

There has been speculation that drugs such as cyclosporine may be effective in modifying pathological hypertrophy in human heart disease.7,8 Our observations and prior studies in patients with pressure overload indicate that this speculation is likely to be incorrect. This rodent model of experimental hypertrophy was used because ascending aortic stenosis produces progressive severe hypertrophy due to pressure overload that closely simulates the hypertrophic phenotype that occurs in humans in response to common disorders of valvular aortic stenosis, aortic constriction, and hypertension. It is noteworthy that rodent models of pressure-overload hypertrophy, including the model of ascending aortic constriction,11 have provided test systems that have accurately predicted the effects of pharmacological agents on cardiac remodeling and outcome in major randomized human heart failure trials.30 The clinical experience of the use of cyclosporine in human cardiac transplantation also provides insight into its potential effect on pathological hypertrophy. In patients who develop hypertension and left ventricular hypertrophy after cardiac transplantation, morphometric studies of patients subjected to cyclosporine versus noncyclosporine immunosuppression strongly suggest that long-term cyclosporine treatment does not prevent or suppress pathological pressure-overload hypertrophy in patients.31,32

In summary, we observed that cyclosporine treatment fails to inhibit left ventricular hypertrophy or cardiac expression of ANF in aortic stenosis mice. In contrast to transgenic models with forced expression of activated forms of calcineurin or NFAT, this countermeasure is insufficient to modify pathological hypertrophy secondary to pressure overload. Furthermore, the elevation of left ventricular calcineurin activity is not a requirement for the development of severe pressure-overload hypertrophy in this model.

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