Reversibility of Adverse, Calcineurin-Dependent Cardiac Remodeling

Jeff M. Berry, Vien Le, David Rotter, Pavan K. Battiprolu, Bennett Grinsfelder, Paul Tannous, Jana S. Burchfield, Michael Czubryt, Johannes Backs, Eric N. Olson, Beverly A. Rothermel, Joseph A. Hill

Rationale: Studies to dissect the role of calcineurin in pathological cardiac remodeling have relied heavily on murine models, in which genetic gain- and loss-of-function manipulations are initiated at or before birth. However, the great majority of clinical cardiac pathology occurs in adults. Yet nothing is known about the effects of calcineurin when its activation commences in adulthood. Furthermore, despite the fact that ventricular hypertrophy is a well-established risk factor for heart failure, the relative pace and progression of these 2 major phenotypic features of heart disease are unknown. Finally, even though therapeutic interventions in adults are designed to slow, arrest, or reverse disease pathogenesis, little is known about the capacity for spontaneous reversibility of calcineurin-dependent pathological remodeling.

Objective: We set out to address these 3 questions by studying mice engineered to harbor in cardiomyocytes a constitutively active calcineurin transgene driven by a tetracycline-responsive promoter element.

Methods and Results: Expression of the mutant calcineurin transgene was initiated for variable lengths of time to determine the natural history of disease pathogenesis, and to determine when, if ever, these events are reversible. Activation of the calcineurin transgene in adult mice triggered rapid and robust cardiac growth with features characteristic of pathological hypertrophy. Concentric hypertrophy preceded the development of systolic dysfunction, fetal gene activation, fibrosis, and clinical heart failure. Furthermore, cardiac hypertrophy reversed spontaneously when calcineurin activity was turned off, and expression of fetal genes reverted to baseline. Fibrosis, a prominent feature of pathological cardiac remodeling, manifested partial reversibility.

Conclusions: Together, these data establish and define the deleterious effects of calcineurin signaling in the adult heart and reveal that calcineurin-dependent hypertrophy with concentric geometry precedes systolic dysfunction and heart failure. Furthermore, these findings demonstrate that during much of the disease process, calcineurin-dependent remodeling remains reversible. (Circ Res. 2011;109:407-417.)

Key Words: heart failure • hypertrophy • remodeling

Strong epidemiological evidence links left ventricular hypertrophy with adverse cardiovascular events, including heart failure and death.1–4 Consistent with this, current therapies that improve clinical outcomes are often associated with regression of ventricular hypertrophy.5–7 However, whereas significant strides have been made recently in elucidating the molecular circuitry governing pathological cardiac remodeling,8 few therapies in clinical use target hypertrophic growth mechanisms directly.

Calcineurin is a cytoplasmic protein phosphatase implicated in the pathogenesis of cardiac hypertrophy and heart failure.9 Calcineurin dephosphorylates transcription factors of the NFAT (nuclear factor of activated T cells) family, resulting in their translocation to the nucleus and triggering of a complex transcriptional program.10 Calcineurin also targets other proteins to activate nontranscriptional signaling cascades. Consistent with widespread involvement in heart disease, studies in animal models have demonstrated a critical role for calcineurin in biomechanical stress-induced hypertrophic remodeling,11,12 and calcineurin activation has been observed in heart failure in humans.13–15

Studies designed to dissect the role of calcineurin signaling in heart disease have relied, by and large, on genetic manipulations in mice. In this context, gain- and loss-of-function manipulations are typically initiated at or before birth. However, recent studies have highlighted the fact that the effects
of mechanisms triggered in adulthood oftentimes differ from those seen when the same mechanisms are triggered during development, when a host of other events are taking place. Furthermore, the great majority of clinically relevant disease states involving calcineurin occur in adults. However, little is known about the effects of calcineurin activation in adult animal models.

Pathological ventricular remodeling is a dynamic process involving changes in cardiomyocyte size and shape, alterations in gene expression and substrate metabolism, changes in intracellular calcium homeostasis, deposition of excess extracellular matrix, sarcomere recruitment, and more. In many animal models, pathological stress elicits a hypertrophic growth response that culminates ultimately in contractile dysfunction and heart failure. In humans, however, in whom disease-related stress initiates long after cardiac development is complete, this pathogenic progression has been questioned. Furthermore, the extent to which ventricular hypertrophy is a risk factor for systolic dysfunction may depend on specific phenotypic features, such as whether its geometry is concentric or eccentric. Given all this, it is important to determine the natural history of load-induced remodeling and to tease out molecular events governing the progression of hypertrophy and failure.

In light of recent advances in our understanding of signaling cascades governing pathological cardiac remodeling, the hypertrophic growth response per se has emerged as a viable therapeutic target. This strategy hinges critically, however, on the notion that pathological remodeling is reversible and not just preventable. Yet little is known at present regarding the extent to which calcineurin-dependent remodeling is reversible. Finally, clinically relevant antiremodeling therapies, both pharmacological and mechanical, are only modestly efficacious, and hence it is important to tease out the diseased myocyte’s capacity for spontaneous recovery.

Thus, we set out to address three questions: (a) what are the effects of calcineurin activation initiated during adulthood; (b) is ventricular hypertrophy a requisite precursor of calcineurin-dependent heart failure, and if so, what are its features; and (c) is the calcineurin-dependent phenotype reversible in the absence of antiremodeling therapy? To accomplish this, we engineered mice harboring in cardiomyocytes a constitutively active calcineurin transgene driven by a tetracycline-responsive promoter element. Expression of the mutant calcineurin transgene was initiated in adulthood for variable lengths of time to determine the natural history of disease pathogenesis and associated molecular and functional events, and to identify markers of disease reversibility.

**Methods**

A detailed Methods section is available in the Online Data Supplement available at http://circres.ahajournals.org.

**Animal Care**

Transgenic mice that conditionally express a constitutively active calcineurin mutant, specifically in cardiomyocytes, were generated using the α-mysosin heavy chain promoter and the tetracycline transactivator off-promoter system. Both male and female animals ages 5 to 8 weeks were studied using protocols approved by the animal care and use committee of the University of Texas Southwestern Medical Center.

**Echocardiography**

Echocardiograms were performed using a Sonos 5500 system with a 15-MHz probe, and M-mode and 2-dimensional (2-D) parasternal short-axis images were obtained. Left ventricular mass was calculated by the cubed method, as previously described.

**Immunoblot Analyses**

Immunoblots were performed on protein lysates prepared from left ventricular tissue. All antibodies used are presented in the Online Data Supplement.

**Quantitative Real-Time Reverse Transcription Polymerase Chain Reaction (RT PCR)**

RNA was extracted from snap-frozen left ventricular tissue and used to prepare cDNA. Real-time PCR was performed using an ABI 7000 system. All primers used are presented in the Online Data Supplement.

**Histology and Cardiomyocyte Cross-Sectional Area**

Selected hearts were perfusion fixed in paraformaldehyde and subjected to cutting and staining as described previously. Cardio-myocyte area was measured from hematoxylin and eosin-stained images and from wheat germ agglutinin-stained images. Fibrosis was measured from picrosirius red-stained images. Hydroxyproline analysis was performed on the basis of previously described methods.

**In Vivo Electrophysiology Studies**

In vivo electrophysiology studies were performed on sedated mice using a multipolar electrode catheter placed via the right jugular vein as previously described.

**Statistical Methods and Data Handling**

All data are presented as mean ± standard deviation (SD). Comparison of data between 2 groups was performed using the Mann–Whitney U test. The authors had full access to, and take full responsibility for, the integrity of these data. All authors have read and agree to the manuscript as written.

**Results**

To test the effects of calcineurin activation in the adult mammalian heart, we generated transgenic mice that conditionally express a constitutively active calcineurin mutant specifically in cardiomyocytes. We used the tetracycline-inhibited transactivator system under control of the αMHC promoter (αMHC-tTA) to control expression of a constitutively active form of the human calcineurin A subunit (CnA*) (Figure 1A). Several lines of teto-CnA* mice were generated.
and then crossed with αMHC-tTA mice. A line of double transgenic mice (tTA/CnA*) was selected for analysis, which manifested increased cardiac mass by 8 weeks of age, similar to that previously described for conventional αMHC-calcineurin transgenic mice. 10 For subsequent experiments, all mice including breeding pairs were administered doxycycline via drinking water so that expression of the CnA* transgene was suppressed in utero and in early postnatal life.

Doxycycline was withdrawn from several tTA/CnA* mice at age 8 weeks, and hearts were harvested at age 16 weeks. Western blot analysis of protein from ventricular tissue confirmed expression of a truncated calcineurin protein of the expected molecular weight in tTA/CnA* mice (Figure 1B, Supplemental Figure 1A). This protein was not evident in hearts from single transgenic αMHC-tTA mice or from tTA/CnA* mice that continued to receive doxycycline, confirming conditional expression of CnA*.

We investigated a downstream target of calcineurin signaling in order to confirm increased calcineurin activity in tTA/CnA* hearts. The expression of regulator of calcineurin (RCAN1.4) is directly controlled by calcineurin via the Nfat pathway. 29,30 We observed a robust increase in RCAN1.4 in hearts of tTA/CnA* mice in comparison with that of single transgenic αMHC-tTA mice (Figure 1C, Supplemental Figure IA), occurring within 5 days of doxycycline withdrawal (Figure 1D, Supplemental Figure IB). This augmentation in RCAN1.4 protein levels was not evident when doxycycline was continued in tTA/CnA* mice (Figure 1C). Thus, doxycycline was effective in suppressing the expression and activity of CnA* in double transgenic tTA/CnA* mice.

Calcineurin Activation in the Adult Heart Triggers Pathological Hypertrophy

To test the effects of prolonged calcineurin activation in adult heart, doxycycline was withdrawn from tTA/CnA* mice at 5 weeks of age, and hearts were harvested at 21 weeks of age (Figure 2A). For comparison, a second group of tTA/CnA* mice was maintained on doxycycline to suppress CnA* throughout the study. First, echocardiograms were performed biweekly to assess serial changes in cardiac size and function. Calcineurin signaling in CnA* ON mice triggered a significant decline in fractional shortening consistent with impaired ventricular systolic function (Figure 2B). Calcineurin activation elicited a 34% increase in left ventricular (LV) internal diameter at end-diastole (LVIDd OFF 3.23 ± 0.40 mm, \( n = 10 \); LVIDd ON 4.34 ± 1.07 mm, \( n = 14 \), \( P < 0.01 \)), and an even greater increase in LV internal diameter at end-systole (LVIDs OFF 1.04 ± 0.36 mm, LVIDs ON 2.93 ± 1.30, \( P < 0.01 \), Figure 2B and 2C). The greatest decline in contractile function occurred between 2 and 6 weeks after removing doxycycline. No significant decline in fractional shortening occurred in tTA/CnA* mice that were maintained on doxycycline in the CnA* OFF group.

Necropsy analysis revealed a significant increase in cardiac mass after 16 weeks of calcineurin activation (Figure 2D and 2E), and 3 animals (of 17) in the CnA* ON group died before study completion (no deaths of 10 mice occurred in the CnA* OFF group). Lung weights were similar between the 2 groups, although the 2 CnA* ON mice with the largest hearts were noted to have lung weights twice that of controls.

Cardiomyocyte hypertrophy was evident on microscopic examination of sections of ventricular tissue. Cardiomyocyte cross-sectional area increased significantly in CnA* ON mice (266 ± 82 \( \mu \)m\(^2\), \( n = 240 \) cells, 4 hearts) in comparison with CnA* OFF mice (185 ± 42 \( \mu \)m\(^2\), \( n = 240 \) cells, 4 hearts, \( P < 0.01 \)) (Figure 2F and 2G, Supplemental Figure II). We also noted prominent interstitial fibrosis in hearts subjected to 16 weeks of calcineurin activation (Figure 2H). Quantification of fibrosis from histological images revealed more fibrosis in hearts of CnA* ON mice (ON 6.0 ± 1.5%, \( n = 5 \); OFF 3.2 ± 2.0%, \( n = 6 \), \( P = 0.02 \)).

We next set out to determine the phenotype of calcineurin-induced ventricular remodeling after just 8 weeks of calcineurin activation (Figure 3A). Single transgenic αMHC-tTA littermates were used as controls, and all mice were subjected to the same schedule of doxycycline. Hearts were harvested 8 weeks after doxycycline removal, a time point associated with significant declines in fractional shortening on the basis...
of our initial study (Figure 2C). Eight weeks of calcineurin activation in adult hearts triggered profound pathological hypertrophy (Figure 3). Analysis of echocardiographic images revealed significant declines in systolic function (Figure 3B), robust increases in ventricular mass, left ventricular wall thickness, and chamber dimension (Figure 3C). Of note, we observed a subtle increase in cardiac mass in single-transgenic tTA mice in comparison with wild-type littermates, but there was no significant decline in fractional shortening (Supplemental Figure III). We therefore used single-transgenic tTA mice as controls for comparison with double-transgenic mice. (Head-to-head comparisons of LV
mass estimated by echo and measured on necropsy revealed a high correlation; see Supplemental Figure IV.)

Postmortem analysis revealed consistent results; heart weight:body weight ratios increased 48% ($P<0.05$) in tTA/CnA* mice in comparison with hearts from control mice (Figure 3D). To evaluate for activation of the fetal gene program, a marker of pathological remodeling in heart, we extracted protein and RNA from ventricular tissue 8 weeks after removing doxycycline. Calcineurin signaling triggered dramatic increases in $\beta$ myosin heavy chain ($\beta$MHC) protein and mRNA (Figure 3E and 3F, Supplemental Figure IC). There was also a significant increase in BNP expression, but no statistically significant changes in ANF or SERCA2a expression were observed.

**Calcineurin-Induced Ventricular Hypertrophy Precedes Systolic Dysfunction**

In animal models in which calcineurin is activated early in life, hypertrophic growth of the heart precedes contractile dysfunction and heart failure. In humans, however, in whom disease-related stress initiates long after cardiac development is complete, this pathogenic progression has been questioned. Does ventricular hypertrophy progress routinely to heart failure, or does ventricular hypertrophy develop secondarily, as a
consequence of systolic dysfunction? Furthermore, the extent to which ventricular hypertrophy is a risk factor for systolic dysfunction may depend on specific phenotypic features, such as whether it is concentric or eccentric.\(^3\) To test these important questions, we studied 8-week-old male mice with serial echocardiograms performed biweekly during calcineurin activation. First, we measured left ventricular mass and wall thickness by echocardiogram, observing robust increases within 2 weeks (Figure 4A and 4B). Next, we evaluated ventricular size and performance. Interestingly, there was no significant decrease in fractional shortening at 2 weeks despite the development of ventricular hypertrophy (Figure 4C). Fractional shortening was significantly decreased by 4 weeks, but left ventricular chamber dimension remained unchanged until 8 weeks (Figure 4D). Thus, calcineurin activation in adult mice triggered ventricular hypertrophy followed by systolic dysfunction, and left ventricular chamber dilation occurred last. There was no significant difference in heart rates in these mice at the time the echocardiograms were performed (Supplemental Figure V).

In order to evaluate the relationship between gene expression and ventricular remodeling, we collected RNA from mice at 1 week and 8 weeks after removing doxycycline. Just 1 week after removing doxycycline, we observed a robust increase in RCAN1.4 expression consistent with activation of calcineurin (Figure 4E). Immunoblots for RCAN1.4 also confirmed robust calcineurin activation at 1 week (data not shown). There was, however, no significant change in expression of fetal gene markers at this early time point. After

![Figure 4. Calcineurin-induced ventricular hypertrophy precedes systolic dysfunction, and fetal gene activation is delayed after calcineurin activation.](image-url)
by hydroxyproline assay. Collagen content within the LV did not increase significantly after 8 weeks of calcineurin activation (ON 0.9±0.2 mg/g, n=3; OFF 0.9±0.2, n=3; P=NS) despite significant ventricular dilation and declines in systolic function (Figure 5). By contrast, after 16 weeks of calcineurin signaling, collagen content was significantly increased both by hydroxyproline assay (ON 1.6±0.1 mg/g, n=2; OFF 0.80±0.05, n=2, P<0.05) and analysis of picrosirius red-stained images (ON 6.0%±1.5%, n=5; OFF 3.2%±2.0%, n=6; P=0.02). Collagen content in hearts of ON/OFF mice was similar to control levels (ON/OFF 4.0%±2.0%, n=6). Surprisingly, we were unable to induce ventricular tachyarrhythmia in ON mice despite the presence of significant interstitial fibrosis (Online Supplement, Supplemental Figure VIII). In aggregate, these findings suggest that ventricular fibrosis is a relatively late-arriving feature of the adverse remodeling phenotype, developing after both ventricular hypertrophy and altered contractile function are present.

From these data, it is unclear whether cardiac mass decreased when calcineurin signaling was extinguished or whether hearts in ON/OFF mice simply stopped growing when CnA* was turned off. To test this, we performed 2 additional, parallel experiments on tTA/CnA* mice. For study A, CnA* was activated at age 8 weeks, and mice were euthanized at age 16 weeks. For study B, CnA* was also activated at age 8 weeks, and echocardiograms were performed at age 16 weeks to confirm depressed ventricular function and CnA* was suppressed until age 24 weeks, at which time mice were euthanized. As in the previous experiments, echocardiography confirmed recovery of ventricular function and a decrease in LV mass when CnA* was turned off in study B (Figure 6A and 6B). Analysis of protein from left ventricular tissue confirmed robust activation of calcineurin in tTA/CnA* hearts at the time of sacrifice in study A (Figure 6C). By contrast, calcineurin activity was not elevated in tTA/CnA* hearts at study completion in study B.
successful suppression of CnA* during the second half of the experiment. Importantly, heart weights at the completion of study B were significantly lower than those at the completion of study A, despite having been subjected to the same 8 weeks of CnA* activation (Figure 6D).

Activation of the genes coding for βMHC and BNP was detected after 8 weeks of calcineurin activation (Figure 3E). After 8 weeks of recovery in study B, βMHC and BNP mRNA returned to control levels (Figure 6E). Interestingly, those mice with limited recovery of fractional shortening (less than 50% after 8 weeks of recovery) also had normal expression of βMHC (included in Figure 6E). Conversely, BNP expression remained elevated in some but not all mice with limited recovery. Together, these data lend strong support to the concept that calcineurin-dependent remodeling of the heart can be reversed even without antiremodeling therapy.

Discussion
In recent years, calcineurin has emerged as a major mechanism of pathological cardiac remodeling, active in numerous forms of heart disease. As such, this molecule has generated much interest as a target of therapy. Here, we addressed several questions relevant to the therapeutic targeting of calcineurin in heart disease: what are the effects of calcineurin activation in the fully developed adult heart; does calcineurin-dependent hypertrophy precede contractile dysfunction, and, if so, what are its phenotypic features; to what extent are these phenotypes reversible; can reversal be accomplished in the absence of therapy? To accomplish this, we engineered a mouse model that provides high-fidelity, spatiotemporal control of calcineurin activation. From these experiments, we report the following significant findings: (a) calcineurin activation in adulthood triggers robust pathological remodeling; (b) ventricular hypertrophy, concentric in nature, precedes systolic dysfunction and tissue fibrosis; (c) ventricular fibrosis develops after both hypertrophy and contractile dysfunction have arisen; (d) hypertrophic growth, and contractile dysfunction are each partially and spontaneously reversible. Together, these findings shed new light on the adverse remodeling response elicited by calcineurin and raise yet further the prospects of targeting this molecule for therapeutic gain.

Calcineurin Signaling Is Maladaptive in Adult Heart
Heart disease is the leading cause of morbidity and mortality in the industrialized world, contributing importantly to ever-expanding health care expenditures. The pathogenesis of many forms of heart disease involves a set of complex remodeling processes, many of which are independent risk factors for adverse clinical events. For example, the
presence of left ventricular hypertrophy in patients with hypertension is associated with substantially increased morbidity and mortality.\textsuperscript{1} Also, a decline in ejection fraction after myocardial infarction is a major predictor of increased risk of developing heart failure or sudden death.\textsuperscript{33} In many of these events, persistent or excessive activation of the calcineurin signaling pathway is thought to play a significant role.\textsuperscript{21}

Previous models of calcineurin activation in neonatal murine hearts demonstrated an almost 3-fold increase in cardiac mass at only 3 to 12 weeks of age.\textsuperscript{10} Working with adult myocytes, we observed significantly less hypertrophy despite the presence of robust calcineurin activation for 16 weeks. In addition, adult mice manifested less than 10% mortality at 16 weeks, which contrasts with the greater than 50% mortality at 16 weeks observed when calcineurin is activated in neonatal mice.\textsuperscript{34} Together, these findings are consistent with a model in which pathological signaling events activated early in life interact with developmental pathways, often compounding the resulting adverse phenotype.

Our model of conditional activation of calcineurin provided an opportunity to investigate the time course of events that occur with calcineurin signaling. We found that ventricular hypertrophy is an early event in the progression of calcineurin-induced heart failure. Left ventricular mass, measured noninvasively by echocardiography, was significantly increased after just 2 weeks of calcineurin activation, whereas left ventricular cavity size and ejection fraction remained normal. Conversely, we found that tissue fibrosis and fetal gene activation are late-appearing aspects of the phenotype, emerging only after significant contractile dysfunction has developed. A dramatic increase in $\beta$MHC expression was apparent after several weeks of calcineurin activation. Despite an increase in RCAN1.4 during the 1st week, confirming calcineurin activation, we did not observe a significant increase in $\beta$MHC at this early time point. This suggests that calcineurin triggers a cascade of signaling events that later leads to the rise in $\beta$MHC. Yet when calcineurin was turned off, $\beta$MHC returned to control levels. The contribution of $\beta$MHC expression to systolic dysfunction remains uncertain.

These findings lend further support to the notion that calcineurin-induced hypertrophy is maladaptive, because a decline in systolic function ultimately developed in these mice without any additional stressors, such as myocardial infarction or pressure overload. Furthermore, these data are consistent with previous findings that inhibition of calcineurin-dependent signaling in the setting of pressure overload blunts hypertrophy without adverse effects on cardiac function or clinical events.\textsuperscript{9,35}

**Calcineurin as a Therapeutic Target**

Although preventive strategies will undoubtedly remain cornerstones of therapy, many patients do not present to clinical attention until after significant structural heart disease has already developed. It is therefore critical to parse the various pathological elements and determine and quantify their potential for reversal. Some, such as myocyte death, have essentially no potential for recovery. Others, however, such as cellular hypertrophy, contractile dysfunction, and fibrosis hold potential for significant reversibility. Prior to this study, however, nothing was known about the kinetics of development of the multiple phenotypes elicited by calcineurin, their emergence in adult heart, and their potential for reversal.

Both pharmacological and device-based therapies improve clinical outcomes in association with reverse remodeling.\textsuperscript{5,7,36} and calcineurin signaling mechanisms may prove to be new targets for recovery of cardiac function. For example, calcineurin suppression by systemic administration of cyclosporine A is capable of reversing cardiac hypertrophy,\textsuperscript{37} although cyclosporine A may trigger hypertrophy in some models of heart disease.\textsuperscript{38} Rapamycin treatment will reverse hypertrophy induced by pressure overload\textsuperscript{39,40} and in AKT transgenic mice.\textsuperscript{41} Our results here extend these observations by demonstrating the reversibility of some of the effects of calcineurin signaling. Furthermore, that this reversal occurs when myocyte-specific calcineurin activity is suppressed points to a cell-autonomous mechanism of reverse remodeling.

Variable degrees of reverse remodeling were observed in ON/OFF mice, but left ventricular geometry did not emerge as a robust predictor of the potential for recovery of function; mice with the greatest LV dilation or lowest percent fractional shortening following 8 weeks of calcineurin activation sometimes demonstrated significant reverse remodeling when calcineurin activation was turned off. Thus, other factors may mark, or even determine, the potential for recovery of ventricular function. Interstitial fibrosis emerged as a late manifestation of calcineurin-triggered remodeling, possibly the result of activation of secondary signaling cascades. Excessive fibrosis in some hearts may have limited reverse remodeling when calcineurin signaling was extinguished.

A major objective of our study was to determine whether hypertrophy was reversible if the underlying cause were removed. Because some evidence suggests that the capacity for cardiac plasticity and repair declines with age, we chose to study young adult mice in an effort to obviate confounding, age-dependent changes in plasticity. In other words, we set out specifically to study young adults—with presumably maximal capacity for reversibility—in order to determine whether such reversibility is possible in the setting of a robust signaling cascade (viz calcineurin). Moving forward, having established for the first time that such reversibility of remodeling is, indeed, possible, it will be of interest to test for age-dependent declines in cardiac plasticity.

Increased calcineurin activity has been reported in the hearts of patients with multiple forms of structural heart disease.\textsuperscript{13–15} Furthermore, inhibition of calcineurin via either pharmacological or transgenic strategies blunts hypertrophic growth in a variety of animal models of heart disease.\textsuperscript{51,12,35,42} Importantly, suppression of hypertrophic growth by calcineurin inhibition is not associated with impairment of cardiac function or increased mortality.\textsuperscript{9} Consistent with this, data reported here demonstrate that calcineurin-induced cardiac hypertrophy results, in fact, in impaired ventricular function. These findings, then, add to a growing literature implicating the hypertrophic growth response per se as a maladaptive response to stress and a viable target for therapeutic intervention.\textsuperscript{9}
Conclusion
Calcineurin activation in the adult heart triggers pathological remodeling, which is, at least in part, reversible. Whereas certain forms of hypertrophic growth are thought to represent compensatory responses to environmental stress, calcineurin-induced remodeling appears to be uniquely maladaptive, progressing ultimately to systolic dysfunction, heart failure, and premature mortality. This progression, however, is reversible, suggesting that novel calcineurin-targeting therapies may prove effective in further improving outcomes and quality of life for patients with heart disease.

Acknowledgments
We thank Dr Rhonda Bassel-Duby for critical support and Ms Beverley Huet for expert assistance with the statistical analyses.

Source of Funding
This work was supported by grants from the National Institutes of Health (HL-075173 to J.A.H.; HL-090144 to J.A.H.; HL-090842 to J.A.H.; HL-072016 to B.A.R.; HL-097768 to B.A.R.), American Heart Association (AHA) (0640084N to J.A.H.; 0655202Y to J.A.H.; HL-072016 to B.A.R.; HL-097768 to B.A.R.), American Diabetes Association (7–08-MN-21-ADA to J.A.H.), Deutsche Forschungsgemeinschaft (BA 2258/1–1 to J.B.), and the AHA—Jon Holden DeHaan Foundation (0970518N to J.A.H.).

Disclosures
None.

References
Novelty and Significance

What Is Known?

- Left ventricular hypertrophy is associated with adverse cardiovascular events, including heart failure and death.
- Calcineurin is a cytoplasmic protein phosphatase implicated in the pathogenesis of cardiac hypertrophy.

What New Information Does This Article Contribute?

- Calcineurin signaling in the adult heart triggers ventricular hypertrophy with markers of pathological remodeling.
- Calcineurin-induced ventricular hypertrophy precedes the development of systolic dysfunction and heart failure.
- Calcineurin-induced cardiac hypertrophy reverses when calcineurin signaling is turned off.
- Fetal gene expression and ventricular fibrosis, each a late manifestation of pathological remodeling, manifest significant reversibility.

Strong epidemiological evidence links left ventricular hypertrophy with adverse cardiovascular events, and heart failure therapies that improve clinical outcomes are often associated with regression of hypertrophy. Calcineurin may play a role in the pathogenesis of hypertrophic heart disease, yet little is known regarding the mechanisms of calcineurin-induced ventricular hypertrophy in the adult heart. Data reported here demonstrate that calcineurin signaling in adult cardiomyocytes triggers pathological ventricular hypertrophy and heart failure. Hypertrophy, heart failure, and other aspects of the pathological remodeling response reverse when calcineurin activation is eliminated.

This study is significant for defining the time course and natural history of remodeling events in adult heart triggered by calcineurin signaling. Ventricular hypertrophy precedes the development of systolic dysfunction and heart failure. A robust increase in beta myosin heavy chain expression occurs, but this is not apparent during the first week of calcineurin signaling. Beta myosin heavy chain expression returns to baseline levels as calcineurin-induced hypertrophy reverses. Future studies are warranted to investigate calcineurin and its downstream effectors as therapeutic targets to prevent, and possibly reverse, relevant features of the pathologically remodeled ventricle.
Reversibility of Adverse, Calcineurin-Dependent Cardiac Remodeling

Jeff M. Berry, Vien Le, David Rotter, Pavan K. Battiprolu, Bennett Grinsfelder, Paul Tannous, Jana S. Burchfield, Michael Czubryt, Johannes Backs, Eric N. Olson, Beverly A. Rothermel and Joseph A. Hill

Circ Res. 2011;109:407-417; originally published online June 23, 2011;
doi: 10.1161/CIRCRESAHA.110.228452

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2011 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/109/4/407

Data Supplement (unedited) at:
http://circres.ahajournals.org/content/suppl/2011/06/23/CIRCRESAHA.110.228452.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation Research can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation Research is online at:
http://circres.ahajournals.org/subscriptions/
SUPPLEMENTAL MATERIAL

Reversibility of Adverse, Calcineurin-Dependent Cardiac Remodeling

Jeff M. Berry, MD¹, Vien Le, MD¹, David Rotter, PhD¹, Pavan K. Battiprolu, PhD¹, Bennett Grinsfelder¹, Paul Tannous, MD, PhD¹, Jana S. Burchfield, PhD¹, Michael Czubryt, PhD², Johannes Backs, MD², Eric N. Olson, PhD², Beverly A. Rothermel, PhD¹, Joseph A. Hill, MD, PhD¹,²
**Detailed Methods**

**Animal Care**

We engineered double transgenic mice (C57BL/6 background) harboring a truncated form of the calcineurin A subunit lacking the C-terminal autoinhibitory domain (CnA*)\(^1\). Temporal control of expression was accomplished using the tetracycline transactivator off promoter (TetO) system\(^2\). Multiple lines of TetO-CnA* transgenic mice were generated. These animals were crossed with mice harboring the tetracycline-inhibited transactivator (tTA) under control of the \(\alpha\)-myosin heavy chain promoter (\(\alpha\)MHC-tTA)\(^3\) to yield double transgenic mice (tTA/CnA*) for use in experiments. Both male and female animals aged 5 to 8 weeks were studied using protocols approved by the animal care and use committee of UT Southwestern Medical Center.

**Echocardiography**

Echocardiograms were performed on conscious, gently restrained mice using a Sonos 5500 system and 15 MHz linear probe. A short axis view of the left ventricle at the level of the papillary muscles was obtained, and M-mode recordings were obtained from this view. Measurements of interventricular septum thickness (IVS), left ventricular internal diameter (LVID), and left ventricular posterior wall thickness (LVPW) were made from 2D parasternal short axis views in diastole. Left ventricular mass was calculated by the cubed method as 1.05 \(\times\) \(((IVS + LVID + LVPW)^3 - LVID^3)\) (mg)\(^4\). Left ventricular internal diameter at end-diastole (LVIDd) and end-systole (LVIDs) were measured from M-mode recordings. Fractional shortening was calculated as \((LVIDd - LVIDs) / LVIDd\) (%).

**Immunoblot analyses**

Hearts were harvested and snap frozen in liquid nitrogen. Protein lysates were prepared from left ventricular tissue homogenized in lysis buffer (0.1% Triton X-100, 2% glycerol, 10mM Tris, 1mM Na bisulfite, 1mM NaF, protease inhibitors, pH=7.0) and centrifuged (9,000 rpm, 10 min). For extraction of myosin protein, a different lysis buffer was used (0.3M KCl, 0.1M KH\(_2\)PO\(_4\), 50mM K\(_2\)HPO\(_4\), 10mM EDTA, protease inhibitors). Protein concentration of lysates was estimated using the Bradford method, and equal quantities of protein were loaded per gel lane and separated by electrophoresis. Proteins were transferred to a PVDF membrane and equivalence of protein loading and transfer confirmed by Ponceau stain. Proteins of interest were detected using primary antibody and HRP-conjugated secondary antibody.
Antibodies used were as follows: anti-calcineurin Aβ (AB1697, Chemicon International, Temecula, CA); anti-myosin (skeletal, slow; M8421, Sigma-Aldrich, St. Louis, MO), anti-RCAN (custom-made, Gilead, Bromfield, CO); goat anti-mouse HRP-conjugated antibody (1721011, BioRad, Hercules, CA); goat anti-rabbit HRP-conjugated antibody (1721019, BioRad, Hercules, CA).

Quantitative real-time PCR
RNA was extracted from snap frozen left ventricular tissue using Trizol reagent according to the manufacturer’s instructions. RNA concentration was estimated from each sample using a Nanodrop ND-1000 spectrophotomer. Two µg RNA of each sample was used to make cDNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). Real-time PCR was performed on an ABI 7000 system in triplicate for each sample. Relative quantities of each transcript were determined by normalizing to cyclophilin A.

Primers for real-time PCR reactions were as follows: Cyclophilin A:
CAGACGCCACTGTGCTTTT / TGTTTTGGAACCTTTGTCTGCAA; 
βMHC:CTACAGGCTGGGCTTACCT / TCTCCTTCTCAGACTTCCGC; 
BNP: CACCGCTGGGAGGTCACT / GTGAGGCCTTGGTCCTTCAA; 
ANF: CATCACCGTTGGGCTTTTCT / TGGGCTCCAATCCTGTCAATC; 
SERCA 2a: CCATCTGCTTGTCCATGTCACT / CAAATGGTTTAGGAAGCGGTTACT

Histology and cardiomyocyte cross-sectional area
Selected hearts were perfusion fixed in paraformaldehyde and subjected to cutting and staining as described previously. Hematoxylin and eosin-stained tissue sections from 4 hearts in each group were studied at 200x magnification. Six randomly selected fields were studied per heart, and all myocytes cut in short axis with a visible nucleus were measured. Cell borders were planimetered using ImageJ software by an operator who was blinded to treatment group. This experiment was repeated using WGA-stained images from 4 hearts.

Picrosirius red stained tissue sections were studied to assess fibrosis. Six randomly selected fields were studied per heart. The area of tissue staining positive for fibrosis was measured using ImageJ software by an operator who was blinded to treatment group. Results are reported as the percent area of tissue staining positive for fibrosis.
Hydroxyproline analysis was performed based on previously described methods\textsuperscript{6}. Briefly, hearts were harvested, the left ventricle dissected free, and the tissue lyophilized. Equal quantities of lyophilized material were suspended in 6 N HCl and hydrolyzed at 120°C followed by neutralization with 4 N NaOH. Samples and standards (L-hydroxyproline, Sigma) were incubated for 20 minutes (RT) with chloramine T, followed by addition of Erlich reagent (3.75g of \textit{p}-dimethylaminobenzaldehyde, 15 mL of l-propanol, 6.5 mL of perchloric acid (60\%) in 25 mL for 20 min (60°C). Absorbances were read at 558 nm, and values were calculated from a standard curve generated for each analysis. Results are expressed as microgram of hydroxyproline per milligram of dried tissue sample.

\textit{In vivo electrophysiology studies}

\textit{In vivo} electrophysiology studies were performed as previously described\textsuperscript{7}. Mice were sedated by intraperitoneal administration of pentobarbital (0.050 mg/gm). The right jugular vein was exposed, and a 1.1 F octapolar electrophysiology catheter (Millar EPR-800) was inserted transvenously into the right jugular vein and advanced to the right atrium or right ventricle using electrogram tracings for guidance. The EP catheter included eight electrodes with 1.0 mm electrode spacing, and the distal electrode pair was used to stimulate while recording from all other electrodes. A standard 6 lead surface ECG was recorded during the study. Programmed ventricular stimulation was performed with one, two, and three extrastimuli following an eight beat drive train. Each extrastimulus coupling interval was decreased by 10 msec intervals until refractory. The same protocol was performed on all mice while observing for ventricular tachyarrhythmia of 4 beats or more.

\textbf{Statistical methods and data handling}

All values are presented as mean ± SD. Comparison of data between groups was performed using the Mann-Whitney U test. Comparison of fractional shortening over time between groups was performed using two-way repeated measures ANOVA. Holm-Sidak \textit{post hoc} testing was used to correct for multiple comparisons. Using the Bland-Altman analysis method, the agreement between LV mass determined echocardiographically and necropsy heart weight was calculated as the mean (bias) ± error (2 standard deviations). For statistical comparisons, significance was taken as \( p < 0.05 \). All statistical analyses were performed using SigmaStat v3.1 software.
The authors had full access to, and take full responsibility for, the integrity of these data. All authors have read and agree to the manuscript as written.
Supplemental Data

During the reverse remodeling experiment, there was variability in the degree of reverse remodeling when doxycycline was restarted (Online Figure VI). In an effort to gain insight into mechanisms, we examined echocardiographic characteristics of mice just prior to reinitiating doxycycline to identify potential markers of reversibility. Mice demonstrating significant reverse remodeling (%FS>50% at study completion) had a slightly higher %FS after 8 weeks of calcineurin activation (%FS: responders 40.2±6.0%, non-responders 36.4±2.8%, p=0.13). No other specific parameters of ventricular hypertrophy or chamber dimension were predictive of the extent of reverse remodeling (LVIDd: responders 3.9±0.5 mm, non-responders 4.1±0.3 mm, p=NS; LV posterior wall thickness: responders 1.1±0.2 mm, non-responders 1.2±0.2 mm, p=NS; ratio of wall thickness-to-LVIDd: responders 0.28±0.07, non-responders 0.28±0.06, p=NS) (Online Figure VII).

Given the presence of significant interstitial fibrosis with prolonged calcineurin activation, we hypothesized that these mice would be susceptible to ventricular arrhythmia. We have shown previously that mice subjected to severe transverse aortic constriction (sTAC) develop dilated cardiomyopathy that is associated with propensity to arrhythmia upon ventricular extra-stimulation7. Surprisingly, we were unable to induce ventricular arrhythmia in mice after 16 weeks of calcineurin activation; in contrast, sTAC, which elicited a similar cardiomyopathic phenotype, was associated with readily triggered ventricular arrhythmia (Online Figure VIII). These data suggest that aspects of the electrophysiological remodeling response elicited by calcineurin activation differ from that provoked by elevated afterload.
**Supplemental Table**

Left ventricular hypertrophy precedes systolic dysfunction with calcineurin activation.

Echocardiographic measurements of left ventricular size and function. Left ventricular hypertrophy develops after two weeks of calcineurin activation (age 10 weeks). A decline in fractional shortening is not apparent until age 12 weeks. Left ventricular chamber dilation is just apparent at age 16 weeks.

<table>
<thead>
<tr>
<th>Age (weeks)</th>
<th>8</th>
<th>10</th>
<th>12</th>
<th>14</th>
<th>16</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mice</td>
<td>ON</td>
<td>OFF</td>
<td>ON</td>
<td>OFF</td>
<td>ON</td>
</tr>
<tr>
<td>N</td>
<td>9</td>
<td>9</td>
<td>9</td>
<td>9</td>
<td>9</td>
</tr>
<tr>
<td>LVIDd (mm)</td>
<td>3.31</td>
<td>3.30</td>
<td>3.47</td>
<td>3.63</td>
<td>3.81</td>
</tr>
<tr>
<td>LVIDs (mm)</td>
<td>1.26</td>
<td>1.27</td>
<td>1.38</td>
<td>1.50</td>
<td>1.86*</td>
</tr>
<tr>
<td>FS (%)</td>
<td>62.0</td>
<td>61.7</td>
<td>60.1</td>
<td>58.8</td>
<td>51.2*</td>
</tr>
<tr>
<td>LV mass (g)</td>
<td>132</td>
<td>116</td>
<td>158*</td>
<td>127</td>
<td>186*</td>
</tr>
<tr>
<td>IVSd (mm)</td>
<td>1.03</td>
<td>0.93</td>
<td>1.10*</td>
<td>0.89</td>
<td>1.11*</td>
</tr>
<tr>
<td>LVPWd (mm)</td>
<td>1.10</td>
<td>1.03</td>
<td>1.20*</td>
<td>0.98</td>
<td>1.23*</td>
</tr>
</tbody>
</table>

LVId, left ventricular internal diameter at end-diastole; LVIDs, left ventricular internal diameter at end-systole; FS, fractional shortening; LV mass, left ventricular mass calculated from 2D echo images using cubed method; IVSd, interventricular septal thickness at end-diastole; LVPWd, left ventricular posterior wall thickness at end-diastole; * p <0.05.
Supplemental Figure Legends

Online Figure I: Removal of doxycycline triggers increased RCAN1.4 and βMHC protein in tTA/CnA* mice. A. Representative immunoblots of LV lysates prepared from hearts harvested 7 days after removal of doxycycline and probed for calcineurin (CnA), RCAN, and tubulin. B. Representative immunoblots of LV lysates from tTA/CnA* hearts harvested 4 or 5 days after removal of doxycycline and probed for CnA, RCAN, and tubulin. C. Representative immunoblot of LV lysates prepared 8 weeks after removal of doxycycline and probed for βMHC.

Online Figure II: Calcineurin activation in adult heart triggers cardiomyocyte hypertrophy. A. Representative histological images of wheat germ agglutinin (WGA)-stained left ventricular tissue sections revealing cardiomyocyte size at study completion. Bar = 20 µm. B. Mean cardiomyocyte cross-sectional area measured from WGA-stained tissue sections. 60 cells were measured from 4 hearts. ON, calcineurin activated for 16 weeks; OFF, calcineurin suppressed for 16 weeks. * p<0.01.

Online Figure III: Expression of tTA protein triggers increased cardiac mass and no change in function. A. Mean heart weight to body weight ratio of mice sacrificed at age 16 weeks. B. Mean fractional shortening measured from M-mode tracings obtained at age 16 weeks. WT, wild-type (n=7), tTA, single transgenic for tTA (n=7), tTA/CnA*, double transgenic for both tTA and CnA* transgenes (n=10). *p<0.05.

Online Figure IV: Left ventricular mass calculated from 2D echocardiographic images accurately predicts heart weight. A: LV mass was determined by echocardiography on the same day that hearts were harvested and weighed. Line of equality is shown. B: Bland-Altman plot showing the difference in measurements as a function of the mean of measurements for each heart. The bias and error for echocardiographically determined LV mass compared to total heart weight was -3.1 ± 49.4 mg. This is consistent with previously published data in which the cubed formula slightly over-estimated LV weight measured at necropsy⁴. (Collins et al. Am J Physiol Heart Circ Physiol 2001; 280: H1954-62.)

Online Figure V: Average heart rate of mice during serial echocardiograms was not different for control (OFF) and experimental (ON) mice.
Online Figure VI: Mice demonstrate variable reverse remodeling when calcineurin activity is extinguished. Graph showing fractional shortening for individual mice during 8 weeks of calcineurin activation followed by 8 weeks of recovery. Those mice with fractional shortening greater than 50% at study completion were categorized as responders.

Online Figure VII: Echocardiographic data in responders and nonresponders prior to reverse remodeling. A: Mean fractional shortening measured from M-mode tracings obtained at 8 weeks after removing doxycycline and at 16 weeks (8 weeks after restarting doxycycline. B: Mean left ventricular internal diameter in diastole (LVIDd), left ventricular posterior wall thickness in diastole (LVPWd), and LVPWd-to-LVIDd ratio measured 8 weeks after removing doxycycline in responders and nonresponders.

Online Figure VIII: Calcineurin-induced cardiomyopathy was not associated with ventricular arrhythmia. A. Representative in vivo electrophysiology study showing inducible ventricular tachyarrhythmia with extra-stimulation in a mouse subjected to severe transverse aortic constriction (sTAC). Lead I, surface electrocardiogram lead; RV EGM, right ventricular electrogram, RA EGM, right atrial electrogram, S1/S2/S3, ventricular pacing stimuli. B. Frequency of inducible ventricular arrhythmia in mice during in vivo electrophysiology studies.
Supplemental References


Online Figure I

A

7 days off Dox

<table>
<thead>
<tr>
<th></th>
<th>tTA</th>
<th>tTA/CnA*</th>
</tr>
</thead>
<tbody>
<tr>
<td>CnA*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RCAN 1.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RCAN 1.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>tubulin</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

B

<table>
<thead>
<tr>
<th>tTA/CnA*</th>
<th>Days off Dox</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5 days</td>
</tr>
<tr>
<td>CnA*</td>
<td></td>
</tr>
<tr>
<td>RCAN 1.1</td>
<td></td>
</tr>
<tr>
<td>RCAN 1.4</td>
<td></td>
</tr>
<tr>
<td>tubulin</td>
<td></td>
</tr>
</tbody>
</table>

|             | 4 days        |
| CnA*        |               |
| RCAN 1.1    |               |
| RCAN 1.4    |               |
| tubulin     |               |

C

IB: βMHC
Protein Loading

250
150

Online Figure I
Online Figure II

A

OFF

ON

B

Myocyte Cross-sectional area, µm²

Myocyte Cross-sectional area, µm²

OFF

ON

Online Figure II
Heart Weight/Body Weight (mg/gm) | Fractional Shortening (%)
---|---
WT | tTA | tTA/CnA* | WT | tTA | tTA/CnA*

* p < .05

p = NS
Online Figure IV

**A**

Heart Weight (HW, mg) vs. LV Mass by Echo (mg)

**B**

Difference in HW and LV Mass by Echo (mg) vs. Mean of HW and LV Mass by Echo (mg)
Online Figure V

Heart Rate (bpm)

Baseline           2                  4                  6               8 weeks

ON
OFF
Online Figure VII

A

Fractional Shortening (%)

Nonresponders
Responders

p = 0.13

B

<table>
<thead>
<tr>
<th>Metric</th>
<th>8 weeks</th>
<th>16 weeks</th>
</tr>
</thead>
<tbody>
<tr>
<td>LVIDd (mm)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LVPWd (mm)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LVPWd:LVIDd (ratio)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Online Figure VII
Online Figure VIII

The figure shows electrographic recordings from different leads (Lead I, RV EGM, RA EGM) with annotations indicating the time points (S1, S2, S3) and a time scale of 500 msec. A bar graph illustrates the percentage of mice with inducible VT (%), with p = 0.011. The table below summarizes the number of mice with VT in different conditions:

<table>
<thead>
<tr>
<th>Condition</th>
<th>Sham</th>
<th>sTAC</th>
<th>CnA OFF</th>
<th>CnA ON</th>
</tr>
</thead>
<tbody>
<tr>
<td>Data</td>
<td>1 of 14</td>
<td>5 of 8</td>
<td>0 of 5</td>
<td>0 of 5</td>
</tr>
</tbody>
</table>