Calcineurin/NFAT Coupling Participates in Pathological, but not Physiological, Cardiac Hypertrophy

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Abstract—Calcineurin (PP2B) is a calcium/calmodulin-activated, serine-threonine phosphatase that transmits signals to the nucleus through the dephosphorylation and translocation of nuclear factor of activated T cell (NFAT) transcription factors. Whereas calcineurin-NFAT signaling has been implicated in regulating the hypertrophic growth of the myocardium, considerable controversy persists as to its role in maintaining versus initiating hypertrophy, its role in pathological versus physiological hypertrophy, and its role in heart failure. To address these issues, NFAT-luciferase reporter transgenic mice were generated and characterized. These mice showed robust and calcineurin-specific activation in the heart that was inhibited with cyclosporin A. In the adult heart, NFAT-luciferase activity was upregulated in a delayed, but sustained manner throughout eight weeks of pathological cardiac hypertrophy induced by pressure-overload, or more dramatically following myocardial infarction-induced heart failure. In contrast, physiological hypertrophy as produced in two separate models of exercise training failed to show significant calcineurin-NFAT coupling in the heart at multiple time points, despite measurable increases in heart to body weight ratios. Moreover, stimulation of hypertrophy with growth hormone–insulin-like growth factor-1 (GH-IGF-1) failed to activate calcineurin-NFAT signaling in the heart or in culture, despite hypertrophy, activation of Akt, and activation of p70 S6K. Calcineurin Aβ gene–targeted mice also showed a normal hypertrophic response after GH-IGF-1 infusion. Lastly, exercise- or GH-IGF-1–induced cardiac growth failed to show induction of hypertrophic marker gene expression compared with pressure-overloaded animals. Although a direct cause-and-effect relationship between NFAT-luciferase activity and pathological hypertrophy was not proven here, our results support the hypothesis that separable signaling pathways regulate pathological versus physiological hypertrophic growth of the myocardium, with calcineurin-NFAT potentially serving a regulatory role that is more specialized for maladaptive hypertrophy and heart failure. (Circ Res. 2004;94:110-118.)

Key Words: signaling ■ calcineurin ■ hypertrophy ■ heart failure ■ transcription

Cardiac hypertrophy is defined as an enlargement of the heart associated with an increase in cardiac myocyte cell volume that occurs in response to diverse pathophysiological stimuli such as hypertension, ischemic heart disease, valvular insufficiency, infectious agents, or mutations in sarcomeric genes.1 Hypertrophic growth of the myocardium is thought to preserve pump function, although prolongation of the hypertrophic state is a leading predictor for the development of arrhythmias, sudden death, and heart failure.2,3 However, not all forms of cardiac hypertrophy are necessarily pathological, as athletic conditioning can stimulate heart growth without deleterious consequences.4 A number of studies have been dedicated to elucidating the molecular mechanisms underlying the hypertrophic growth process of the myocardium.5,6 One pathway that has received attention is mediated by the calcium/calmodulin-activated protein phosphatase, calcineurin (PP2B). Calcineurin is activated by sustained elevations in intracellular calcium, which facilitates binding to its primary downstream effector, nuclear factor of activated T cells (NFAT).7 NFAT transcription factors are normally hyperphosphorylated and sequestered in the cytoplasm, but rapidly translocate to the nucleus after calcineurin-mediated dephosphorylation.7 Cardiac-specific activation of calcineurin or its downstream effector NFAT are sufficient to induce a robust hypertrophic response in transgenic mice.8 Furthermore, genetic inhibition of calcineurin or NFAT has shown the pathway to be necessary for a full hypertrophic response in a number of rodent models.9 Some reports have also shown elevations in calcineurin protein levels or phosphatase activity in failing or hypertrophic human hearts, suggesting that this pathway might regulate pathological remodeling and failure.10–12

In contrast to the proposed pathological role for calcineurin-NFAT signaling in the heart, a signaling pathway
initiated by insulin-like growth factor-1 (IGF-1) has been hypothesized to mediate physiological and developmental growth of the myocardium. IGF-1 binds to its receptor on the cell surface leading to the activation of phosphoinositide 3-kinase (PI3K), which in turn promotes activation of Akt through phosphoinositide-dependent protein kinase-1. Akt then facilitates activation of mammalian target of rapamycin (mTOR), leading to p70 S6 kinase activation and augmented protein synthesis. Transgenic mice overexpressing activated mTOR, leading to p70 S6 kinase activation and augmented protein synthesis, have been generated using activated adenoviral vector (Nutropin AQ, Genentech) and Long R3-IGF-1 (JRH Biosciences). Hypertrophy or dilated heart failure remain an area of ongoing controversy.

Materials and Methods

NFAT-Luc Construction and Generation of Transgenic Mice

Nine copies of an NFAT binding site from the IL-4 promoter (5′-TGGAATT-3′) were positioned 5′ to a minimal promoter from the α-myosin heavy chain gene (−164 to +16) and inserted upstream of the luciferase reporter in pGL-3 Basic (Promega) to create NFAT-luc. The NFAT-luciferase transgene was injected into newly fertilized oocytes to generate transgenic mice (FVBN background), which gave mice that were phenotypically normal. To create NFAT-luc, the same construct was cloned into the NotI sites of pAC-CMVpLpA from which the CMV promoter was removed. Recombinant adenovirus was generated in HEK293 cells using previously described methods. All procedures performed in animals were approved by the Institutional Animal Care and Use Committee.

Cell Culture

All in vitro experiments utilizing AdNFAT-luc or a similarly designed AdMEF-2-luc were performed in neonatal ventricular myocytes isolated from 1- to 2-day-old rats; infection and culture conditions have been previously reported.

In Vivo Surgical and Exercise Protocols

Pathological hypertrophy was induced by constriction of the transverse aortic arch. In short, the transverse aortic arch was visualized through a median sternotomy and 7-0 silk ligature was tied around the aorta (27-gauge constriction) between the right brachiocephalic and left common carotid arteries. To generate heart failure, NFAT-luc mice were subjected to permanent ligation of the left anterior descending (LAD) coronary artery for 21 days. Protocols have been previously described. Growth hormone (GH)-IGF-1 infusion model of hypertrophy was previously described. Growth hormone (Nutropin AQ, Genentech) and Long R3-IGF-1 (JRH Biosciences) were infused at 4 mg/kg each, twice a day via subcutaneous injection.

Molecular Analyses

Western blotting was performed as previously described. Luciferase assays from hearts were performed as previously described. Dot blotting for mRNA levels of hypertrophic marker genes was described previously.

Statistical Analysis

Differences between experimental groups were analyzed by a two-tailed Student’s t test using Excel software.

Results

Construction and Characterization of NFAT-Luciferase Transgenic Mice

The traditional calcineurin enzymatic assay is wrought with both technical and theoretical difficulties (see Discussion). A surrogate measure of calcineurin activity is through analysis of NFAT transcriptional responsiveness. In this study, we generated transgenic mice containing an NFAT binding site-dependent luciferase reporter as a means of examining calcineurin-NFAT signaling in the heart. The transgene includes nine concatamerized high-affinity NFAT binding sites from the IL-4 promoter and a minimal promoter (Figure 1A). Seven founder lines were established, two of which...
(lines 15.1 and 15.14) displayed calcineurin-inducible reporter activity in the heart (see next section). Line 15.1 was chosen for all subsequent analyses given its relatively high level of expression in the heart.

NFAT-luciferase transgenic mice displayed detectable activity in most tissues surveyed at 3 weeks of age, with highest expression occurring in the brain, kidney, and heart, each of which are sites of considerable calcineurin protein expression (Figure 1B). Line 15.14 transgenic mice displayed a very similar profile of basal expression in each of these tissues, albeit with lower absolute activity per microgram of protein (data not shown). NFAT-luciferase activity was assessed from hearts of line 15.1 mice at different developmental times, both pre- and postnatally. Interestingly, NFAT-luciferase activity peaked during developmental maturation of the heart, whereas the adult heart showed relatively lower activity (Figure 1C).

NFAT-Luciferase Reporter Activity Is Calcineurin Responsive

To verify the specificity of the NFAT-luciferase reporter, these mice were crossed with activated calcineurin transgenic mice, as well as treated with the calcineurin-specific inhibitor cyclosporin A (CsA). At 2 and 6 weeks of age, the activated calcineurin transgene, which is expressed only within myocytes, produced a 6- to 10-fold increase in NFAT reporter activity in the heart that was inhibited after 36 hours of CsA administration (Figure 2A). The calcineurin transgene produced a 2-fold increase in heart weight to body weight ratios, and this increase was not affected by short CsA treatment, indicating that reporter activity likely reflects cardiac calcineurin signaling and not the hypertrophic state of the heart (Figure 2B). Adult cardiac myocytes were also purified after enzymatic disassociation, from mice previously treated with or without CsA, as a means of further verifying that myocytes themselves can express the transgene and that this expression is calcineurin-regulated (Figure 2C).

NFAT-Luciferase Activity in Pathological Hypertrophy

Acute pressure overload is typically used as a means of inducing a pathological profile of cardiac hypertrophic growth and ventricular remodeling.5,6 Whereas initiation of pressure overload–induced hypertrophy (first 2 days) is associated with immediate early gene activation, less is known of the signaling factors or genes that sustain the long-term hypertrophic growth of the myocardium and its transition to dilated failure. To address these issues, NFAT-luciferase transgenic mice were subjected to transverse aortic constriction (TAC) for various lengths of time, followed by analysis of cardiac luciferase activity. Interestingly, cardiac calcineurin-NFAT activity was not increased within 24 hours, suggesting a delay in the initiation phase of this pathway (Figure 3A). However, by day 2 and thereafter, NFAT-luciferase activity was elevated by 2- to 3-fold for up to 8 weeks (Figure 3A). This activation profile, combined with the fact that NFAT reporter activity is elevated before the onset of definable hypertrophy (Figures 3B and 3C), suggests that calcineurin-NFAT signaling functions as a delayed, but sustained signal for pathological hypertrophy. These data are similar to our previous study that used a calmodulin immunoprecipitation technique as a means of measuring calcineurin activation in the heart. Lim et al28 demonstrated a 2-day delay in calcineurin-calmodulin association after aortic banding in the rat, but after this time activation was maintained for at least 6 weeks.

Calcineurin-NFAT Signaling Is Upregulated in Failing Hearts

Although pathological hypertrophy has many potential etiologies, a common clinical endpoint is congestive heart failure. Previous reports have shown elevated calcineurin activity in
ventricular tissue from failing human hearts, yet others have not seen an association. In this study, NFAT-luciferase transgenic mice were subject to permanent left anterior descending coronary artery (LAD) occlusion, creating a myocardial infarct (MI) model of heart failure. Twenty-one days afterward, these mice showed substantial loss of left ventricular tissue, scarring, and interstitial fibrosis (Figure 4E). Assessment of heart and lung weight to body weight ratios and fractional shortening measured by echocardiography showed two distinct phenotypes of mice: those with overt failure (n/11005/3) and those that were less severely affected (n/11005/5) (Figures 4B through 4D). Ventricular tissue was harvested from the noninfarcted area for assessment of NFAT-luciferase activity. Compared with sham-operated mice, nonfailing hearts showed a 1.7-fold increase in cardiac NFAT activity, similar to mice undergoing hypertrophy in response to pressure overload (Figure 4A). In contrast, severely failing hearts displayed a significantly higher level of NFAT-luciferase activity (5-fold) compared with sham controls (Figure 4A). These results indicate that the magnitude of calcineurin-NFAT signaling correlates with the severity of the underlying pathological condition.

**Exercise-Induced Hypertrophy Fails to Upregulate NFAT-Luc Reporter Activity**

The data described in the previous sections suggest that calcineurin-NFAT signaling is associated with growth and remodeling of the myocardium after pathological stimulation. However, the role that calcineurin-NFAT signaling plays in mediating adaptive or physiological growth of the myocardium is less defined. In this study, separate cohorts of NFAT-luciferase reporter transgenic mice were exercised using either voluntary free wheel running or swimming for various lengths of time. All mice analyzed underwent a similar degree of exercise stimulation (similar number of wheel revolutions...
Calcineurin-NFAT/MEF2 Signaling Is Not Regulated by IGF-1–Akt Signaling

That mice undergoing swimming showed Akt activation is consistent with the observation that competitive male athletes have elevated IGF-1 levels in the heart. To more carefully evaluate the potential association between IGF-1–PI3K-Akt signaling and calcineurin-NFAT signaling an NFAT-luciferase reporter adenovirus was generated for in vitro studies in cultured cardiomyocytes (AdNFAT-luc). To verify the specificity of this reporter, cultured neonatal rat cardiomyocytes were infected with AdNFAT-luc alone or in combination with viruses encoding activated calcineurin (AdΔCnA) or activated NFATc3 (AdΔNFAT). Coinfection with AdΔCnA or AdΔNFAT resulted in a 200- and 30-fold activation of NFAT-luciferase activity, respectively (Figure 6A). The activity induced by AdΔCnA coinfection, but not AdΔNFAT, was completely inhibited with CsA, further verifying specificity (Figure 6A). Cultured cardiomyocytes infected with the NFAT-luc reporter were also subjected to serum stimulation to examine the time course of activation. Similar to the NFAT-luciferase reporter transgenic mice, a somewhat delayed profile of activation was observed in culture, such that maximal activity was not observed for 24 to 36 hours (Figure 6B) (see Discussion).

The transcription factor myocyte enhancer factor 2 (MEF-2) is also activated by calcineurin. Infection of neonatal cardiomyocytes with a MEF-2–dependent luciferase reporter adenovirus also demonstrated responsiveness to activated calcineurin and partial inhibition with CsA, suggesting that analysis of MEF2 could function as an additional surrogate for calcineurin activation (Figure 6C). Using these two reporters, IGF-1 treatment for 24 hours had no significant effect on activity, despite inducing Akt phosphorylation (Figures 6D and 6E). Moreover, overexpression of Akt with a recombinant adenovirus did not affect either NFAT or MEF-2 reporter activity, in contrast to the massive increase observed with AdΔCnA coinfection (Figures 6A and 6C). By comparison, serum stimulation induced an approximate 5-fold activation of both reporters (Figure 6C). These results suggest that IGF-1–Akt signaling does not activate calcineurin-NFAT signaling, further suggesting a specialization in signaling.

To extend the results observed in vitro, an in vivo model of IGF-1 signaling was performed in the NFAT-luciferase reporter mice. Ross and colleagues previously reported that 14 days of GH/IGF-1 infusion produced a myocardial growth response. NFAT-luciferase mice were injected two times a day with GH/IGF-1 (each at 4 mg/kg), resulting in a 35% increase in body weight, whereas vehicle-injected littermate reporter mice showed only a 3% increase over this time (Figure 7A). Analysis of heart-to-tibia-length ratios showed that GH/IGF-1 injection augmented heart growth, although NFAT-luciferase activity was not altered (Figure 7A). GH/IGF-1 infusion produced robust activation of both Akt and p70 S6K in the hearts of injected mice, further validating the effectiveness of this protocol (Figure 7B). Collectively, these results indicate that cardiac growth driven by IGF-1–Akt signaling does not utilize calcineurin-NFAT.

These results suggest that IGF-1–Akt signaling may induce a different molecular program compared with a pathological stimulus such as pressure overload. To more directly address this interpretation, molecular markers of cardiac hypertrophy were analyzed by mRNA dot blotting. The data show that TAC stimulation for 14 days induced expression of β-myosin heavy chain (β-MHC), atrial natriuretic factor (ANF), b-type natriuretic peptide (BNP), and skeletal α-actin in the heart. In contrast, mice subjected to 20 days of swimming or 14 days of GH/IGF-1 infusion had no induction of these same marker genes in the heart, despite Akt and p70 S6K activation (Figure 7C). These results support the hypothesis that different molecular pathways underlie pathological versus physiological cardiac hypertrophy.

Finally, calcineurin Aβ gene–targeted mice were analyzed for their ability to hypertrophy after GH/IGF-1 infusion. Previously, calcineurin Aβ-null mice were shown to have a substantial defect in their ability to undergo cardiac hypertrophy after a pathological stimulus due to pressure overload. However, GH/IGF-1 infusion induced the same relative level of cardiac hypertrophy between calcineurin Aβ-null mice and their strain-matched wild-type controls (Figure 7D). These results indicate that GH/IGF-1 stimulation does not depend on optimal calcineurin activity for the induction of cardiac hypertrophy.

Discussion

Advantages of an In Vivo Reporter System

In recent years, a number of groups have constructed and characterized transgenic mice carrying reporter elements as a way of directly assessing biological activity in vivo. Reporter
transgenic mice have been characterized for the transcription factors myocyte enhancer factor-2 (MEF2), estrogen receptor, NFκB, and LEF/TCF. These systems allow for the determination of the spatiotemporal activity of transcription factors or their upstream signaling effectors in a quantitative manner. By comparison, calcineurin-NFAT activity has been traditionally measured with an enzymatic assay from protein lysates. However, the enzymatic phosphatase assay is plagued by both technical and theoretical shortcomings. For example, calcineurin activity is extremely sensitive to oxidation, and commonly used lysis buffers lack reducing agents. The calcineurin assay requires addition of calcium and saturating levels of calmodulin, which essentially nullifies any differences in activity due to calmodulin association. Lastly, it is still unclear how measurement of calcineurin activity from protein lysates reflects in vivo activity, if at all. An alternative method for measuring calcineurin activity consists of calmodulin immunoprecipitation followed by Western blotting for calcineurin protein association. Although this later assay has revealed data similar to that reported with the NFAT-luciferase reporter transgene in this study, it is also less than ideal.

Analysis of NFAT subcellular distribution or transcriptional activity is arguably the most reliable means of assessing calcineurin activity, especially given the intimate relationship between these two factors. However, the activation profile of NFAT is also modulated by counter-acting kinases such as c-Jun N-terminal kinases, p38, protein kinases A, glycogen synthase kinase 3, and casein kinase. These considerations suggest that although the NFAT-luciferase reporter mice can serve as a calcineurin assay surrogate in the heart, the effects of other signaling pathways likely modulate the magnitude and duration of the read-out. Finally, whereas the NFAT-luciferase reporter transgene reveals important correlative data suggesting a role for this pathway in various disease states of the heart, it does not prove a direct cause-and-effect relationship.
Immediate Versus Delayed Activation of Calcineurin in Pathological Hypertrophy

Our data showing continued activation of the NFAT-luc reporter throughout pressure-overload hypertrophy and infarct-induced failure strengthen the hypothesis that calcineurin-NFAT functions to maintain the hypertrophic profile of the heart. This hypothesis is also supported by the fact that hypertrophied and failing hearts have altered intracellular Ca²⁺ handling,²⁶ potentially serving as part of the stimulus for calcineurin-NFAT activation. However, it is uncertain why significant calcineurin-NFAT activation is not observed until 24 to 36 hours after stimulation in vitro and in vivo. This is in contrast to in vitro reports using calcium ionophores, which stimulate NFAT translocation within 10 minutes.³⁷ One possible explanation lies in the mechanisms whereby NFAT factors are phosphorylated by the kinases discussed earlier, directly antagonizing effects of calcineurin. It is conceivable that, although NFATs initially accumulate in the nucleus immediately after an appropriate calcium signal, the actual transcriptional response is integrated over substantially longer periods of time that require maintained presence of NFAT. Indeed, many of the stimuli that activate calcineurin in cardiac myocytes also activate mitogen-activated protein kinases (MAPKs) and PKA, which would temporarily antagonize NFAT nuclear accumulation. However, it is possible that these kinase-dependent signaling pathways are partially desensitized within 1 to 2 days, thus permitting significant NFAT nuclear accumulation thereafter.

Role of Calcineurin-NFAT in Pathological Versus Physiological Hypertrophy

In most models of pathological hypertrophy studied to date, inhibition of calcineurin-NFAT signaling has yielded either a reduction in the hypertrophic response and/or a delay in the progression from hypertrophy to heart failure.³⁸ The data presented in this study extend this paradigm to demonstrate that calcineurin-NFAT signaling is activated in a sustained manner during both TAC-induced pressure overload and myocardial infarction–induced heart failure. However, very little is presently known regarding the role of calcineurin-NFAT signaling in regulating physiological hypertrophy or adaptive growth of the myocardium. Our results indicate that calcineurin-NFAT is not activated after either voluntary wheel-running or swimming, despite the observation of significant cardiac hypertrophy. In fact, swimming exercise even produced a significant and reproducible reduction in NFAT-luciferase activity in the heart at certain time points. Also of note, direct infusion of GH-IGF-1, which is thought to underlie adaptive or physiological growth, had no effect on NFAT-luciferase activity in response to 2% fetal bovine serum.²⁹,³⁰,³¹ The data from our experiments support the hypothesis that calcineurin signaling, particularly calcineurin-NFAT activation, is not relevant to physiological hypertrophy. However, it is possible that calcineurin activity is partitioned between effectors based on the magnitude or duration of the stimulus. Indeed, calcineurin signaling has been previously shown to underlie exercise-induced activa-
known role in regulating the physiological hypertrophy program of the myocardium, potentially through a transcriptional regulatory partner such as AP-1 or GATA4. It is also possible that the NFAT-luciferase reporter line described in this study might be subject, in part, to nonspecific regulation through another factor that recognizes the multimerized NFAT site. However, levels of endogenous cardiac MCIP1 mRNA were not altered in mice subjected to swimming or GH-IGF-1 infusion (data not shown). Because MCIP1 expression is also directly regulated by NFAT activation in the heart, this result further supports the hypothesis that calcineurin-NFAT are not primary mediators of the physiological growth response.

Finally, the data presented in this study suggest that calcineurin inhibition would be desirable in potentially treating certain forms of maladaptive hypertrophy. However, given the relatively toxic profile of existing calcineurin inhibitory drugs, novel therapeutic agents would be needed. Also of concern, inhibition of calcineurin signaling may render the heart more susceptible to apoptotic cell death because this pathway has been previously implicated in the survival of cardiac myocytes after hypoxic stimuli, as extensively detailed previously.42

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