NFAT Transcription Factors Are Critical Survival Factors That Inhibit Cardiomyocyte Apoptosis During Phenylephrine Stimulation In Vitro

William T. Pu, Qing Ma, Seigo Izumo

Abstract—Biomechanical stress on the heart results in activation of numerous signaling cascades, leading to cardiomyocyte hypertrophy, apoptosis, and ultimately, heart failure. The Ca\(^{2+}\)/CaM-dependent phosphatase calcineurin is an essential mediator of cardiac hypertrophy, and in most but not all studies, calcineurin inhibition attenuated cardiac hypertrophy in vivo. However, calcineurin inhibition has been reported to have adverse effects on cardiac remodeling and cardiomyocyte apoptosis. Calcineurin regulates the activity of a number of downstream targets, including the transcription factors NFAT, MEF2, and NF-\(\kappa\)B, and the apoptotic factor Bad. To evaluate the contribution of NFAT activation by calcineurin to cardiomyocyte responses to hypertrophic stimulation, we used adenovirus to express VIVIT, a selective peptide inhibitor of calcineurin-mediated NFAT activation. We found that selective NFAT inhibition during phenylephrine stimulation inhibited hypertrophy but resulted in increased cardiomyocyte apoptosis. In contrast, nonselective inhibition of calcineurin by cyclosporin A did not cause cardiomyocyte apoptosis after phenylephrine stimulation. Cyclosporin A suppressed the effect of VIVIT on cardiomyocyte apoptosis. These results demonstrate that during phenylephrine stimulation calcineurin activates both pro- and antiapoptotic pathways in cardiomyocytes, and that NFAT activity is a critical component of the antiapoptotic pathway that regulates whether the outcome of calcineurin activation is cardiomyocyte apoptosis or survival. (Circ Res. 2003;92:725-731.)

Key Words: apoptosis ■ cardiac hypertrophy ■ calcineurin ■ NFAT ■ phenylephrine
All five NFAT isoforms are expressed in cardiomyocytes. To probe the role of NFAT proteins in cardiomyocyte hypertrophy, we used a previously characterized peptide that selectively antagonizes NFAT activation by calcineurin to probe the role of NFAT proteins in cardiomyocyte hypertrophy. We found that in cultured cardiomyocytes, NFAT activation is essential for hypertrophy in response to stimulation by the α-adrenergic agonist phenylephrine (PE). Surprisingly, we also found that inhibition of NFAT activation during PE stimulation led to increased cardiomyocyte apoptosis, and this effect could be blocked by nonselective calcineurin inhibition with CsA. These results indicate that NFATs act as critical survival factors in the setting of cardiac hypertrophy and heart failure. Our results also raise questions about the mechanisms underlying the disappointing effect of calcineurin inhibition on apoptosis and cardiac remodeling despite its demonstrated activity in blocking cardiac hypertrophy.

**Materials and Methods**

**Reagents**

VIVIT-GFP and human NFATc4 were provided by A. Rao. HA-tagged NFATc4 was obtained from G. Crabtree. Ne4, a constitutively activated mutant of NFATc4, was generated from human NFATc4 by using PCR mutagenesis to add an N-terminal FLAG tag and to delete the N-terminal 317 amino acids, which include the regulatory domain of NFATc4. Ne4 mut, a point mutant of Ne4 in which Arg430 of NFATc4 has been changed to alanine, was constructed by site-directed mutagenesis (QuikChange, Stratagene) based on mutagenesis data for NFATc1. The inability of this mutant to bind DNA was confirmed by gel-shift analysis and by NFAT reporter assays (data not shown). The NFAT-luciferase reporter was previously described. All PCR-generated constructs were verified by DNA sequencing.

**RT-PCR**

Reverse transcription from DNase-treated total RNA was performed using SuperScript II (Invitrogen). PCR was performed under non-saturating conditions using NFAT isoform-specific primer pairs for 20 cycles in the presence of 32P-dATP. PCR products were resolved on a nondenaturing polyacrylamide gel and visualized by autoradiography.

**Adenovirus**

Adenoviruses were generated using the AdEasy system (Stratagene) and purified on CsCl gradients. Viruses were titered by the toxic infectious dose method and used at a multiplicity of infection of 5 to 10.

**Cardiomyocyte Culture**

Neonatal rat ventricular cardiomyocytes were dissociated using collagenase and trypsin and purified from nonmyocytes by centrifugation on a Percoll gradient as previously described. Cardiomyocytes were plated at a density of 0.5 to 1×10^4/cm^2 in 5% horse serum, and switched to serum-free media 16 hours after plating. Cells were infected with adenovirus on the day after plating, proteins were allowed to express for 48 hours, and cells were then stimulated with 10 μmol/L PE. Where indicated, antagonists CsA (1 μg/mL; Sigma) or z-VAD-fmk (100 μmol/L; Calbiochem) were added 30 minutes before PE addition. Adult cardiomyocytes were dissociated by retrograde perfusion with collagenase as previously described.

**Microscopy**

Cell viability was determined 48 hours after PE stimulation by staining with the membrane permeable nucleic acid stain Syto 13 (Molecular Probes) and the membrane impermeable DNA stain propidium iodide. Randomly selected fields were acquired using an MDS290 digital imaging system (Kodak) for later analysis. At least 10 randomly selected 200× fields were counted per sample. To determine cell surface area, myocytes were fixed with 10% buffered formalin followed by staining with α-actinin antibody (Sigma) and DAPI. Cells were imaged as above using at 400× magnification and measured using NIH Image. At least 100 cells were measured per sample. Nuclear translocation was measured 8 hours after stimulation with 10 μmol/L PE. These conditions were chosen to circumvent cell death seen at longer time points. After fixation with 4% PFA, cells were stained with an affinity purified NFATc2 antibody (gift from Dr A. Rao, Harvard Medical School, Boston, Mass) or monoclonal anti-HA antibody (Covance) and imaged using a confocal microscope (BioRad). To quantitate the fraction of cells with nuclear HA-NFATc4 nuclear localization, over 100 consecutive cardiomyocytes transfected with both HA-NFATc4 and VIVIT-GFP (or GFP) were counted. TUNEL staining was performed per the manufacturer’s recommendations (Roche). DNA internucleosomal fragmentation was measured as described previously.

**Statistical Analysis**

Statistical analysis was performed using StatView (SAS Institute). Unless otherwise noted, means derived from 3 independent samples were compared by ANOVA with the Scheffé’s F procedure for post-hoc comparisons. Values are displayed as mean±SEM.
Results

NFAT Isoform Expression in Cardiomyocytes

Five NFAT isoforms have been identified. NFATc1 (also known as NFAT2), NFATc2 (NFAT1), NFATc3 (NFAT4), and NFATc4 (NFAT3) share a common calcineurin docking motif and are regulated by calcium-calmodulin, whereas NFAT5 is not regulated by calcium-calmodulin or calcineurin. In order to determine the distribution of NFAT isoforms in cardiomyocytes, we generated isoform-specific primers and amplified NFATc1 to 4 and NFAT5 by RT-PCR. All five NFAT isoforms were detected in total RNA from whole heart and could be amplified with comparable efficiency under nonsaturating conditions from total RNA from a dissociated adult mouse cardiomyocyte preparation that was composed of at least 90% cardiomyocytes (Figure 1). These results indicate that all known NFAT isoforms are expressed at the RNA level in cardiomyocytes.

NFAT Inhibition With VIVIT Peptide

The redundancy of NFAT isoform expression in cardiomyocytes suggested that analysis of a necessary role for NFATs in cardiac hypertrophy using traditional knockout approaches might be difficult. To circumvent these potential problems, we chose to use a dominant-negative approach using a previously described peptide inhibitor of NFAT activation.13 The VIVIT peptide was found to be 25 times more effective at inhibiting NFAT binding to calcineurin than a peptide spanning the naturally occurring calcineurin binding site of NFATc2.13 To test the effectiveness of VIVIT-GFP in blocking NFAT activation, we cotransfected Jurkat cells with VIVIT-GFP or GFP and an NFAT-dependent reporter. In this system, activation of the reporter requires activation of AP-1 as well as calcineurin; therefore, cells were stimulated by the calcium ionophore ionomycin as well as the PKC agonist phorbol 12-myristate 13-acetate (PMA). As described previously,13 VIVIT-GFP blocked the activation of the NFAT reporter as effectively as the calcineurin antagonist cyclosporin A (CsA), whereas GFP alone had no effect (Figure 2B).13 In comparison to CsA, which nonselectively antagonizes calcineurin catalytic activity, VIVIT peptide is more selective, because it did not interfere with the activation of other calcineurin targets such as NF-kB.13 We used adenovirus to express VIVIT-GFP or GFP in cultured cardiomyocytes (Figure 2C). In response to stimulation with PE and other hypertrophic agonists, NFAT isoforms translocate to the nucleus in cultured neonatal cardiomyocytes.9-11 To measure the effect of VIVIT-GFP expression on NFAT activation, we cotransfected VIVIT-GFP and HA-NFATc4 and monitored the subcellular local-
NFAT Activation Is Necessary for Cardiac Hypertrophy in Response to PE

We examined the response of cardiomyocytes to PE stimulation in the presence of VIVIT-GFP. Control cardiomyocytes expressing GFP hypertrophied normally after PE stimulation, as assessed by the increase in cell surface area (Figure 3A, left panels). In contrast, PE stimulation did not increase the cell surface area of VIVIT-GFP–expressing cardiomyocytes (Figures 3A, right, and 3B). VIVIT-GFP–expressing cardiomyocytes remained competent to hypertrophy, because hypertrophy in response to the protein kinase C agonist PMA was not affected by expression of VIVIT-GFP (data not shown). The inhibition of cardiomyocyte hypertrophy in response to PE by VIVIT-GFP is consistent with prior findings that overexpression of calcineurin inhibitory proteins or a dominant-negative NFAT mutant, or treatment with CsA, blocked cardiomyocyte hypertrophy,1,10,19–22 and demonstrates an essential role for NFAT in cardiac hypertrophy induced by PE but not by PMA.

NFAT Activation Promotes Cellular Survival During PE Stimulation

While studying the effect of VIVIT on cardiac hypertrophy, we noticed that PE stimulation resulted in more than a 2-fold increase in the prevalence of dead cells in VIVIT-GFP–expressing cardiomyocytes (Figures 4A and 4B). This effect was not seen in unstimulated cells expressing VIVIT-GFP, and was unrelated to GFP overexpression or adenoviral infection because control cells infected with GFP-expressing adenovirus showed baseline levels of cell death in the presence or absence of PE (Figures 4A and 4B). In addition, we obtained similar results using an adenovirus that expresses a tandemly repeated VIVIT peptide without a GFP tag (data not shown).

To confirm that the decrease in cell viability in PE-treated, VIVIT-GFP–expressing cells was due to inhibition of NFAT signaling, we asked whether or not expression of a constitutively activated NFATc4 mutant protein (Nc4)1 would rescue cells from the effects of VIVIT-GFP plus PE. We found that compared with expression of β-galactosidase in control cells, expression of Nc4 significantly reduced the effect of VIVIT-GFP on cell death (Figure 4C). In contrast, in the context of VIVIT-GFP plus PE treatment, cells expressing an NFATc4 mutant deficient in DNA-binding (Nc4 mt) had an incidence of cell death that was indistinguishable from control cells expressing β-galactosidase (Figure 4C). These results demonstrate that the effect of VIVIT-GFP on cell viability was due to NFAT inhibition, and that cell survival after PE stimulation requires NFATc4 activation and DNA-binding. Consistent with an antiapoptotic role for NFAT proteins, expression of Nc4 significantly reduced cardiomyocyte cell death due to apoptotic stress by H2O2 or staurosporine (Figure 4D).

Calcineurin Inhibition Blocks VIVIT and PE-Induced Cell Death

Prior studies using nonselective calcineurin inhibitors in PE-stimulated cardiomyocytes did not describe an alteration in cellular viability. We found that CsA treatment of unstimulated or PE-stimulated cells had no effect on cell death in GFP-expressing control cardiomyocytes (Figure 4E). In contrast, the increased cell death due to PE plus VIVIT-GFP was blocked by CsA (Figure 4E). This result indicates that calcineurin activation of other downstream targets is necessary for cell death due to NFAT inhibition.
Cell Death in the Presence of VIVIT Plus PE Is Due to Apoptosis

We asked if the increased cell death seen in cardiomyocytes treated with VIVIT-GFP and PE was due to apoptosis. Terminal dUTP nick end-labeling (TUNEL) of VIVIT-GFP– and PE-treated cardiomyocytes demonstrated increased TUNEL-positive nuclei compared with controls (Figure 5A). Analysis of DNA isolated from cardiomyocytes after treatment with VIVIT-GFP and PE showed increased internucleosomal DNA fragmentation (DNA laddering), consistent with increased apoptosis (Figure 5B). Treatment of cardiomyocyte cultures with the caspase inhibitor z-VAD-fmk blocked the increase in cell death observed after VIVIT-GFP and PE treatment (Figures 5C and 5D). Collectively, these results demonstrate that PE stimulation in the setting of NFAT inhibition by VIVIT-GFP resulted in increased cardiomyocyte apoptosis, and that apoptosis was the principal mechanism of increased cell death.

Because PE stimulation of VIVIT-GFP–expressing cardiomyocytes increased apoptosis, we asked whether or not the failure of VIVIT-GFP–expressing cells to hypertrophy in response to PE was due to activation of apoptotic pathways. We found that addition of z-VAD-fmk to VIVIT-GFP–expressing cardiomyocytes did not restore the capacity of these cells to hypertrophy in response to PE. The surface area of VIVIT-GFP–expressing cells treated with PE and z-VAD-fmk was 1003±53 μm², compared with 973±55 μm² in the absence of z-VAD-fmk (P>0.05; unpaired t test). Thus, increased apoptosis and attenuation of hypertrophic responses are distinct phenotypes that result from selective NFAT inhibition.

Figure 4. Increased cardiomyocyte death due to PE stimulation and NFAT inhibition. A, Cardiomyocytes expressing GFP or VIVIT-GFP were stimulated with vehicle or PE. After 48 hours, all cells were stained with Syto 13 (green), and inviable cells were stained with propidium iodide (red). Scale bar=50 μm. B, NFAT inhibition resulted in increased cell death during PE stimulation. Cell death is expressed as the ratio of propidium iodide stained nuclei to all nuclei. In unstimulated cells, VIVIT did not significantly alter cell death. In the presence of PE, VIVIT expression significantly increased cell death compared with LacZ or GFP controls (P<0.001). C, Cardiomyocytes infected with VIVIT-GFP plus LacZ, constitutively activated NFATc4 (Nc4), or a non–DNA-binding point mutant (Nc4 mt), were stimulated with PE and assayed for cell death. Compared with control cells expressing LacZ, cells expressing Nc4 had a lower incidence of cell death (P=0.04). In contrast, a non–DNA-binding point mutant (Nc4 mt) did not reduce cell death induced by VIVIT+PE. D, Overexpression of Nc4 protects cardiomyocytes from apoptotic stress. Cardiomyocytes overexpressing LacZ or Nc4 were treated with H₂O₂ (200 μmol/L) or staurosporine (STS, 2 μmol/L). Cell death was significantly decreased by Nc4 compared with LacZ (P<0.05; n=3; unpaired t test). E, Unstimulated or PE-stimulated cardiomyocytes expressing GFP or VIVIT-GFP were cultured in the presence or absence of CsA. CsA treatment had no effect on cell death. In PE-stimulated cells, VIVIT-GFP expression increased cell death compared with GFP-expressing controls (P=0.004). This effect was diminished by CsA (P=0.003 compared with VIVIT-GFP+PE-CsA).

Figure 5. Cell death due to PE stimulation and NFAT inhibition was due to increased cardiomyocyte apoptosis. A, Neonatal cardiomyocytes expressing GFP or VIVIT-GFP and treated with vehicle or PE were assayed for apoptosis by TUNEL staining and confocal imaging. Green, GFP. Red, TUNEL. Scale bar=50 μm. B, Low molecular weight DNA was collected from cells treated as in A, separated on an agarose gel, and visualized by staining with ethidium bromide. C, Cell death is inhibited by the broad spectrum caspase inhibitor zVAD-fmk. Green, Syto 13. Red, propidium iodide. D, Quantitative analysis of C. Cell death was determined as in Figure 2. PE stimulation of VIVIT-expressing cells increased cell death (P<0.001 vs unstimulated cells), and this effect was blocked by zVAD-fmk (P<0.001 vs VIVIT+PE). Scale bar=50 μm.
Discussion

Essential Role for NFAT in Cardiomyocyte Hypertrophy

Multiple lines of evidence indicate that calcineurin activation is essential for cardiomyocyte hypertrophy (reviewed in Reference 2). However, a necessary role for NFAT in cardiac hypertrophy is less well established. By overexpressing a dominant-negative protein consisting of the N-terminus of NFATc4 blocked hypertrophy of cultured cardiomyocytes treated with cardiotropin-1 or endothelin-1. In addition, genetic knockout of NFATc3 but not NFATc4 attenuated the hypertrophic response to angiotensin II and pressure-overload.

Antiapoptotic Function of NFAT During Cardiomyocyte Hypertrophy

While inhibition of NFAT activation did not alter the survival of unstimulated cardiomyocytes, we found that PE stimulation of cardiomyocytes overexpressing the dominant-negative NFAT inhibitory peptide underwent apoptosis at an increased rate. This effect could be blocked by expression of constitutively activated NFATc4, but not by a similar NFATc4 mutant protein that failed to bind DNA. These results indicate that NFAT activation protects cardiomyocytes from apoptosis during stimulation by PE.

PE stimulates the hypertrophic response in cardiomyocytes by activating the heterotrimeric G protein Gq. Gq activation has been associated with cardiomyocyte apoptosis both in vitro and in vivo. Whereas physiological levels of Gq activation protected cardiomyocytes from apoptotic stress, high levels of Gq stimulation induced by adenoviral overexpression of activated Gq led to cardiomyocyte apoptosis in cultured cardiomyocytes. In transgenic mice, overexpression of Gq resulted in compensated cardiac hypertrophy, and imposition of additional hemodynamic loads (pregnancy or pressure overload) resulted in decompensated heart failure associated with increased cardiomyocyte apoptosis. The calcineurin-NFAT pathway is activated by Gq stimulation, and our results indicate that NFAT activation may account at least in part for the protective effects of physiological levels of Gq activation.

Pro- and Antiapoptotic Consequences of Calcineurin Activation

We found that treatment of cardiomyocytes with the nonselective calcineurin antagonist CsA did not alter cardiomyocyte cell death during PE stimulation. In addition, CsA rescued cardiomyocytes from increased cell death associated with selective NFAT inhibition during PE stimulation. These results are consistent with a model in which calcineurin activates both pro- and antiapoptotic pathways in cardiomyocytes, and NFAT is a critical component of the antiapoptotic pathway (Figure 6). This model is consistent with data from other cell types, in which calcineurin activation has been found to have both proapoptotic and antiapoptotic consequences, sometimes in the same cell type. In cardiomyocytes, calcineurin likewise has been found previously to both promote and inhibit apoptosis.

Our results suggest that NFAT is a critical regulator of the balance between pro- and antiapoptotic consequences of calcineurin activation. Importantly, NFAT transcriptional activity is also regulated by its interaction with a number of other transcription factors such as AP-1, MEF2, and GATA4, each of which is regulated by additional signaling pathways. In addition, mitogen-activated protein kinases directly phosphorylate NFAT and modify its activity. Thus, NFAT may integrate inputs from multiple signaling pathways to regulate cardiomyocyte apoptosis during the hypertrophic response.

Our results have implications for therapeutic strategies designed to treat heart failure. Although calcineurin inhibition resulted in attenuation of cardiac hypertrophy in most animal models, recent studies have suggested that calcineurin inhibition by CsA may adversely affect cardiac remodeling and increase cardiomyocyte apoptosis in vivo. Our results indicate that the increased apoptosis may be due in part to decreased NFAT activity and suggest that therapeutic strategies of calcineurin inhibition that spare NFAT function may avert these deleterious consequences of nonselective calcineurin inhibition.

Acknowledgments

W.T.P. was supported by grants from the Hood Foundation and the National Heart, Lung, and Blood Institute (NHLBI). S.I. was supported by NHLBI grants RO1 AG61716 and HL65742. The authors thank A. Rao for reagents and advice.

References

2. Wilkins BJ, Molkentin JD. Calcineurin and cardiac hypertrophy: where have we been? Where are we going? J Physiol. 2002;541:1–8.
3. Yang G, Meguro T, Hong C, Asai K, Takagi G, Karoor VL, Sadoshima J, Vatner DE, Bishop SP, Vatner SF. Cyclosporine reduces left ventricu-
ular mass with chronic aortic banding in mice, which could be due to apoptosis and fibrosis. *J Mol Cell Cardiol.* 2001;33:1505–1514.


NFAT Transcription Factors Are Critical Survival Factors That Inhibit Cardiomyocyte Apoptosis During Phenylephrine Stimulation In Vitro
William T. Pu, Qing Ma and Seigo Izumo