Raf-1 Kinase Is Required for Cardiac Hypertrophy and Cardiomyocyte Survival in Response to Pressure Overload

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Background—Cardiac hypertrophy is a common response to pressure overload and is associated with increased mortality. Mechanical stress in the heart results in the activation of the small GTPase ras and the Raf-1/MEK/ERK signaling cascade in addition to other signaling pathways.

Methods and Results—in an attempt to determine the requirement for the serine/threonine kinase Raf-1 in the pathogenesis of cardiac hypertrophy, we generated transgenic mice with cardiac-specific expression of a dominant negative form of Raf-1 (DN-Raf). DN-Raf mice appeared normal at birth, were fertile, and had normal cardiac structure and function in the absence of provocative stimulation. In response to pressure overload, cardiac extracellular signal–regulated kinase (ERK) activation was inhibited, but c-Jun N-terminal kinase (JNK) activation and p38 mitogen-activated protein kinase (MAPK) activation were normal. DN-Raf mice were sensitized to pressure overload and the development of cardiomyocyte apoptosis, and ≥35% of animals died within 7 days of aortic banding. Surviving DN-Raf animals were markedly resistant to the development of cardiac hypertrophy and hypertrophic gene induction in response to transverse aortic constriction.

Conclusions—These results establish that Raf-1 kinase activity is essential for cardiac hypertrophy and cardiomyocyte survival in response to pressure overload. (Circulation. 2004;110:718-723.)

Key Words: proto-oncogene proteins c-raf ■ protein kinases ■ cardiomegaly ■ signal transduction

Human cardiac hypertrophy often develops as a by-product of hypertension or valvular heart disease. Adult cardiomyocytes are unable to divide and respond to stress and growth stimuli by increasing their rate of protein synthesis, resulting in increased cell volume.1 Growth of individual cardiomyocytes results in thickening of the heart. Cardiac hypertrophy is a potent risk factor for the development of cardiac arrhythmias, diastolic dysfunction, congestive heart failure, and death.2,3

Pressure overload activates several signaling pathways in cardiac tissue. Pressure overload causes the local release of ligands, including angiotensin II and endothelin-1 in the heart.1,4 Angiotensin II and endothelin-1 bind to G protein–coupled receptors on the surface of cardiomyocytes. Many G protein–coupled receptors, including the receptors for endothelin-1 and angiotensin II, activate the small GTPase ras by mechanisms that may involve activation of protein kinase C or transactivation of the epidermal growth factor receptor.5 GTP-bound ras, in turn, recruits the Raf-1 serine/threonine kinase, or the highly related B-Raf, to the plasma membrane, where it is activated in a complex, multistep process.6–8 Active Raf-1 or B-Raf triggers a kinase cascade that includes MEK1/2 (hereafter referred to as MEK) and ERK1/2 mitogen-activated protein kinase (MAPK) (hereafter referred to as ERK).9–11 ERK is a pleiotropic kinase that phosphorylates many targets, including transcription factors, cytoskeletal proteins, apoptotic proteins, cell cycle enzymes, and translational factors.12

Pressure overload also causes the activation of intrinsic “stretch” receptors in cardiomyocytes that likely include the integrin family of transmembrane proteins.13–15 Activation of integrins stimulates the nonreceptor tyrosine kinase, focal adhesion kinase (FAK).16–18 Activated FAK recruits the Grb2 adapter and other signaling proteins.13,14,19,20 Grb2 promotes the activation of ras via its interaction with the guanine nucleotide exchange factor SOS.20,21 Finally, pressure overload may lead to the local release of growth factors that bind to cell surface receptor tyrosine kinases, which activate the ras cascade via Grb2 and SOS.22

The relative role of signaling molecules in the pathogenesis of cardiac hypertrophy is often investigated by use of small mammals. Several signaling proteins, including Gαq/G11, were shown to be important mediators of the hypertrophic response in genetically modified mice.23 Recent work by our group showed that Grb2 function is required for pressure overload–induced cardiac hypertrophy.24 In contrast, we and others showed that p38α and p38β MAPK are not required.
for hypertrophy and, surprisingly, may have an antihypertrophic function in vivo.\textsuperscript{24,25}

The role of Raf/MEK/ERK signaling in the pathogenesis of cardiac hypertrophy is controversial. In work with cultured cardiomyocytes, the Raf/MEK/ERK pathway has been variously observed to be essential for hypertrophy,\textsuperscript{26} to have an anti hypertrophic effect,\textsuperscript{27} or to regulate hypertrophic gene induction but not actual cell growth.\textsuperscript{28,29} In work with transgenic mice, Bueno and colleagues\textsuperscript{30} demonstrated that cardiac-specific expression of activated MEK resulted in robust concentric hypertrophy. Activated MEK transgenic mice developed a compensated form of cardiac hypertrophy with preserved ventricular contractile function. In addition, cardiac tissue from activated MEK transgenic mice was partially resistant to apoptotic stimuli. This antiapoptotic effect may be due to the ability of ERK to inactivate caspase-9.\textsuperscript{31}

Although overexpression of activated MEK resulted in cardiac hypertrophy, it is uncertain whether Raf-I/MEK/ERK activation is required for this process.

Methods

Generation of Transgenic Mice

A cDNA encoding dominant negative human Raf-I (DN-Raf) (K375H)\textsuperscript{32,33} with a FLAG epitope tag was inserted into the vector pBl (gift of Jeffrey Robbins, University of Cincinnati, Cincinnati, Ohio) containing the pIBI (gift of Jeffrey Robbins, University of Cincinnati, Cincinnati, Ohio) containing the α-myosin heavy chain (α-MHC) promoter and an SV40 polyadenylation site, as previously described.\textsuperscript{34} Linearized DNA was injected into the pronuclei of 1-cell C57Bl/6J embryos. Progeny were analyzed by PCR to detect transgene integration.\textsuperscript{35} Multiple lines were obtained, and integration of the transgene was analyzed by Southern blotting. The highest-expressing line, with integration of 4 copies of the transgene (DN-Raf-4x), was in this study. Transgenic DN-Raf-4x mice were compared with nontransgenic littermates in every experiment.

All research involving the use of mice was performed in strict accordance with protocols approved by the Animal Studies Committee of Washington University School of Medicine.

Protein Analysis

Cytosolic extracts of ventricular tissue were separated by SDS-PAGE, and proteins were electrophoretically transferred to nitrocellulose filters that were blocked with milk, washed, and then incubated with primary antibody.\textsuperscript{36} Primary antibodies used included the following: rabbit polyclonal anti-Raf-I and anti-FLAG antibodies (Santa Cruz Biotechnology), rabbit polyclonal anti-phospho-p38 MAPK, anti-total-p38 MAPK, anti–cleaved caspase-3, anti-phospho-JNK, anti–total-JNK, and anti-ERK1/2 antibodies (Cell Signaling Incorporated). Filters were extensively washed and then were incubated with horseradish peroxidase–conjugated anti-rabbit secondary antibody (Amersham). Bands were visualized with the ECL system (Amersham).\textsuperscript{37}

Protein Kinase Assays

In vitro ERK activity assays were performed by use of a kit from Cell Signaling Incorporated. In brief, anti-phospho-ERK immunoprecipitates were derived from ventricular cytosolic lysates obtained 1 or 7 days after transverse aortic constriction (TAC). The specific substrate protein Elk-1, 200 μmol/L ATP, and kinase reaction buffer were added to the immunoprecipitates. Kinase reactions were terminated after a 30-minute incubation period, and proteins were separated by SDS-PAGE and then analyzed by immunoblotting with a specific anti-phospho-Elk-1 antibody.

Transverse Aortic Constriction

TAC was performed as previously described.\textsuperscript{35,37} The surgeon was blinded in all cases to the transgenic status of the mice. After 1 or 7 days, surviving animals were subjected to transthoracic echocardiography; then animals were killed, and the hearts were dissected out and weighed.

Transthoracic Echocardiography

Transthoracic echocardiography was performed in awake mice by use of an Acuson Sequoia 256 Echocardiography System equipped with a 15-MHz (15L8) transducer, as described previously (Acuson, a Siemens Company).\textsuperscript{35,36}

Ascending aortic blood velocity was determined by Doppler echocardiography. A 5-MHz hand-held Doppler ultrasound probe (Acuson) was used to obtain transcutaneous aortic blood velocity waveforms in unanesthetized mice. Approximately 10 to 15 ascending and transverse aortic waveforms were acquired for each mouse. Aortic blood velocity measurements were used to determine the pressure gradient induced by transverse aortic constriction.

The echocardiographer was blinded in all cases to the transgenic status of the mice.

Terminal Deoxynucleotidyl Transferase–Mediated dUTP Nick-End Labeling Assay

Animals were euthanized, and the heart was excised, fixed overnight at 4°C in 10% formalin in PBS, embedded in paraffin, and sectioned with a microtome. Terminal deoxynucleotidyl transferase–mediated dUTP nick-end labeling (TUNEL) assay was performed on 5-μm sections (TdT-FragEL DNA Fragmentation Detection Kit). Sections were mounted on coverslips and evaluated by fluorescence microscopy.

Analysis of Gene Expression

RNA was purified from quick-frozen cardiac tissue with Trizol (Invitrogen). The TaqMan Gold RT-PCR kit was used according to the manufacturer’s instructions (PE Biosystems). Quantitative PCR was performed as previously described with specific primers and fluorescence probes for atrial natriuretic factor (ANF) and β-myosin heavy chain (β-MHC).\textsuperscript{24} mRNA levels were compared at various time points after correction by use of concurrent GAPDH message amplification with GAPDH probe and primers used as an internal standard.

Statistical Analysis

All data are reported as mean±SEM. Statistical analysis was performed by 2-tailed Student t test, ANOVA, and Kaplan-Meier log-rank test, where applicable. A value of P<0.05 was considered statistically significant.

Results

Generation of Transgenic Mice With Cardiac-Specific Expression of DN-Raf

To test whether Raf-I is an essential regulator of cardiac hypertrophy, transgenic mice were generated with cardiac-specific expression of a dominant negative mutant form of Raf-I. In this mutant, the ATP-binding site lysine residue is mutated to histidine (K375H).\textsuperscript{32,33} The α-MHC promoter was linked to the cDNA encoding DN-Raf with a FLAG epitope tag, and 3 independent lines of transgenic mice in the C57Bl/6J strain were generated. Mice from all 3 lines were viable, fertile, and had normal cardiac function at baseline as determined by echocardiography (data not shown). The line with the greatest number of integrated copies of the transgene (≈4 copies, DN-Raf-4x) was used for physiological studies. Expression of DN-Raf was robust in DN-Raf-4x transgenic cardiac tissue, and DN-Raf protein levels exceeded wild-type
Inhibition of ERK Activity in DN-Raf Transgenic Cardiac Tissue

The ability of the dominant negative mutant form of Raf-1 to inhibit ERK activity was examined. DN-Raf-4x and nontransgenic mice were subjected to pressure overload with TAC or to a sham operation. One and 7 days after banding, cardiac tissue was removed, and ERK activity was analyzed by in vitro kinase assay. In nontransgenic mice, ventricular ERK activity was present at low levels at 1 day (Figure 2) and 7 days (data not shown) after a sham operation and was dramatically increased after TAC. In contrast, ERK activity was markedly reduced in DN-Raf-4x mice after sham operation or TAC at both time points (Figure 2). To determine whether the effect of DN-Raf was specific to the Raf-1/MEK/ERK signaling cascade, JNK phosphorylation was analyzed in DN-Raf-4x cardiac tissue and was found to increase normally 1 day and 7 days after TAC (data not shown). In addition, p38 MAPK phosphorylation increased normally in DN-Raf-4x cardiac tissue after TAC (data not shown).

Reduced Survival and Increased Cardiomyocyte Apoptosis in DN-Raf Transgenic Mice After TAC

In response to TAC, only 65% of DN-Raf-4x (11 of 17) mice survived, and these survival rates were much lower than those observed in nontransgenic littermates. Indeed, 100% of nontransgenic mice (15 of 15) survived the TAC procedure for 7 days (Figure 3A). Comparison of the Kaplan-Meier survival curves for the 2 groups by log-rank test revealed a statistically significant difference in survival probability (χ² = 6.2, P = 0.012). Although the cause of death could not be determined for most of the DN-Raf-4x mice after TAC, echocardiographic analysis of 2 mice before death revealed poorly contractile, dilated hearts.

One possible explanation for the high mortality observed in DN-Raf-4x mice after TAC is an increased rate of cardiomyocyte apoptosis. We previously demonstrated that DN-14-3-3 transgenic mice, which have impaired cardiac ERK activation but enhanced p38 MAPK activation, develop overwhelming cardiomyocyte cell death several days after TAC. Apoptosis in cardiac tissue was assessed in the present study by determining the amount of active cleaved caspase-3 (p17) in ventricular cytosolic lysates. DN-Raf-4x cardiac tissue had markedly increased cleaved caspase-3 protein levels one day after TAC compared with nontransgenic mice (Figure 3B). Similar results were seen in DN-Raf-4x cardiac tissue obtained 7 days after TAC (data not shown).

To confirm that the increase in cleaved caspase-3 protein observed in DN-Raf-4x ventricular lysates correlated with cardiomyocyte apoptosis, TUNEL was performed on tissue sections. These assays revealed that 2.34 ± 0.17% of cardiomyocytes were apoptotic in DN-Raf-4x mice 1 day after TAC, but only 0.68 ± 0.07% of nontransgenic cardiomyocytes were TUNEL positive after TAC (P = 0.0002) (Figure 3C).

Impaired Hypertrophic Response of DN-Raf Transgenic Mice to TAC

Seven days after TAC, hearts were isolated from surviving animals, and the ratios of left ventricular weight to body weight (LVW/BW) and biventricular weight to body weight (BVW/BW) were calculated as a measure of cardiac hypertrophy. As expected, LVW/BW increased by 41% in nontransgenic C57Bl/6J mice, from 3.2 ± 0.2 to 4.5 ± 0.7 mg/g (P < 0.05) (Figure 4A). DN-Raf-4x transgenic mice were partially resistant to the development of cardiac hypertrophy after TAC (Figure 4A). LVW/BW increased by 13% in DN-Raf-4x transgenic mice, from 3.1 ± 0.05 to 3.5 ± 0.1 mg/g (P = NS) (Figure 4A). BVW/BW increased by 32% in nontransgenic C57Bl/6J mice, from 4.1 ± 0.5 to 5.4 ± 0.8 mg/g (P < 0.01). In contrast, BVW/BW increased by only 8% in
DN-Raf-4x transgenic mice, from 3.9±0.05 to 4.2±0.1 mg/g (P=NS).

Echocardiographic analysis confirmed that DN-Raf-4x mice were resistant to the development of cardiac hypertrophy in response to pressure overload. Echocardiographically derived left ventricular mass index increased by 35% in nontransgenic mice and 12% in DN-Raf-4x mice 7 days after TAC (Table).


The reduced ability of DN-Raf mice to exhibit cardiac hypertrophy in response to TAC was not a result of less robust aortic constriction. Peak blood velocities at the site of aortic constriction were determined by transthoracic Doppler echocardiography 7 days after TAC and were used to estimate pressure gradients. The average estimated pressure gradient was 67±6.4 mm Hg in nontransgenic mice and was 59±1.9 mm Hg in DN-Raf transgenic mice (P=NS).

The size of individual cardiomyocytes was next analyzed to determine whether the impaired hypertrophic response in DN-Raf hearts was due to cell death or to reduced cellular growth. Computerized analysis of photomicrographs of tissue sections demonstrated that the cross-sectional area of cardiomyocytes from nontransgenic animals was markedly greater after TAC than after sham operation (Figure 4B). In contrast, the increase in cardiomyocyte area seen in DN-Raf specimens after TAC was minimal (Figure 4B). These results establish that the impaired cardiac hypertrophic response observed in surviving DN-Raf-4x animals was indeed a result of reduced cell growth.

**Impaired “Fetal” Gene Expression in DN-Raf Mice After TAC**

Gene expression analysis revealed that DN-Raf-4x mice were resistant to TAC-induced alterations in cardiac gene expression. In response to TAC, cardiac ANF gene expression was robustly induced in nontransgenic mice but not in DN-Raf-4x
mice (Figure 5A). In addition, cardiac β-MHC gene expression was markedly induced in nontransgenic mice but not in DN-Raf-4x mice after TAC (Figure 5B).

Discussion

The Raf-1/MEK/ERK signaling cascade is involved in cell growth and proliferation in nearly all eukaryotic cell types. In the present study, we analyzed the role of Raf-1 in the development of mechanical stress–induced cardiac hypertrophy by use of mice with cardiac-specific expression of DN-Raf. Transgenic mice appeared normal and were fertile in the absence of experimental manipulation but were deficient in cardiac ERK activation. In addition, many DN-Raf-4x transgenic mice did not tolerate acute pressure overload, and >35% of animals died within 7 days of the TAC procedure with cardiac contractile dysfunction and presumed cardiogenic shock. Surviving animals were resistant to pressure overload–induced cardiac hypertrophy. These results complement those in cultured neonatal and adult rat cardiomyocytes in which Raf-1 activity is required for ligand-induced cardiomyocyte growth. They also support data that showed that cardiac-specific expression of activated MEK resulted in robust concentric hypertrophy with maintained ventricular contractile function.

Previously, we demonstrated that p38MAPK activity is not required for hypertrophy and may in fact inhibit the development of cardiac hypertrophy. Taken together, one model that emerges is that the Raf-1/MEK/ERK pathway promotes, but the p38 MAPK pathway inhibits, cardiac growth, survival, and contractility.

The fact that Raf-1 is required for cardiac hypertrophy does not explain how the Raf-1/MEK/ERK cassette promotes cardiomyocyte growth. Recently, Wang and Proud demonstrated that ERK activity promotes the growth of rat adult cardiomyocytes by activating ribosomal protein S6 kinase 1 (S6K1) and by phosphorylating the eIF4E-binding protein-1 (4E-BP1). In this way, ERK directly regulates the translational machinery and promotes protein synthesis. Analysis of cardiac S6K1 activity in DN-Raf-4x mice may provide further insights into the role of the Raf-1/MEK/ERK cascade in the pathogenesis of cardiac hypertrophy.

Although our data suggest that Raf-1 activity blocks pressure overload–induced cardiomyocyte apoptosis, the precise biochemical mechanism for this effect is uncertain. Recent work with cultured noncardiac cells showed that Raf-1/MEK/ERK signaling pathway blocks apoptosis in part because ERK phosphorylates caspase-9 on threonine-125. This phosphorylation was found to inhibit caspase-9 activation despite the presence of cytosolic cytochrome c. These results suggest that the Raf-1/MEK/ERK cascade acts downstream of the mitochondrial effects of the proapoptotic
BH3-domain protein BAX that promotes cytochrome c release. In contrast, p38 MAPK phosphorylates p53 in some cell types, increasing the transcriptional activity of p53 and inducing the expression of BAX.\textsuperscript{40,41}

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