Extracellular Signal-Regulated Kinases 1 and 2 Regulate the Balance Between Eccentric and Concentric Cardiac Growth


Rationale: An increase in cardiac afterload typically produces concentric hypertrophy characterized by an increase in cardiomyocyte width, whereas volume overload or exercise results in eccentric growth characterized by cellular elongation and addition of sarcomeres in series. The signaling pathways that control eccentric versus concentric heart growth are not well understood.

Objective: To determine the role of extracellular signal-regulated kinase 1 and 2 (ERK1/2) in regulating the cardiac hypertrophic response.

Methods and Results: Here, we used mice lacking all ERK1/2 protein in the heart (Erk1−/− Erk2fl/fl-Cre) and mice expressing activated mitogen-activated protein kinase kinase (Mek)1 in the heart to induce ERK1/2 signaling, as well as mechanistic experiments in cultured myocytes to assess cellular growth characteristics associated with this signaling pathway. Although genetic deletion of all ERK1/2 from the mouse heart did not block the cardiac hypertrophic response per se, meaning that the heart still increased in weight with both aging and pathological stress stimulation, it did dramatically alter how the heart grew. For example, adult myocytes from hearts of Erk1−/− Erk2fl/fl-Cre mice showed preferential eccentric growth (lengthening), whereas myocytes from Mek1 transgenic hearts showed concentric growth (width increase). Isolated adult myocytes acutely inhibited for ERK1/2 signaling by adenosiral gene transfer showed spontaneous lengthening, whereas infection with an activated Mek1 adenovirus promoted constitutive ERK1/2 signaling and increased myocyte thickness. A similar effect was observed in engineered heart tissue under cyclic stretching, where ERK1/2 inhibition led to preferential lengthening.

Conclusions: Taken together, these data demonstrate that the ERK1/2 signaling pathway uniquely regulates the balance between eccentric and concentric growth of the heart. (Circ Res. 2011;108:176-183.)

Key Words: hypertrophy ■ signaling ■ heart ■ genetically altered mouse ■ ventricular remodeling ■ MAPK

The myocardium undergoes cellular and ventricular chamber remodeling and/or hypertrophy as a means of augmenting or maintaining cardiac output in response to increased workload or pathological insults. Cardiac hypertrophy is an independent risk factor for heart failure, arrhythmias, sudden cardiac death, and cardiovascular morbidity and mortality.1,2 The fundamental response of the myocardium to an increase in afterload is termed concentric hypertrophy, which consists of an increase in ventricular wall thickness without chamber enlargement.3 In contrast, conditions that increase preload, including significant valvular regurgitation and volume overload, promote chamber dilatation with no increase or even a decrease in left ventricular wall thickness, a process involving eccentric hypertrophy.

The concentric and eccentric hypertrophy of the entire myocardium is mainly the result of different modes of cardiomyocyte cell growth.4 In eccentric hypertrophy, there is preferential assembly of contractile protein units in series, leading to a relatively greater increase in the length than the width of myocytes. Concentric hypertrophy results from an assembly of contractile protein units in parallel with a relative increase in the width of individual cardiac myocytes.5,6 Concentric and eccentric growth likely result from orchestrated activation of specific intracellular signaling pathways,
although the identity and mechanisms whereby these signaling pathways differentially regulate myocyte growth are not currently known.

The mitogen-activated protein kinases (MAPKs) have been implicated as focal mediators of cardiac hypertrophy in both cell culture and genetically modified mouse models.7 For example, the extracellular signal-regulated kinases 1 and 2 (ERK1/2) branch of the greater MAPK signaling cascade appears to induce a unique form of concentric cardiac hypertrophy in transgenic mice caused by expression of activated MAPK kinase (Mek)1.8 Moreover, dozens of studies conducted in cultured cardiac myocytes have suggested that ERK1/2 signaling is necessary for promoting hypertrophic growth.9 Signaling through the ERK1/2 cascade is classically initiated at the cell membrane by activation of the small G protein Ras that then recruits the MAP3K Raf-1 to the plasma membrane, where it is activated,10 ERK1/2 also become activated in cardiac myocytes in response to many stimuli, such as G protein–coupled receptor agonists, receptor tyrosine kinase agonists, cytokines, reactive oxygen species, and stretch.7 These various stimuli initiate MAP3Ks, which then phosphorylate and activate the dual-specificity kinases MEK1 and MEK2 (MAP2Ks) that serve as dedicated kinases for ERK1/2 phosphorylation and activation.11

Interestingly, although transgenic mice overexpressing an activated form of Mek1 develop concentric hypertrophy, inhibition of ERK1/2 by overexpression of dual-specificity phosphatase (DUSP)6, which inactivates ERK1/2 in the heart, did not reduce cardiac hypertrophy following multiple pathological stimuli.12 These results suggest that although activation of MEK1-ERK1/2 signaling can program cardiac hypertrophy, ERK1/2 signaling may not be needed for cardiac hypertrophy induced by pathological stimuli. These seemingly contradictory findings may be attributable to the type of cardiac growth that results from ERK1/2 activation or inactivation, such as concentric versus eccentric cardiac growth. Indeed, here, we show that ERK1/2 activation specifically leads to concentric cardiac growth at the level of the cardiac myocyte, whereas inhibition of ERK1/2 preferentially permits eccentric growth. Through the combined use of Erk1- and Erk2-null mice, activated Mek1-expressing transgenic mice, and adult cardiomyocytes in culture, we show that ERK1/2 regulate the ability of myocytes to grow either in width (concentric) or in length (eccentric). Thus, the MEK1-ERK1/2 pathway may be the first identified signaling pathway capable of specifically directing the mode of cardiomyocyte hypertrophy.

Methods

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

Animal Models

Erk1−/− animals have been described previously.13 Erk2-loxP (fl) mice were described recently.14 The Nkx2.5-Cre knock-in mice,15 as well as transgenic mice in which a nuclear-localizing Cre cDNA was placed under the control of the mouse α-myosin heavy chain (αMHC) promoter, have also been described previously.16 Echocardiography for assessment of ventricular size and function was performed in 2% isoflurane-anesthetized mice.16

Western Blotting

Generation of protein samples from tissue, along with Western blotting and chemifluorescent detection, have been described previously.8 All antibodies were obtained from Cell Signaling Biotechnology (Beverly, Mass).

Histology and Staining

Hearts were collected at the indicated times, fixed in 10% formalin-containing PBS, and embedded in paraffin. Serial 5-μm heart sections were cut and stained with hematoxylin/eosin or Masson trichrome.

Invasive Hemodynamic Studies

Invasive hemodynamics in the closed-chest mouse was performed with a 1.4F Millar catheter (Millar Instruments, Houston, Tex) as described previously.17

Studies in Isolated Cardiomyocytes

Adult ventricular myocytes were isolated as described previously.18 For histology or immunofluorescence, cells were fixed and stained with antibodies for sarcomeric α-actinin (Sigma). Contractility was assessed as described previously.19

Results

Loss of ERK1/2 From the Heart Induces Hypertrophy and Early Adult Lethality

Previous studies have shown that Erk1-null mice have normal cardiac structure and function,12 although it was not possible to examine Erk2-null mice because of embryonic lethality.14,20 To circumvent this problem and to unequivocally determine the necessary function of both ERK1/2 in the heart as hypertrophic mediators, we crossed Erk1−/− mice with Erk2fl/fl targeted mice and a cardiac Cre-recombinase–expressing mouse line. We used both Nkx2.5-Cre knock-in mice and transgenic mice expressing Cre under the control of the mouse αMHC promoter.16 Western blotting showed absence of ERK1 protein (upper band) and very efficient depletion of ERK2 protein (lower band) in the same mutant hearts with either the
Erk1/2 gene–deleted mice develop eccentric hypertrophy. A, Western blots for the indicated proteins from heart extracts of Nkx2.5-Cre mice, Erk1−/−/Erk2fl/fl mice (1), Erk1−/−/Erk2fl/flNkx2.5-Cre mice (2), and Erk1−/−/Erk2fl/flNkx2.5-Cre mice (3). JNK1/2 indicates c-Jun N-terminal kinase; p, phosphorylated. B, Survival plot showing high early mortality in Erk1−/−/Erk2fl/flNkx2.5-Cre (black squares) and Erk1−/−/Erk2fl/flNkx2.5-Cre mice (blue circles) but not in control Erk1−/−/Erk2fl/flNkx2.5-Cre littermates (red triangle). C and D, Heart weight normalized to body weight (HW/BW) at 60 and 90 days of age in control Nkx2.5-Cre (white bar), Erk1−/−/Erk2fl/fl mice (red bar) (1), Erk1−/−/Erk2fl/flNkx2.5-Cre mice (blue bar) (2), and Erk1−/−/Erk2fl/flNkx2.5-Cre mice (black bar) (3). *P<0.05 vs Nkx2.5 Cre mice. Number of mice analyzed is indicated in the bars. E, Quantitative RT-PCR for the hypertrophic markers Nppa, Nppb, Acta1, and Myh7 in the indicated groups of mice (n=5). F, Transverse hematoxylin/eosin-stained heart sections from the indicated mice at 60 and 90 days.

Nkx2.5-Cre or αMHC-Cre lines (Figure 1A). No other changes were observed in ERK5, p38, or c-Jun N-terminal kinase protein levels or phosphorylation, although MEK1/2 were hyperphosphorylated with ablation of Erk1/2, highlighting a negative-feedback mechanism (Figure 1A).21

Whereas mice of the genotype Cre, Erk1−/−Cre, Erk2fl/fl Cre, and Erk1−/−Erk2fl/fl had normal lifespans, Erk1−/−Erk2fl/flCre (both αMHC-Cre and Nkx2.5-Cre) began dying after 60 days of age and none survived beyond 7 months (Figure 1B and data not shown). Interestingly, surviving Erk1−/−/Erk2fl/flCre mice showed spontaneous cardiac hypertrophy as defined by an increase in heart weight normalized to body weight (Figure 1C and 1D). This increase in heart weight also correlated with induction of the hypertrophic gene program, such as increased mRNA for Nppa, Nppb, Acta1, and Myh7 (Figure 1E). Histological analysis of these hearts showed ventricular dilation by 60 to 90 days of age with areas of mild fibrosis, as assessed with Masson trichrome staining (Figure 1F and 1G; Online Figure I, B and D). However, analysis of cardiac histological sections and isolated cardiomyocytes did not show changes in myofiber organization or sarcomeric structure (Online Figure I, E and F). Analysis of apoptosis by TUNEL staining in these null hearts did not show increased cell death at baseline in 60- to 90-day-old mice (Online Figure I, A and C). Thus, loss of Erk1/2 from the heart induces pathology and a form of hypertrophy without increased cell death.

Careful assessment of cardiac ventricular chamber dimensions showed dilation beginning even at 30 days of age in Erk1−/−Erk2fl/flNkx2.5-Cre mice, which became more pronounced by 60 and 90 days with both Cre lines (Figure 2A). Corresponding with ventricular dilation, cardiac ventricular performance, as measured by echocardiography, was significantly and progressively reduced at 30, 60, and 90 days of age in Erk1−/−Erk2fl/flNkx2.5-Cre mice (both Nkx2.5-Cre and αMHC-Cre lines) (Figure 2B). Analysis of cardiac contractility with a Millar catheter also showed a severe reduction in function in Erk1−/−/Erk2fl/flNkx2.5-Cre mice at 60 days of age at baseline and with increasing dosages of dobutamine (Figure 2C). However, Erk1−/−/Erk2fl/flNkx2.5-Cre mice were not in overt heart failure at the age of 60 days because left ventricular end diastolic filling pressures were not elevated, nor was lung edema observed (Figure 2D and data not shown). Myocytes isolated from hearts of Erk1−/−/Erk2fl/flNkx2.5-Cre mice even showed enhanced contractility when compared to control cardiomyocytes (Online Figure II). However, with just 30 more days of age (90 days) double-null mice developed overt failure with significantly reduced cardiac function and pulmonary edema, as demonstrated by increased lung weight/body weight ratio (Figure 2E). Although resting ECG recordings were normal in the Erk1−/−/Erk2fl/flNkx2.5-Cre mice (data not shown), there may be some contribution of arrhythmia to the lethality as these mice aged and dilatory growth became extreme. Thus, we believe that overt heart failure is the primary cause of lethality in Erk1−/−/Erk2fl/flNkx2.5-Cre mice, as also evidenced by panting and reduced mobility with aging (Online Movie).

Loss of ERK1/2 From the Heart Does Not Reduce Stress-Induced Hypertrophy

Although Erk1−/−/Erk2fl/flNkx2.5-Cre mice showed spontaneous cardiac hypertrophy with aging, it was still formally possible that loss of ERK1/2 from the heart might attenuate stress-induced cardiac hypertrophy, given the countless studies that have suggested a necessary role for this signaling pathway in transducing growth of cultured cardiomyocytes. To address this issue, we first subjected 45-day-old Erk1−/−/Erk2fl/flNkx2.5-Cre mice to 14 days of angiotensin II/phenyleph-
Erk1/2 gene–deleted mice showed significantly more cardiac hypertrophy after TAC and a greater reduction in FS but no change in LVEDD (Figure 3D through 3F; Online Figure III, B through D). These results again suggest that ERK1/2 are not required for cardiac hypertrophy, and, if anything, a loss of ERK1/2 from the heart produced slightly more growth with pathological stimulation.

The loss of ERK1/2 also did not prevent physiological exercise–induced hypertrophy (Online Figure IV, A through C), a stimulus that itself tends to induce eccentric heart growth. Indeed, Erk1/2 gene–deleted mice developed significantly more cardiac hypertrophy with swimming exercise compared with controls, which was associated with greater chamber dilation and an even greater increase in myocyte length without an increase in myocyte thickness (Online Figure IV, A through D).

Loss of ERK1/2 Induces Preferential Eccentric Cardiomyocyte Growth

Hearts from Erk1/2 gene–deleted mice showed robust dilatation and loss of whole-organ function, even though isolated Erk2fl/fl-Nkx2.5-Cre mice showed robust dilation and loss of body weight (LW/BW) ratio in the same group of mice shown in A at 90 days of age with the corresponding legend (N = 4 to 5 mice; *P < 0.05 vs Cre).

Figure 2. Cardiac structural and functional assessment in Erk1/2 gene–deleted mice. A and B, Assessment of LVEDD and FS% by echocardiography in the indicated groups of mice at 30, 60, and 90 days of age. Number of mice analyzed is indicated in the bars. *P < 0.05 vs Cre at each corresponding age.

C and D, Invasive hemodynamic measurement of cardiac output assessed at baseline and during infusion of escalating doses of dobutamine as the maximal rate of pressure change in the left ventricle (Max dP/dt) and left ventricular end diastolic pressure (LVEDP) in control (blue) and Erk1/2 gene–deleted mice (red) (n = 6; *P < 0.05). E, Lung weight to body weight (LW/BW) ratio in the same group of mice shown in A at 90 days of age with the corresponding legend (N = 4 to 5 mice; *P < 0.05 vs Cre).
myocytes from these hearts were not functionally compromised. This suggested that loss of ERK1/2 from the heart induced failure resulting from extreme ventricular remodeling, dilation, and increased wall tension but not caused by myocyte dysfunction, as is typically observed in most other forms of heart failure. To further understand the cellular mechanism underlying these observations, we first isolated adult myocytes from hearts of Erk1\(^{-/-}\) Erk2\(^{fl/fl-Nkx-Cre}\) mice and compared them with myocytes from transgenic mice expressing activated (a) MEK1 (greater ERK1/2 activation). Remarkably, adult myocytes from hearts of Erk1\(^{-/-}\) Erk2\(^{fl/fl-Nkx-Cre}\) mice were significantly longer, whereas aMEK1 myocytes were wider and shorter (Figure 4A and 4B). Indeed, the length/width ratios between these 2 genotypes was even more pronounced, suggesting that ERK1/2 activation promotes concentric myocyte growth, whereas loss of ERK1/2 results in a default program of eccentric growth (Figure 4C). This growth relationship was even more pronounced after Ang II/PE stimulation, where myocytes from hearts of Erk1\(^{-/-}\) Erk2\(^{fl/fl-Nkx-Cre}\) mice showed an even greater increase in cell length, although some increase in width was observed (Figure 4D and 4E). Similarly, as discussed above, swimming exercise caused significantly greater elongation of Erk1\(^{-/-}\) Erk2\(^{fl/fl-Nkx-Cre}\) cardiomyocytes compared with controls, without an increase in width (Online Figure IV, D).

To investigate the mechanism of cellular growth associated with ERK1/2 activity in adult myocytes, we performed 2 additional assays. First, we isolated adult rat cardiomyocytes and infected them with recombinant adenoviruses encoding β-galactosidase (control) or dominant-negative (dn) Mek1 and Dusp6 together to achieve complete inhibition of ERK1/2 signaling over 3 days in serum-free cultures (Figure 5A). Reciprocally, we infected myocytes with a recombinant adenovirus encoding aMEK1 to induce constitutive ERK1/2 signaling over 3 days (Figure 5A). Myocytes were then immunostained for α-actinin and measured for length and width (Figure 5B). Remarkably, inhibition of ERK1/2 produced longer myocytes, whereas activation of ERK1/2 produced wider myocytes, with even greater differences in length-width ratios (Figure 5C and 5D). Although this was a static cell culture–based model system, it nonetheless again suggested that ERK1/2 were regulating the intrinsic growth response of the myocyte to discriminate between length and width.
width. As a control, coinfection of Ad-Dusp6 with Ad-aMEK1 fully inhibited ERK1/2 phosphorylation, showing that Dusp6 dominates over aMek1 (Online Figure V, A). More importantly, Ad-aMEK1-induced cardiomyocyte concentric growth was fully blocked by Ad-Dusp6 coinfection, conclusively indicating that aMek1 only functions through ERK1/2 in programming growth and that aMek1 has no function on its own apart from ERK1/2 (Online Figure V, B and C).

As a second, potentially more “physiological” assay, we used engineered heart tissues (EHTs) wherein neonatal rat cardiomyocytes are reconstituted in an anisotropic myocardial syncytium under defined mechanical load. After 7 days in culture, EHTs were infected with a combination of adenoviral vectors encoding dnMek1 and Dusp6 to inhibit ERK1/2 signaling or a control green fluorescent protein (GFP)-encoding virus (Figure 5E). The EHTs were subsequently transferred to a stretch device and submitted to unidirectional cyclic stretch for an additional 5 days. Unlike the in vivo situation, EHTs are not affected by confounding compensatory mechanisms such as neurohumoral stimulation. In addition, mechanical load on the cardiomyocytes in EHTs are similar between groups, and any alteration in morphology is only associated with an intrinsic change in the cardiomyocyte growth program. On culture day 12, EHTs were dispersed with a collagenase mixture, and cells were fixed and stained with sarcomeric α-actinin to assess cell dimensions (Figure 5F). In accordance with our observations in vivo, the ex vivo inhibition of ERK1/2 signaling in the EHT system also resulted in a significant increase in cell length and a significant reduction in cell width (Figure 5F and 5G). These results again demonstrate the ability of the ERK signaling cascade to directly modulate cell size, independently of effects on contractility.

Discussion

Our results suggest a model whereby ERK1/2 signaling is necessary to prevent eccentric cardiomyocyte growth. Indeed, Erk1/2 double-null ventricular myocytes lengthen and become slightly thinner with aging and pathological stimulation to cause whole-organ eccentric growth. Antithetically, activation of ERK1/2 with aMek1 preferentially programmed concentric cardiac growth, while, at the same time, partially inhibiting eccentric growth. These results suggest a refined hypothesis whereby ERK1/2 are not technically necessary for whole-organ hypertrophy, as measured by heart weight normalized to body weight, but are necessary for promoting a properly coordinated growth response that also allows myocyte thickening and addition of sarcomeres at the periphery (Figure 6). Thus, ERK1/2 signaling is necessary for facilitating a select type of cardiac growth in vivo, concentric hypertrophy, while, at the same time, preventing eccentric growth and addition of sarcomeres in series (Figure 6).

The model of growth proposed above is also consistent with previous work in which we inhibited the ERK1/2 pathway in vivo using a transgene to overexpress Dusp6 in the heart. Dusp6 transgenic mice showed complete loss of ERK1/2 phosphorylation and activity, although the cardiac hypertrophic response was not reduced following TAC or agonist infusion. In fact, Dusp6transgenic mice subjected to long-term TAC showed even larger increases in heart weights compared with control mice, an increase that was associated with chamber dilation, suggesting greater eccentric growth. Thus, although we had not previously measured cellular lengths and widths in Dusp6 transgenic hearts, their increased propensity toward dilation with pathological stimulation suggests a similar mechanism in play, whereby loss of ERK1/2 signaling promotes cardiomyocyte lengthening.

To our knowledge the MEK1-ERK1/2 signaling pathway appears to be unique in its ability to regulate both the length and width decision of a myocyte with aging or stress stimulation. Previously, activation of the MEK5-ERK5 signaling pathway was reported to produce eccentric cardiac hypertrophy by lengthening myocytes, although the reciprocal relationship of increased width was not examined in Mek5 or Erk5 gene-deleted mice. Moreover, many transgenic mouse models of cardiomyopathy and heart failure lead to spontaneous dilation with myocyte lengthening, such as with myocyte enhancer factor-2 overexpression. The data we present with the MEK1-ERK1/2 pathway are unique because activation of this pathway leads to myocyte thickening, whereas loss leads to myocyte lengthening without cellular dysfunction, suggesting it is a primary effect. Moreover, growth of adult myocytes in culture over 3 days showed the exact same relationship, and even myocytes from beating and cyclically stretched EHTs showed the same effect. Because nearly all other transgenic models of heart failure appear to cause secondary dilation, we believe that the MEK1 effect in driving concentric hypertrophy is highly unique and suggestive of a true biological signaling pathway whose role is to program myocyte thickening.
Ventricular dilation can result from either myocardial cell elongation through an addition of contractile protein units in series or myocyte slippage and changes in wall architecture.27 Either event, or a mixture therein, changes the geometry of the ventricle by increasing the internal radius and thinning of the free wall, resulting in greater wall tension and secondary neuroendocrine stress signaling.28 In fact, according to Laplace’s law, our echocardiographic measurements in hearts from Erk1\(^{-/-}\)/Erk2\(^{+/+}\)-Cre mice suggest an almost doubled load at the end of diastole as compared with control mice. This significantly increased load could explain the reduced load at the end of diastole as compared with control mice.

These hearts showed even better functional performance in isolation. Such results from Erk1/2-deleted adult myocytes are in stark contrast to the observed slowing of contraction in myocyte or, more provocatively, could even lead to mechanical dysfunction.29 However, this reduction in whole-organ function and secondary disease manifestations are unlikely the result of a primary defect in myocyte contractility in the absence of ERK1/2, because adult myocytes from these hearts showed even better functional performance in isolation. Such results from Erk1/2-deleted adult myocytes are in stark contrast to the observed slowing of contraction in unloaded myocytes from other models of heart failure.30 Such a dramatic increase in series sarcomeres without matching concentric growth of sarcomeres on the periphery of the fibers likely promotes whole-organ dysfunction through dilation and dramatically increased wall tension with less myocyte–myocyte connectivity.

Concentric hypertrophy usually results from pressure overload, whereas eccentric hypertrophy usually results from situations of volume overload or forms of exercise. However, almost all hypertrophic stimuli appear to activate and induce phosphorylation of ERK1/2.7 Together, these observations may suggest 2 divergent points of view. The more simplistic view suggests that the ERK pathway is activated in all types of hypertrophy to program concentric growth of myocytes. Therefore, in pressure overload, the MEK1-ERK1/2 pathway would work with other signaling pathways to add sarcomeres in parallel and increase the width of the cells, whereas in volume-overload situations, the ERK pathway would work against the machinery that drives the cell toward elongation. Alternatively, it is possible that volume overload does not lead to continual recruitment and activation of ERK1/2 in the myocyte or, more provocatively, could even lead to mild inhibition of ERK1/2 to permit preferential addition of sarcomeres in series. Unfortunately, the relationship between types of cardiac growth (concentric versus eccentric) and the associated profile of ERK1/2 activation has not been carefully mapped in vivo at multiple time points, although Toischer et al recently reported ERK1/2 activation at 24 hours of pressure-overload hypertrophy (with concentric myocyte growth) but not with an eccentric hypertrophy–promoting shunting procedure.31

The precise intracellular targets that are phosphorylated by ERK1/2 to mediate concentric growth and suppress eccentric growth have not been identified. In theory, ERK1/2 might phosphorylate one or more regulatory proteins that control sarcomeric assembly, such that ERK1/2 activation inhibits the construction of series units, whereas assembly of sarcomeres in parallel is enhanced. In addition to affecting assembly of sarcomeres, ERK1/2 might regulate the site of new protein synthesis within the sarcomeres, either at the periphery of a fiber area or within the middle of a fiber to produce lengthening (Figure 6). In addition to acute regulation of sarcomerogenesis, ERK1/2 activation might affect the type of hypertrophy through a transcriptional mechanism that secondarily affects how myocytes grow. ERK1/2 might also impact other signaling pathways that control other aspects of cardiomyocyte growth, although analysis of AKT, GSK3β, JAK/STAT, and calcineurin did not reveal differences among control, Mek1 transgenic, or Erk1\(^{-/-}\)/Erk2\(^{+/+}\)-Cre mice (Online Figure VI).

Interestingly, autophosphorylation of ERK1/2 on Thr188, which directs ERK1/2 to phosphorylate nuclear targets, did not affect the growth response,32 suggesting that MEK1-ERK1/2 signaling within the cytoplasm may be more germane in controlling the type of growth that occurs. Indeed, ERK1/2 are known to directly phosphorylate the cytoplasmic ribosomal proteins p90 ribosomal S6 kinase (p90RSK) and p70S6K that could control protein synthesis.33 Clearly, additional studies are needed to identify the ERK1/2 effectors in hypertrophy and study them through gain- and loss-of-function approaches in vivo to assess their roles in concentric and eccentric hypertrophic growth.

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Disclosures

None.

References

**Novelty and Significance**

**What Is Known?**

- An increase in afterload or preload induces cardiac concentric and eccentric hypertrophy, respectively.
- The extracellular signal-regulated kinases (ERK) pathway is activated by stimuli inducing concentric hypertrophy.
- Constitutive activation of the ERK pathway in the heart results in concentric hypertrophy.

**What New Information Does This Article Contribute?**

- Increases in MEK1/ERK1/2 signaling directly programs myocyte thickening in vitro and in vivo.
- Decreased or absent ERK1/2 signaling induces myocyte lengthening and eccentric hypertrophic growth.
- Mice lacking ERK1/2 from the heart show eccentric cardiac growth at baseline and with stimulation.

We studied mice lacking all ERK1/2 protein in the heart and mice expressing activated Mek1 in the heart to evaluate the role of the ERK 1/2 signaling cascade in regulating the cardiac hypertrophic response. Whereas activation of the ERK pathway induced concentric hypertrophy, inhibition of this pathway resulted in eccentric hypertrophy. Using cardiomyocytes isolated from these mouse models, and using ex vivo culture models we show that these effects were mediated directly by ERK signaling. Greater ERK1/2 signaling directly programmed myocyte thickening, whereas inhibition of ERK1/2 signaling promoted myocyte lengthening. Thus, this study sheds light on the molecular mechanisms that are partially responsible for the differential hypertrophic response of the heart to an increase in preload versus afterload.
Extracellular Signal-Regulated Kinases 1 and 2 Regulate the Balance Between Eccentric and Concentric Cardiac Growth

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SUPPLEMENTAL MATERIAL

Supplemental material and methods

Animal models
The Erk1\(^{-/-}\) animals have been described before.\(^1\) Erk2 loxP (fl) targeted mice were generated as recently described.\(^2\) The Nkx2.5-Cre knock-in mice,\(^3\) and transgenic mice in which a nuclear-localizing Cre cDNA was placed under the control of the mouse \(\alpha\)-MHC promoter were also described.\(^4\) Erk1\(^{+/+}\) and Erk2\(^{0/0}\) were crossed to generate Erk1\(^{-/-}\) Erk2\(^{0/0}\) mice. The generated Erk1\(^{-/-}\) Erk2\(^{0/0}\) mice were crossed with either Nkx2.5-Cre or \(\alpha\)-MHC-Cre mice. Cre mice were back-crossed 6 generations into Erk1\(^{-/-}\) Erk2\(^{0/0}\) background. The Nkx2.5-Cre and \(\alpha\)-MHC-Cre mice had normal and identical cardiac structure and function. Throughout the study we used Cre mice, Erk1\(^{-/-}\) Erk2\(^{0/0}\) mice, and Erk1\(^{-/-}\) Cre littermates as controls because no differences were observed in cardiac function or morphology at baseline in any group. Only male mice were used for the study. All experiments involving animals were approved by the Institutional Animal Care and Use Committee at Cincinnati Children’s Hospital Medical Center.

Isolation of mouse cardiomyocytes
Adult ventricular myocytes were isolated as previously described.\(^5\) Briefly, hearts were removed from heparinized (0.1 cc) and anesthetized mice (Nembutal; 100 mg/kg) and cannulated for retrograde perfusion. Ventricular cardiac myocytes were isolated by liberase blendzyme (Roche) digestion followed by a brief mechanical disassociation. Post digestion the myocytes were resuspended in MEM (Modified Eagles Medium) plus 5% FBS (fetal bovine serum). Myocytes were counted and approximately 20,000 cells were plated on laminin-coated coverslips.

Western Blotting
Generation of protein samples from tissue, along with Western blotting and chemifluorescent detection were described previously.\(^6\) All antibodies were obtained from Cell Signaling Biotechnology (Beverly, MA).

Immunostaining
Adult ventricular myocytes were isolated as previously described.\(^5\) Following isolation, cardiomyocytes were attached to laminin coated glass coverslips. Immunocytochemistry was
performed following fixation with 4% paraformaldehyde with \( \alpha \)-actinin for sarcomeric staining (Sigma).

Histology
Hearts were collected, fixed in 10% formalin (PBS buffered), dehydrated, and embedded in paraffin. Global heart architecture was determined from transverse (short axis) 5-\( \mu \)m deparaffinized sections stained with H&E. Fibrosis was detected with Masson’s trichrome staining. Fibrosis was quantified using the MetaMorph software package (Molecular devices Inc.).

Pressure overload, osmotic minipumps, echocardiography and invasive hemodynamics for cardiac function
All mice were anesthetized with 2% Isoflurane by inhalation. Echocardiography was performed in M-mode using a Hewlett Packard SONOS 5500 instrument equipped with a 15 MHz transducer as described previously.\(^4\) Invasive hemodynamics was performed using the closed-chest approach by cannulating the right carotid artery with a 1.4F Millar pressure transducing catheter placed through the aorta and into the left ventricle (Millar Instruments, Houston, Tex) as previously described \(^7\) and further analyzed using Labchart software. Cardiac hypertrophy was induced by either transverse aortic constriction (TAC) to produce pressure overload as previously described,\(^8\) or by using Alzet osmotic minipumps (Durect corp.) that continuously infused angiotensin II (432 \( \mu \)g/kg/d) and phenylephrine (100 mg/kg/d) for 2 weeks as previously described.\(^9\)

Apoptosis detection
TUNEL was performed as previously described\(^{10}\) by end labeling using terminal deoxynucleotidyl transferase with the CardioTACS staining kit (Trevigen), according to the manufacturer’s instructions.

Swimming exercise
To assess exercise-induced physiological hypertrophy, mice were subjected to swimming as previously described.\(^{10}\) The protocol consisted of a conditioning period (10-minute-increment increases each day in swimming time) until two 90-minute sessions were achieved daily. Eleven days of swimming was performed before the experiment was terminated and hearts were analyzed.

mRNA expression analysis
RNA was extracted from snap-frozen ventricles of 60 day old mice with the RNA fibrous tissue kit according to the manufacturer’s instructions (Qiagen). Reverse transcriptase (RT) reaction was performed using the SuperScript III first-strand synthesis system for RT-PCR (Invitrogen) and random hexamer primers according to the manufacturer’s instructions. Quantitative real time PCR was performed with sybr-green and taq polymerase master mix (Applied Biosystems). Data was normalized to the expression of GAPDH.

Studies in isolated rat cardiomyocytes
Rat hearts were excised from heparinized (1500 U/kg) and anesthetized (Nembutal; 150 U/kg) adult female Sprague Dawley rats (200g, Harlan, Indianapolis, IN). Ventricular cardiac myocytes were isolated via collagenase-hyaluronidase digestion as previously described.11 Post digestion, the myocytes were resuspended in DMEM (Dulbecco’s Minimum Eagle Media) plus 5% fetal bovine serum (FBS) and approximately 20,000 cells were plated on laminin-coated coverslips and incubated for 2 hours. The media was then replaced with serum-free DMEM and myocytes were transduced with purified recombinant adenoviral vectors.

Engineered heart tissue (EHT) generation
EHTs were prepared as described before.12 Briefly, a reconstitution mixture containing isolated heart cells from neonatal rats (2.5x10⁶ cells in DMEM with 10% fetal calf serum, 1 mmol/l glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin), pH-neutralized collagen type I from rat tails (0.8 mg/EHT), basement membrane protein containing Engelbreth-Holm-Swarm tumor exudate (10% v/v; BD Biosciences), and concentrated serum-containing culture medium (2xDMEM, 20% horse serum, 4% chick embryo extract, 200 U/ml penicillin, and 200 µg/ml streptomycin) was poured into circular molds (inner diameter: 8 mm; outer diameter: 16 mm; height, 5 mm). On culture day 7 spontaneously beating EHTs were transduced with Ad-GFP control virus (MOI 5) or Ad-dnMek1+Ad-Dusp6 (MOI 5) for 6 hours before transferring them to stretch devices to continue culture under phasic load (from 100 to 110% of slack length at 2 Hz) for additional 5 days. Multiplicity of infection (MOI) was defined as the virus concentration needed to achieve 100% transduction in a 10 cm cell culture dish containing 2.5x10⁶ neonatal rat heart cells (MOI 1). Uninfected control EHTs were studied in parallel.
On culture day 12 EHTs were immersed in modified bicarbonate-free Hanks’ Balanced Salt Solution with HEPES (BFHH; NaCl 136.9 mmol/l, KCl 5.36 mmol/l, MgSO$_4$ 0.81 mmol/l, glucose 5.55 mmol/l, CaCl$_2$ 0.0125 mmol/l, KH$_2$PO$_4$ 0.44 mmol/l, Na$_2$HPO$_4$ 0.34 mmol/l, HEPES 20 mmol/l) containing 0.035 mg/ml Liberase Blendzyme III (Roche) and 30 mmol/l BDM for 30 min at 37 °C under continuous shaking. After a second round of digestion and gentle trituration the cell suspension was centrifuged at 200 g for 3 min and subsequently fixed in 4% formaldehyde in the presence of 30 mmol/l BDM. Cell smears were prepared on Superfrost glass slides. Cardiomyocytes were labeled with α-actinin (1:250; Sigma) and DAPI (1 ng/ml). Cell size was assessed by randomly scanning regions of up to 1 mm x 1 mm by automated scanning of 5x5 visual fields with a 40x oil objective (tile scan function; Zeiss LSM 710 NLO). Cell length and area were determined and mean cell width was calculated by dividing cell area by cell length. Only cardiomyocytes with a clear cross striation were included in the analysis.

Statistics

All results are presented as mean ± SEM. Statistical analyses were performed in Microsoft Excel and Prism using student t-test for 2 groups for single condition, or 2-way ANOVA with Bonferroni post-test.

References

7. Liu Q, Chen X, Macdonnell SM, Kranias EG, Lorenz JN, Leitges M, Houser SR, Molkentin JD. Protein kinase C{alpha}, but not PKC{beta} or PKC{gamma}, regulates contractility and


Online Figure I.

A, TUNEL of heart sections in the indicated mice at baseline. Nuclease treatment served as a positive control. B, Assessment of fibrosis by Massons trichrome staining. A section from long-term TAC in wt mouse hearts served as positive control. C and D, quantitative assessment of A and B. n=4 hearts, *p<0.05. E, H&E analysis of heart sections in the indicated mice. F, Immunofluorescent staining of isolated cardiomyocytes from the indicated mouse hearts with anti sarcomeric α-actinin antibody (red).
Online Figure II. A, Assessment fractional shortening (FS) percentage and B, shortening velocity in freshly isolated adult cardiomyocytes from Erk1^-/- Erk2^fl/fl-Nkx-Cre and control Nkx2.5-Cre mice at 60 days of age. The data show increased contractility despite elongation and reduced whole organ heart function. *p<0.05, n=20.
Online Figure III. A, Western blot of heart extracts showing deletion of ERK1 in Erk1\(^{-/-}\) αMHC-Cre mice and deletion of ERK2 in Erk2\(^{fl/fl}\) αMHC-Cre mice without changes in JNK and p38. Phospho-ERK1/2 were also similarly deleted or reduced. B, Gravimetric analysis of heart weight normalized to body weight in αMHC-Cre (white bars), Erk1\(^{-/-}\) αMHC-Cre (grey bars) or Erk2\(^{fl/fl}\) αMHC-Cre (black bars) mice showing no differences at baseline. C and D, Assessment of fractional shortening (FS) and left ventricular end diastolic internal diameter (LVEDD) by echocardiography showing no changes between genotypes at baseline.
Online Figure IV. A, Heart weight/body weight (HW/BW) in control Nkx2.5-Cre and Erk1\textsuperscript{-/-} Erk2\textsuperscript{fl/fl} versus Erk1\textsuperscript{-/-} Erk2\textsuperscript{fl/fl-Nkx-Cre} mice following 11 days of rest or swimming. n=4, *p<0.05 versus control rest, #p<0.05 versus rest Erk1\textsuperscript{-/-} Erk2\textsuperscript{fl/fl-Nkx-Cre} mice). B and C, Assessment of LVEDD and FS% by echocardiography in the indicated groups of mice following 11 days of swimming. n=7, *p<0.05 versus control rest, #p<0.05 versus resting Erk1\textsuperscript{-/-} Erk2\textsuperscript{fl/fl-Nkx-Cre} mice). D, Quantitative analysis of isolated myocyte length and width from hearts of the indicated mice after swimming or at rest. n=600, *p<0.05 versus control rest, #p<0.05 versus rest Erk1\textsuperscript{-/-} Erk2\textsuperscript{fl/fl-Nkx-Cre} myocytes).
Online Figure V. A, Western blot of rat cardiomyocytes showing increased phosphorylation of ERK1/2 following infection with Ad-aMek1, and significantly blocked phosphorylation following the co-addition of Ad-Dusp6. Ad-βgal infection was a control. B, Immunofluorescent staining of isolated rat cardiomyocytes with anti sarcomeric α-actinin antibody (red) at control conditions or following infection with Ad-aMek1 or Ad-aMek1+Ad-Dusp6. C, Quantitative analysis of cell length and width showing that Ad-aMek1 induces an increase in cell width and this increase can be blocked by the addition of Ad-Dusp6. Ad-Dusp6 also induces an increase in cell length. *p<0.05 vs control, n=200. These data indicate that aMEK1 cannot function on its own to cause an increase in cell width without ERK1/2.
Online Figure VI. Western blot of myocyte extracts from control Erk1−/− Erk2fl/fl (1), Erk1−/− Erk2fl/fl-Cre (2) and from acMek Tg (3) mice with the indicated antibodies. There were no significant changes in the activation levels of AKT, GSK3β, STAT3 or calcineurin activation (Ac. Cn) between the various genotypes. A positive control (Hela cells treated by interferon) was added to the last lane for STAT3. The activated calcineurin blot was after immunoprecipitation with calmodulin.