Targeted Deletion of the Extracellular Signal-Regulated Protein Kinase 5 Attenuates Hypertrophic Response and Promotes Pressure Overload–Induced Apoptosis in the Heart

Tomomi E. Kimura,* Jiawei Jin,* Min Zi,* Sukhpal Prehar, Wei Liu, Delvac Oceandy, Jun-ichi Abe, Ludwig Neyses, Arthur H. Weston, Elizabeth J. Cartwright,† Xin Wang†

Rationale: Mitogen-activated protein kinase (MAPK) pathways provide a critical connection between extrinsic and intrinsic signals to cardiac hypertrophy. Extracellular signal-regulated protein kinase (ERK)5, an atypical MAPK is activated in the heart by pressure overload. However, the role of ERK5 plays in regulating hypertrophic growth and hypertrophy-induced apoptosis is not completely understood.

Objective: Herein, we investigate the in vivo role and signaling mechanism whereby ERK5 regulates cardiac hypertrophy and hypertrophy-induced apoptosis.

Methods and Results: We generated and examined the phenotypes of mice with cardiomyocyte-specific deletion of the erk5 gene (ERK5cko). In response to hypertrophic stress, ERK5cko mice developed less hypertrophic growth and fibrosis than controls. However, increased apoptosis together with upregulated expression levels of p53 and Bad were observed in the mutant hearts. Consistently, we found that silencing ERK5 expression or specific inhibition of its kinase activity using BIX02189 in neonatal rat cardiomyocytes (NRCMs) reduced myocyte enhancer factor (MEF)2 transcriptional activity and blunted hypertrophic responses. Furthermore, the inhibition of MEF2 activity in NRCMs using a non-DNA binding mutant form of MEF2 was found to attenuate the ERK5-regulated hypertrophic response.

Conclusions: These results reveal an important function of ERK5 in cardiac hypertrophic remodeling and cardiomyocyte survival. The role of ERK5 in hypertrophic remodeling is likely to be mediated via the regulation of MEF2 activity. (Circ Res. 2010;106:961-970.)

Key Words: cardiac hypertrophy ■ signal transduction ■ genetically modified mice

Cardiac hypertrophy is a virtually universal prerequisite for the development of heart failure. In response to acute or chronic insults the heart initially develops hypertrophic growth. However, sustained stress causes chamber dilation, interstitial fibrosis, and myocyte apoptosis, eventually leading to heart failure or sudden death from arrhythmias. Numerous signaling pathways including mitogen-activated protein kinases (MAPKs) are implicated in mediating the process of cardiac hypertrophy and hypertrophy-induced cardiomyocyte apoptosis.1 In the heart, all 4 classes of MAPKs are activated through either mechanical overload or neurohumoral stimulation.2 However, our understanding of the critical role of individual MAPK in various aspects of hypertrophic remodeling remains fragmented and in many cases controversial.

Extracellular signal-regulated protein kinase (ERK)5 is an atypical MAPK, also known as big MAPK (BMK)1, because it is more than twice the size of the other MAPKs, owing to a very large C-terminal domain.3 It is suggested that the role of this unique C-terminal tail is to regulate myocyte enhancer factor (MEF)2 transcriptional activity and ERK5 subcellular localization.3,4 On exposure to various stimuli, ERK5 is activated by phosphorylation downstream of MEK5.5 An important step in deciphering the function of ERK5 is to identify its downstream targets and MEF2A, -C, and -D are among the best-characterized substrates of ERK5.4,5 Analogous to many MAPKs, ERK5 plays an important role in cell survival, cell proliferation and differentiation. The discovery that ERK5 is an important contributor to cell

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From the Faculty of Life Sciences (T.E.K., J.J., W.L., A.H.W., X.W.) and Faculty of Medical and Human Sciences (M.Z., S.P., D.O., L.N., E.J.C.), University of Manchester, United Kingdom; and Aab Cardiovascular Research Institute (J.-i.A.), University of Rochester School of Medicine and Dentistry, NY.

†These authors contributed equally to this work.

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survival has increased interest in this signaling pathway. In sympathetic neurons, ERK5 is required to mediate the survival response to nerve growth factor by suppressing the expression of Bad and Bim. The analysis of mice with an endothelial-specific deletion of ERK5 provides physiological evidence that ERK5 is essential for the survival of endothelial cells via the activation of MEF2. Moreover, recent studies have demonstrated that ERK5 activation prevents cardiomyocyte apoptosis, likely through the inhibition of a feedback loop of phosphodiesterase 3A/inducible cAMP early repressor. Apart from its role in cardiomyocytes alone does not affect cardiac development. To characterize the role of ERK5 in cardiac hypertrophy we subjected ERK5<sup>cko</sup> mice to hypertrophic stimuli, the mutant mice developed less hypertrophic growth and fibrosis compared to controls (ERK5<sup>f/f</sup>), also increased apoptosis with upregulated expression of p53 and Bad were observed in the mutant heart. Consistently, we found that silencing ERK5 expression or specific inhibition of its kinase activity using a novel compound BIX02189 in neonatal rat cardiomyocytes (NRCMs) reduced MEF2 transcriptional activity and blunted the hypertrophic response. Moreover, the inhibition of MEF2 activity in NRCMs using a non-DNA binding mutant form of MEF2C was found to diminish the ERK5-regulated hypertrophic response. These results clearly reveal an important function of ERK5 in stress-induced cardiac hypertrophic remodeling and cardiomyocyte survival.

**Methods**

Cardiac hypertrophy was induced by transverse aortic constriction (TAC) or chronic infusion of isoproterenol (ISO) at 10 mg/kg per day for 7 days in 8- to 10-week-old male ERK5<sup>f/f</sup> and ERK5<sup>cko</sup> mice as previously described. See the expanded Methods section in the Online Data Supplement (available at http://circres.ahajournals.org) for the following: generation of ERK5<sup>cko</sup> mice, echocardiography, histological analysis, immunoblot analysis, small interfering (si)RNA transfection, adenoviral infection, apoptosis assays, quantitative PCR, luciferase reporter assay, and [3H]leucine incorporation.

**Results**

Characterization of ERK5<sup>cko</sup> Mice

Using α myosin heavy chain promoter driven-Cre transgenic mice, we generated ERK5<sup>cko</sup> mice. Normal levels of ERK5 mRNA were present in brain and liver, whereas ERK5 mRNA was significantly reduced (approximately 86%) in the mutant ventricle (Online Figure I, A). Immunoblot analysis of ventricular extracts from ERK5<sup>cko</sup> mice at 8 weeks old showed more than 80% deletion of ERK5 protein (Online Figure I, B). It was evident that ablation of ERK5 was specific to the heart because protein levels of ERK5 were equivalent in brain, liver and skeletal muscle from both ERK5<sup>cko</sup> and ERK5<sup>f/f</sup> mice (Online Figure I, B). The absence

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of ERK5 in the heart did not cause any compensatory changes in the protein levels of other MAPKs, such as ERK1/2, c-Jun N-terminal kinase (JNK), p38, and MEK5 (Online Figure I, C). Furthermore, cardiac structure and contractile function were examined by histological analysis and echocardiography, respectively, and no differences were found between the two genotypes (data not shown).

**ERK5cko Mice Displayed Less Cardiac Hypertrophic Remodeling on Pressure Overload**

To determine whether ERK5 is required for pressure overload-induced cardiac hypertrophic remodeling, ERK5f/f and ERK5cko mice were subjected to pressure overload stimulation by following 1 week of TAC, ERK5f/f mice showed a 34% increase in heart weight/tibia length (HW/TL). In contrast, ERK5cko mice showed only a 23% increase in HW/TL (Figure 1A and 1B). Consistent with these results, greater cross-sectional area of cardiomyocytes was observed in ERK5f/f-TAC mice (280.05±6.27 μm²), in comparison to 256.51±8.03 μm² in ERK5cko-TAC mice (Figure 1C). Pressure overload–induced hypertrophy is often accompanied by interstitial fibrosis. Based on Masson’s trichrome staining, less ventricular fibrosis was seen in ERK5cko-TAC hearts (Figure 1D). Furthermore, cardiac structure and function were assessed by echocardiography. In response to TAC, ERK5cko mice showed a significant reduction in end-diastolic left ventricular posterior wall thickness compared with the control group. Meanwhile, we also found in the mutant hearts a trend toward a decrease in fractional shortening percentage (FS%) and a markedly slowed aorta maximum velocity, which is a sign of contractile dysfunction (Table). Fetal gene activation is a hallmark of hypertrophic remodeling. As shown in Figure 2, upregulation of the transcripts of the hypertrophic gene markers atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP), skeletal α-actin (Acta1), and β-myosin heavy polypeptide (Myh7) was remarkably blunted in the mutant hearts following TAC; we also found the

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**Table. Echocardiographic Assessment of ERK5f/f and ERK5cko Mice After One Week of TAC**

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<td>dPW (mm)</td>
<td>0.75±0.03</td>
<td>0.99±0.05</td>
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<td>dIVS (mm)</td>
<td>0.88±0.01</td>
<td>1.13±0.08</td>
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<td>LVEDD (mm)</td>
<td>4.02±0.04</td>
<td>3.78±0.18</td>
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<tr>
<td>LVESD (mm)</td>
<td>2.69±0.09</td>
<td>2.95±0.24</td>
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<tr>
<td>FS (%)</td>
<td>34.1±0.5</td>
<td>32.8±1.9</td>
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<tr>
<td>Ao Vmax (cm/sec)</td>
<td>63.4±5.3</td>
<td>65.7±4.3</td>
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<td>Ratio of E/A wave</td>
<td>1.15±0.09</td>
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*P<0.05, ERK5cko-TAC mice vs ERK5f/f-TAC mice.
mRNA level of α myosin heavy polypeptide (Myh6) was compromised in the mutant hearts (Figure 2). Consistent with Masson’s trichrome staining, the fibrosis marker genes connective tissue growth factor (Ctgf), procollagen type I, α2 (Col1α2) and procollagen type III, α3 (Col3α1) were significantly downregulated in ERK5cko-TAC mice compared with the controls (Figure 2). Finally, we investigated the activation of ERK5 and an array of hypertrophic regulators such as ERK1/2, protein kinase (PK)B, p38, and JNK after TAC. ERK5 was activated by TAC in ERK5f/f hearts but not in ERK5cko hearts, meanwhile there was no difference observed in the activation of other hypertrophic regulators between the two genotypes although all were activated following TAC (Figure 3). These results suggest that the blunted hypertrophic response in the mutant mice is likely attributable to the absence of ERK5 in the heart.

**ERK5cko Cardiomyocytes Were More Vulnerable Under Hypertrophic Stress**

Sustained hypertrophic stress causes cardiomyocyte apoptosis which contributes to the transition of pathological hypertrophy to heart failure. To determine the effect of ERK5 on cardiomyocyte viability under hypertrophic stress TUNEL staining and caspase 3 activity assays were performed to detect cardiomyocyte apoptosis. We observed increased TUNEL-positive nuclei along with enhanced caspase 3 activity in the mutant hearts following TAC (Figure 4A through 4C). In accordance with this finding, ERK5cko-TAC mice showed augmented protein levels of Bad and p53, whereas the expression level of Bcl-2 was comparable in both genotypes (Figure 4D). A recent study demonstrated a protective role of ERK5 in sympathetic neurons through the phosphorylation of Ca2+/cAMP response element-binding protein (CREB), thereby suppressing the transcription of Bad. However, we did not observe any change in the protein level and phosphorylation status of CREB in the mutant hearts (Figure 4D). Furthermore, in the two sham groups, no difference was detected in expression levels of these apoptosis-associated proteins (Online Figure II). Together, these data suggest that ERK5 is required for cardiomyocyte survival in response to pressure overload by suppressing the mitochondrion-dependent apoptotic pathway.

**Prolonged Pressure Overload Caused Cardiac Dysfunction in ERK5cko Mice**

To further examine whether the lack of ERK5 in cardiomyocytes predisposes mice to heart failure following long-term pressure overload stimulation, ERK5f/f and ERK5cko mice were subjected to 5 weeks of TAC. Likewise, 5 weeks of TAC also caused compromised HW/TL ratio, smaller myocyte cross-sectional areas, and less fibrosis in ERK5cko mice compared with the controls (Figure 5A through 5C). Analysis of gene expression profile showed that mRNA levels of ANP,
Interstitial fibrosis was also more noticeable in the control hearts (Online Figure IV). Furthermore, the transcript levels of ANP and BNP were found to be remarkably elevated in the ERK5\textsuperscript{ft} hearts, compared with the mutant hearts (Online Figure IV). These results indicate that ERK5 regulates cardiac hypertrophic remodeling not only by pressure overload but also by chronic \( \beta \)-adrenergic stimulation.

Knockdown of ERK5 or Inhibition of Its Activity and Promoted Apoptosis in NRCMs

MEF2 transcription factor is a key regulator of pathological cardiac hypertrophy.\textsuperscript{18} Led by the result that the transcript levels of Ctgf, Myh6, and Acta1 (known to be downstream targets of MEF2\textsuperscript{18–20}) were compromised in ERK5\textsuperscript{cko}-TAC hearts we further examined whether MEF2 transcriptional activity was mediated by ERK5 in cardiomyocytes in response to hypertrophic stimuli. Using the MEK5/ERK5-selective inhibitor (BIX02189, 10 \( \mu \)mol/L), ERK5 phosphorylation was specifically inhibited which led to attenuated MEF2 activity in NRCMs following ISO treatment (Figure 6A and 6B). To corroborate this result, NRCMs were transfected with the reporter plasmid pG5E1bLuc together with a construct encoding Gal4, Gal4-MEF2A, or Gal4-MEF2D. As expected, reduced MEF2 transcriptional activity was detected in BIX02189-treated NRCMs after ISO stimulation (Online Figure V). In parallel, using an siRNA method we specifically suppressed endogenous ERK5 expression by 83% in NRCMs, a similar inhibitory effect on MEF2 transcriptional activity was also found (Figure 6C and 6D; Online Figure V). Furthermore, siERK5-treated NRCMs showed an impaired hypertrophic response which was evident by decreased mRNA levels of ANP and Acta1 and diminished protein synthesis (Figure 7A and 7B). To further critically examine the effect of MEF2 on ERK5-mediated hypertrophic response, an adenovirus containing a non-DNA binding mutant form of MEF2C (Ad-MEF2C-R3T) was used to inhibit MEF2 activity.\textsuperscript{8,21} After ISO stimulation, we observed an increased rate of protein synthesis and elevated transcript level of ANP in Ad-ERK5-infected NRCMs; however, such hypertrophic increases were appreciably blunted by the coinfection of Ad-MEF2C-R3T (Figure 7C through 7E). Combined, these data strongly suggest that MEF2 acts downstream of ERK5 to promote a hypertrophic response.

Discussion

The major finding of this study is that ERK5 plays an important role in promoting hypertrophic remodeling and cardiomyocyte survival. In the absence of ERK5, hypertrophic growth, interstitial fibrosis, and fetal gene reactivation...
were profoundly compromised; meanwhile, increased cardiomyocyte death was seen following hypertrophic stimuli. Further studies in NRCMs treated with either siERK5 or a novel ERK5 kinase inhibitor (BIX02189) have provided clear evidence that MEF2 is a critical component downstream of the ERK5 signaling pathway in regulating hypertrophic response.

Role of ERK5 in Hypertrophic Remodeling
ERK5 is implicated in the induction of cardiac hypertrophy and heart failure in humans; however, our understanding of its involvement in various aspects of cardiac remodeling is limited and, in some cases, controversial. For example, previous studies using transgenic mice with cardiac-specific overexpression of activated MEK5 provided conflicting results, which can be partially explained by the fact that different isoforms (α, β form) of MEK5 were used to generate the transgenic lines. An additional explanation is the transgenic approach, as phenotypes observed from transgenic mouse models are largely associated with the copy number of overexpressed genes. With respect to the fact that ERK5 may exert actions independent of its kinase activity, it was hoped that the analysis of ERK5cko mice would generate more direct information on the specific contribution of ERK5 in the heart. In the present study, phenotypes observed from ERK5cko mice were reminiscent of observations in MEF2D-null mice; they also demonstrated blunted hypertrophic growth and fibrosis, together with the downregulation of mRNA levels of MEF2-regulated genes. In line with these observations, enhanced hypertrophy was seen in mice with cardiac-specific overexpression of MEK2A after TAC. Interestingly, ERK5 and p38 MAPK are able to phosphorylate and activate MEK2A and MEF2C, whereas MEF2D is a specific substrate of ERK5. Of note, the major MEF2 isoforms in the adult heart are MEF2A and MEF2D, which participate in mediating different aspects of pathological cardiac signaling. Recent studies in conventional and cardiomyocyte-specific p38 knockout mouse models suggest that p38 does not promote cardiac hypertrophy and may, in fact, prevent

Figure 4. ERK5 is required for cardiomyocyte survival in response to hypertrophic stress. A, Increased apoptosis in ERK5cko ventricular myocardium after 1 week of TAC was detected by TUNEL assay (scale bar: 50 μm). Arrows point to TUNEL-positive nuclei. B, The bar graph summarizes the percentage of TUNEL-positive nuclei in ERK5cko hearts compared with that in control hearts. C, Caspase 3 activity was measured by caspase activity assay after TAC (n=7). D, Immunoblot analyses of protein levels of Bad, Bcl-2, p53, and CREB and phosphorylation of CREB by specific antibodies. Tubulin expression is the protein loading control. The ratios of Bad/Bcl-2 and p53/tubulin are represented by the bar graphs (n=4 per group). Data are means±SEM.
Considering the above evidence, MEF2 is most likely to perform as a hypertrophic player downstream of ERK5, rather than p38. Furthermore, responding to ISO stimulation, a decrease was found in MEF2 transcriptional activity in NRCMs in which ERK5 was exclusively knocked down or its kinase activation was specifically inhibited. In addition to this, reduced protein synthesis and downregulated mRNA levels of ANP and Acta1 were detected in siERK5-treated NRCMs in which ERK5 was exclusively knocked down or its kinase activation was specifically inhibited. In addition to this, reduced protein synthesis and downregulated mRNA levels of ANP and Acta1 were detected in siERK5-treated NRCMs in which ERK5 was exclusively knocked down or its kinase activation was specifically inhibited. In addition to this, reduced protein synthesis and downregulated mRNA levels of ANP and Acta1 were detected in siERK5-treated NRCMs in which ERK5 was exclusively knocked down or its kinase activation was specifically inhibited.
NRCMs following ISO treatment. Moreover, the finding that the inhibition of MEF2 activity was able to attenuate the ERK5-regulated hypertrophic response strengthens the notion that MEF2 acts downstream of ERK5 in regulating hypertrophy.

CTGF is a key mediator of tissue fibrosis, which is predominantly expressed in fibroblasts; however, during cardiac remodeling it is also secreted by cardiomyocytes.26,27 Several lines of evidence indicate the important role of MEF2 in regulating CTGF expression.19,20,28 In the present study, the decreased Ctgf mRNA level along with less interstitial fibrosis were seen in ERK5cko-TAC hearts, thus it is plausible to propose that absence of ERK5 in cardiomyocytes is likely responsible for such phenotypes via downregulated MEF2 activity. Taken together, these data provide unequivocal evidence that ERK5 is responsible for controlling MEF2 activity in different aspects of hypertrophic remodeling.

In parallel, we also investigated the activation of a number of hypertrophic regulators following TAC stress, including ERK1/2,29 p38,24 JNK17 and PKB.30 However, we did not observe any difference in their activities between the two genotypes; this underpins the notion that ERK5 is an important character in mediating hypertrophic remodeling. It is worth noting that residual hypertrophic growth seen in ERK5cko-TAC mice is likely attributable to the activation of ERK1/2 and PKB, which are known as promoters of hypertrophy.29,30

ERK5 Is Required for Cardiomyocyte Survival

Apart from the altered hypertrophic response, we also detected an increase in apoptotic cardiomyocytes with enhanced Bad and p53 protein levels in TAC treated-mutant hearts, indicating that ERK5 prevents hypertrophy induced-cardiomyocyte loss. The ability of ERK5 to protect endothelial cells from shear stress via inhibition of Bad activation was established by Pi et al.31 Furthermore, evidence has been provided that ERK5 is required to mediate neuronal survival by suppressing Bad expression via a mechanism dependent on CREB transcriptional activity.6 Consistent with this study, we also found upregulated Bad expression in ERK5cko-TAC cardiomyocytes, although we did not observe any change in the protein level and phosphorylation status of CREB. CREB is a prosurvival transcription factor which either acts as an activator for increasing Bcl-2 expression or as a repressor of Bad expression.6,32 Interestingly, cardiomyocyte-specific inactivation of CREB did not cause any increase in apoptosis at basal level12; nevertheless, further studies are...
required to address the role of CREB in protecting cardiomyocytes following stress. Prolonged pressure overload is known to cause an accumulation of p53, which is a pivotal mediator of apoptosis.\textsuperscript{33,34} It has been demonstrated that p53 is able to induce a number of Bcl-2 family genes, including Bad.\textsuperscript{34,35} Thus, the upregulated p53 expression found in ERK5\textsuperscript{cko}-TAC hearts is proposed to be responsible for the increased level of Bad. It is also worth noting that impaired contractility seen in the mutant hearts after TAC is very likely attributed to the increased apoptosis. Although the actual rate of apoptosis is low, it is, however, reasonable to believe that this level of apoptosis is sufficient to cause cardiac dysfunction.\textsuperscript{36} As discussed, it is suggested that ERK5 is necessary for protecting cardiomyocyte against hypertrophic stress and for determining cardiac function during hypertrophic remodeling.

**Novel Pharmacological Inhibitors of the MEK5/ERK5 Pathway**

ERK5 is one of the least-studied MAPK subfamilies, in part, because of the lack of biological/pharmacological tools, including specific inhibitors. Recently, the MEK5/ERK5-selective inhibitors BIX02188 and BIX02189 have been identified. BIX02189 is demonstrated to have greater potency in inhibiting ERK5 activity than BIX02188.\textsuperscript{37,38} In the present study, by applying BIX02189 to NRCMs, we detected that ERK5 phosphorylation was specifically blocked without affecting the activation of other MAPKs; MEK2 transcriptional activity was also impaired. Thus, for the first time, our results provide important information regarding the effect of BIX02189 in cardiomyocytes. It has been demonstrated that ERK5 is required for tumor growth because of its essential role in the development of tumor vasculature.\textsuperscript{39} Selectively targeting the ERK5 pathway by pharmacological compounds is thought to be a promising approach for treating cancers. Although the availability of selective MEK5/ERK5 inhibitors may shed light in developing such a therapeutic strategy, it must nevertheless be considered that ERK5 has a role in many other tissues. Further investigation of these MEK5/ERK5 inhibitors in vivo under basal and various stress conditions is certainly required.

In conclusion, the present study provides convincing evidence that clarifies the in vivo role of ERK5 in the heart. ERK5 regulates hypertrophic remodeling and protects cardiomyocytes from hypertrophic stress mostly likely by regulating the MEK2 transcription activity. The recognition of the functional importance of ERK5 in the heart provides invaluable information regarding the possible beneficial or adverse effects of new therapeutic strategies.

**Acknowledgments**

We thank Dr Roger Snow (Department of Medical Chemistry, Boehringer Ingelheim Pharmaceuticals Inc) for kindly providing us with the ERK5 inhibitor BIX02189.

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**Disclosures**

None.

**References**

ERK5 can be activated in the hearts of animal models by pressure overload and in cultured cardiomyocytes by various hypertrophy-stimulating factors.

Heart failure (HF) is a debilitating condition that remains a major cause of mortality worldwide. Understanding the molecular determinants of hypertrophic remodeling, a virtually universal prerequisite of the development of HF, is a key step in elucidating the pathogenesis of HF. Extracellular signal-regulated protein kinase (ERK)5 has been implicated in the induction of HF. However, its role in various aspects of hypertrophic remodeling has thus far not been elucidated. Using a range of approaches including a cardiac myocyte-specific ERK5 knockout mouse model (ERK5cko), and an MEK5/ERK5-selective inhibitor (BIX02189), we found that ERK5cko mice developed less hypertrophic growth but increased apoptosis along with cardiac dysfunction in response to pressure overload, suggesting the functional importance of ERK5 in protecting against HF. Moreover, we have identified the transcription factor myocyte enhancer factor 2 as a downstream effector of ERK5 in regulating hypertrophic growth and prevention of HF. Overall, the recognition of the functional importance of ERK5 in the heart provides strategies for the first time the in vivo role of ERK5 in regulating myocardial fibroblast function and cardiac hypertrophy.

What New Information Does This Article Contribute?

- The present study elucidates the in vivo role and molecular basis whereby ERK5 regulates cardiac remodeling and protects cardiomyocytes from hypertrophic stress.

What Is Known?

- ERK5 (an atypical mitogen-activated protein kinase) is implicated in the induction of heart failure in humans.
- ERK5 can be activated in the hearts of animal models by pressure overload and in cultured cardiomyocytes by various hypertrophy-stimulating factors.

Novelty and Significance

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Materials and Methods

Generation of ERK5 Cardiac Knockout Mice

The erk5-flox mice (referred to as ERK5floX) were previously generated1. ERK5floX mice were mated with mice expressing Cre under α myosin heavy chain (αMHC) promoter to generate cardiac-specific ERK5 knockout mice (referred to as ERK5cko). The αMHC-Cre line (kindly provided by Dr. MD Schneider, National Heart and Lung Institute, UK) is a well established model that provides efficient Cre recombinase activity in the myocardium. We did not observe any abnormality in cardiac morphology and function in the αMHC-Cre line up to six months of age. All mice used in this study were maintained in a pathogen-free facility at the University of Manchester. The animal studies were performed in accordance with the UK Home Office and institutional guidelines.

Echocardiography

For cardiac morphological and functional analysis, M-mode and Doppler echocardiographic recordings were performed using an Acuson Sequoia C256 system (Siemens) following a protocol described previously2. Parameters of intraventricular septal thickness (IVS), left ventricular posterior wall thickness (LVPW), left ventricular end-diastolic dimension (LVEDD), left ventricular end-systolic dimension (LVESD) and fractional shortening (FS %), aorta maximum velocity (Ao Vmax) and ratio of the E/A-wave were obtained.

Histology

Freshly dissected heart tissue was fixed in 4% paraformaldehyde, dehydrated and embedded in paraffin. 5-7µm thick sections were cut and stained with hematoxylin & eosin or Masson’s trichrome method as described2. To calculate the mean cross-sectional area approximately 150 randomly selected cardiomyocytes were measured. 45 randomly chosen frames from Masson’s trichrome stained sections were quantified to assess the degree of myocardial fibrosis using Image J software.

Preparation of Lysates and Immunoblot Analysis

Proteins were homogenised and extracted from tissues in Triton lysis buffer (20 mM Tris-HCl, pH 7.4, 137 mM NaCl, 2 mM EDTA, 1% Triton X-100, 25 mM β-glycerophosphate, 10% glycerol, 1 mM orthovanadate, 1 mM phenylsulphonyl fluoride, 10 µg/ml leupeptin, 10 µg/ml aprotinin). Protein extracts (30 µg) were subjected to Western blot analysis with antibodies against JNK, p38 MAPK, p53 (Santa Cruz); ERK1/2, PKB, Bcl-2, CREB, phospho-PKB (Ser 473), phospho-ERK1/2, phospho-JNK, phospho-p38 MAPK, phospho-CREB (Ser 133), MEF2C (Cell Signalling); Bad (BD Transduction); ERK5 (Upstate); phospho-ERK5, GFP (Invitrogen) and tubulin (Sigma). Immunocomplexes were detected by enhanced chemiluminescence with anti-mouse, anti-rabbit, or anti-goat immunoglobulin G coupled to horseradish peroxidase as the secondary antibody (Amersham-Pharmacia).

Characterization of Effect of BIX02189 on Neonatal Rat Cardiomyocytes

Primary cultures of neonatal rat cardiomyocytes (NRCMs) were prepared as previously described2. NRCMs were incubated with 10µM BIX02189 (Boehringer Ingelheim) for 2h prior to the addition of isoproterenol (10µM, 30min). Cell extracts were lysed for immunoblotting to determine the effect of BIX02189 on inhibition of the activation of ERK5.

siRNA Transfection

NRCMs were transfected with siRNA (100nM) using Lipofectamine LTX and Plus reagents according to the manufacturer’s instructions (Invitrogen). Rat ERK5 siRNA (5’-AAAGGGTGCGAGCCCTATAT-3’) was purchased from Dharmaco, siRNA negative control (Si Neg) was obtained from Eurogenetec. To assess the specific effect of ERK5 siRNA on silencing ERK5 expression, the protein levels of MEK5 and ERK1/2 were detected by immunoblot analysis 72h post-transfection.
Adenoviral Infection
NRCMs were infected with either Ad-MEF2C-R3T (Seven Hills Bioreagents), or Ad-ERK5 (kindly provided by Jun-ichi Abe, Rochester University), or Ad-MEF2C-R3T combined with Ad-ERK5 at 50 MOI in serum-free medium for 24h, followed by additional 24h incubation of ISO, afterwards cells were then subjected to quantitative real-time PCR analyses or [3H] leucine incorporation assay. Ad-GFP was used for a control experiment.

Apoptosis Assays
TUNEL assay to detect apoptosis was performed on paraffin-embedded heart sections using the *in situ* Cell Death Detection kit (Roche). Triple staining with Hoechst, anti-α-actinin antibody (Sigma), and TUNEL was performed to confirm apoptotic morphology in cardiac nuclei. An average of total 10,000 myocyte cells from random fields was analyzed. The data are obtained from three independent experiments. For caspase activity assay, proteins were homogenised and extracted from heart tissues in the lysis buffer (25mM HEPES pH 7.5, 10mM KCl, 1.5mM MgCl₂, 1mM EDTA, 1mM EGTA, 1mM benzamidine, 1mM dithiothreitol [DTT], 1mM phenylsulphphonyl fluoride, 1% Triton X100). Protein extracts (50 µg) were incubated with 200µM DEVD-AMC caspase-3 specific fluorogenic substrate (Alexis Biochemicals) for 1h in the caspase reaction buffer (20mM HEPES, 100mM NaCl, 10mM DTT, 0.1% CHAPS, 10% w/v sucrose). Cleavage of the substrate was measured by spectrofluorometer at 380 nm excitation and 460 nm emission wavelengths. To evaluate the effect of ERK5 knockdown or the inhibition of its kinase activation on apoptosis, BIX02189-treated or siERK5-treat NRCMs were stimulated with sorbitol (Sigma) at 300mM to induce apoptosis³,⁴. After 4h sorbitol stimulation, cell lysates were prepared for measuring caspase 3 activity.

Quantitative Real-Time PCR
Tissues or NRCMs were extracted to prepare total RNA using Trizol reagent, followed by the synthesis of cDNA. Real-time quantitative PCRs were performed using the SYBR-green I Core Kit (Eurogentec). The primers used for ANP, BNP, Myh6, Myh7, Acta1, Ctgf, Col1α2, Col3α1 and GAPDH were obtained from Qiagen. PCR products were detected in the ABI-PRISM 7700 sequence detection system (Applied Biosystems), and the results were analyzed using the 2-ΔΔCT method⁵. The level of expression of mRNA was normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA.

 Luciferase Reporter Assay
To measure MEF2 luciferase activity after blocking ERK5 activation, NRCMs were infected with recombinant adenovirus encoding the MEF2-luciferase reporter gene (AdMEF2-Luc, Seven Hills Bioreagents) at 25 MOI in serum-free medium for 24h, prior to the incubation of BIX02189. After 2h incubation with the inhibitor, the cells were then treated with 10µM isoproterenol for an additional 18h in the presence of BIX02189. To measure MEF2 luciferase activity in the absence of ERK5 expression, 48h post-transfection of siRNA, NRCMs were infected with AdMEF2-Luc for 24h followed by isoproterenol stimulation. After various treatments, aliquots of NRCM lysates were assayed for the MEF2 luciferase activity using the luciferase assay kit (Promega). To further assess whether MEF2 transcriptional activity is regulated by either ERK5 knockdown or the inhibition of ERK5 kinase activation, NRCMs were transiently transfected with the reporter plasmid pG5E1bLuc together with a construct encoding the fusion proteins Gal4-MEF2A, or Gal4-MEF2D³, prior to 2h incubation with BIX02189. After BIX02189 incubation, MEF2 transcriptional activity was measured at 24h post ISO stimulation by the dual-luciferase reporter assay system (Promega). Gal4 was used for a control experiment. A pRL-TK plasmid encoding Renilla luciferase was employed to monitor transfection efficiency. In parallel, siRNA-treated NRCMs were transfected with various plasmid vectors as described above using Lipofectamine LTX and Plus reagents (Invitrogen). Following 24h ISO stimulation, MEF2 transcriptional activity was measured by the dual-luciferase reporter assay system (Promega).
**[3H] Leucine Incorporation**

NRCMs were transfected with siRNA (100nM) for 48h, or infected with various adenoviruses (Ad-GFP, Ad-MEF2C-R3T, Ad-ERK5, or Ad-ERK5 plus Ad-MEF2C-R3T) at 50 MOI for 24h following the isoproterenol treatment (10µM, 24h). The cells were then incubated in the same medium with 1.0µCi/ml [3H] Leucine for an additional 24h. After washing in ice-cold PBS, the cells were precipitated with 10% trichloroacetic acid for 30 minutes at 4°C. The precipitates were then solubilized in 0.4 N NaOH for 1h and neutralized with 1N HCl. Radioactivity was measured in a liquid scintillation counter. Each well was normalized to the total DNA content measured at 260nm.

**Data Analysis**

Data are expressed as mean ± SEM and analyzed using one-way or two-way ANOVA followed by Bonferonni’s post-test where appropriate. Comparisons between two groups were performed using Student’s t-test. P-values <0.05 are considered statistically significant.

**Expanded Discussion**

**The Loss of ERK5 in Cardiomyocytes Does Not Have a Primary Influence on Hypertrophy-Induced Angiogenesis**

Enhanced angiogenesis under pressure overload is a critical feature of adaptive hypertrophic growth. Sustained stress eventually causes deregulation of microvascularization and insufficient oxygenation which contribute to the pathogenesis of heart failure. ERK5 is reported to be a negative regulator of angiogenesis. During embryonic development, deficiency of ERK5 results in upregulated hypoxia inducible factor 1α (HIF-1α) activity and increased expression of the vascular endothelial growth factor (VEGF) which impedes the angiogenesis process and vascular maturation. Interestingly, a recent study reported that cardiomyocyte-specific deletion of HIF-1α caused significantly less microvessels and impaired cardiac function after TAC. Taking these findings into account, we assessed capillary density in TAC-treated ERK5^cko hearts. No difference in the capillary-to-cardiomyocyte ratio was found in the two genotypes (data not shown); suggesting the lack of ERK5 in cardiomyocytes does not have a primary influence on capillary density. However, whether ERK5 expressed in nonmyocytes is involved in regulating capillary density in the process of hypertrophic remodeling needs further study.

**References**

Supplement Figure I. Characterization of cardiac-specific deletion of ERK5. (A) Quantitative RT-PCR analysis of the mRNA levels of ERK5 in the left ventricle (LV), brain and liver, demonstrating a 86% decrease in ERK5 mRNA in LV. (B) Western blot analyses to determine specificity of the deletion of ERK5 in the left ventricle compared to protein extracts from various tissues, tubulin expression served as the protein loading control. The ratio of ERK5 expression to tubulin is shown in the bar graph. Data are presented as mean ± SEM, n=3 for each group. (C) Immunoblot analyses show similar expression levels of ERK1/2, JNK, p38 MAPK and MEK5 in the two genotypes. n.s.: no difference found between two groups.
Supplement Figure II. Immunoblot analyses of protein levels of Bad, Bcl-2, p53, CREB, and phosphorylation level of CREB in the two sham groups. Tubulin expression is the protein loading control. The ratios of Bad/Bcl-2, p53/tubulin and P/T CREB are represented by the bar graphs, n=4 per group. Data are presented as mean ± SEM, n.s.: no difference found between two groups
Supplement Figure III. Quantitative real-time PCR analyses of gene markers associated with hypertrophy and fibrosis after 5 weeks of TAC. The data are derived from three independent experiments performed in triplicate and are normalized to the GAPDH content, n= 5 per group. Data are presented as mean ± SEM.
Supplement Figure IV. The loss of ERK5 attenuated isoproterenol induced-cardiac hypertrophy. (A) ERK5<sup>cko</sup> mice showed blunted cardiac hypertrophy following isoproterenol infusion (scale bar: 20µm). (B) HW/TL ratios of ERK5<sup>f/f</sup> and ERK5<sup>cko</sup> mice were evaluated after 7-day isoproterenol treatment, n=4 to 9 per group. (C) Mean cross-sectional areas of cardiomyocytes in ERK5<sup>f/f</sup> and ERK5<sup>cko</sup> mice were calculated, n=4. (D) Ventricular interstitial fibrosis was minimal in ERK5<sup>cko</sup> hearts detected by Masson’s trichrome-staining of cross-sections. Quantification of the relative fibrotic area is expressed as percentage of the area of fibrosis in the microscope views, n=4. (E) Quantitative real-time PCR analyses of hypertrophic gene markers, ANP and BNP. The data are derived from three independent experiments performed in triplicate and are normalized to the GAPDH content, n=3. Data are presented as mean ± SEM.
Supplement Figure V. The ERK5 knockdown or the inhibition of its kinase activation suppressed MEF2 transcriptional activity in NRCMs. (A) NRCMs were transfected with the reporter plasmid pG5E1bLuc together with constructs encoding Gal4-MEF2A, or Gal4-MEF2D, followed by BIX02189 treatment. After 2h inhibitor treatment, MEF2 transcriptional activity was measured by the dual-luciferase reporter assay system before and after ISO stimulation. A control experiment was performed with pG5E1bLuc and Gal4. As expected, both Gal4-MEF2A and Gal4-MEF2D activities were reduced in BIX02189-treated NRCMs, indicating diminished MEF2 transcriptional activity. (B) siRNA-treated NRCMs were co-transfected with various plasmid vectors as indicated. MEF2 transcriptional activity was measured by the dual-luciferase reporter assay system following ISO stimulation. Firefly luciferase activity was normalized to that of Renilla luciferase. The data are derived from three independent experiments performed in duplicate, n=6. Data are presented as mean ± SEM. *P<0.05 versus vehicle alone (A) or control siRNA alone (B); #P<0.05 versus BIX02189 treated + ISO (A) or ERK5 siRNA treated + ISO (B).
Supplement Figure VI. The ERK5 knockdown or the inhibition of its kinase activation sensitizes NRCMs to apoptosis. BIX02189-treated NRCMs (A); or siERK5-treated NRCMs (B) were stimulated with sorbitol for 4h. Following stimulation, caspase 3 activity was measured by caspase activity assay, n=6. Data are presented as mean ± SEM.