β-Adrenergic receptor stimulation causes cardiac hypertrophy via a Gβγ/Erk-dependent pathway

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Aims Activation of the β1-adrenergic receptor and its G protein, Gs, induces cardiac hypertrophy. However, activation of classic Gs effectors, adenyl cyclases (AC) and protein kinase A, is not sufficient for induction of hypertrophy, which suggests the involvement of additional pathway(s) activated by Gs. Recently, we discovered that Gβγ subunits of Gs induce phosphorylation of the extracellular regulated kinases 1 and 2 (Erk1/2) at threonine188 and thereby induce hypertrophy. Here we investigated whether β-adrenergic receptors might also induce cardiac hypertrophy via ErkThr188 phosphorylation.

Methods and results β-Adrenergic receptor activation induced ErkThr188 phosphorylation in mouse hearts and in neonatal cardiomyocytes. Inhibition of Erk1/2 or overexpression of ErkThr188 phosphorylation-deficient mutants (Erk2T188A and Erk2T188S) significantly attenuated β-adrenergic cardiomyocyte hypertrophy in vitro. Erk activity was stimulated by both isoproterenol and the direct AC activator forskolin, but only isoproterenol induced ErkThr188 phosphorylation. ErkThr188 phosphorylation required Gβγ released from Gs and was prevented by Gβγ inhibition. Similarly, isoproterenol, but not forskolin, induced nuclear accumulation of Erk and cardiomyocyte hypertrophy. Long-term application of isoproterenol in mice caused left ventricular hypertrophy and cardiac remodelling, and this was reduced in Erk2T188S transgenic mice, supporting the physiological relevance of ErkThr188 phosphorylation.

Conclusions Activation of Gs by β-adrenergic receptors leads to (i) canonical Erk1/2 activation via AC, and (ii) release of Gβγ, which then associates with activated Erk1/2 and induces ErkThr188 phosphorylation, causing nuclear accumulation of Erk and ultimately cardiomyocyte hypertrophy. These findings reveal a new pathway critically involved in β-adrenergically mediated cardiac hypertrophy and may yield new therapeutic strategies against hypertrophic remodelling.

Keywords Cardiac hypertrophy • Erk1/2 • G protein • G protein Gβγ-subunits • β-Adrenergic receptor • Phosphorylation

1. Introduction

Elevated sympathetic activity in heart-failure patients is associated with poor survival.1,2 Even though it acutely enhances cardiac contractility, sustained activation of β-adrenergic receptors, particularly of the β1 subtype, promotes contractile dysfunction, ventricular arrhythmias, and congestive heart failure.1,2 The resultant cardiac histology is characterized by cardiomyocyte hypertrophy and concomitant interstitial fibrosis.1,3

The β1-adrenergic receptor (β1-AR) signals through a stimulatory G protein (Gs) that activates adenyl cyclase (AC) via the α-subunit (Gαs) and thereby induces the formation of cAMP and activation of protein kinase A (PKA), the major target of cAMP. PKA phosphorylates several regulatory proteins that are involved in the cardiac contraction–relaxation cycle, such as L-type Ca2+ channels, phospholamban, troponin I, and ryanodine receptor 2 (RyR2).2,4,5

Interestingly, among mouse models that overexpress components of the β1-adrenergic signalling cascade, only mice with cardiac overexpression of β1-adrenergic receptors or of Gαs develop a significant degree of cardiac hypertrophy.6,7 In contrast, transgenic mouse models that overexpress signalling proteins downstream of Gs have little relevance for hypertrophic remodelling. Thus, cardiac overexpression of the predominant cardiac isoforms of AC (AC5 or AC6), overexpression of constitutively active PKA, and knock-in of

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2. Methods

An expanded Methods section including reagents and cDNA constructs is included in the Supplementary material online.

2.1 Mice and rats

Erk2T188S and Erk2T188T transgenic mice (T188S and T188T, respectively) were generated as previously described. Isogenic wild-type FVB/N mice were used as controls. Mice were sacrificed by cervical dislocation. Pregnant Sprague-Dawley rats were purchased from Janvier (Le Genest St Isle, France). Animal care was in accordance with the Committee on the Care and Use of Laboratory Animals (Directive 2010/63/EU of the European Parliament) and the corresponding national legislation.

2.2 Cell culture and transfection

Neonatal rat cardiomyocytes (NRCMs) were isolated by enzymatic dissociation of left ventricles from 1- or 2-day-old Sprague-Dawley rats as described previously. Isogenic wild-type FVB/N mice were used as controls. Mice were sacrificed by cervical dislocation. Pregnant Sprague-Dawley rats were purchased from Janvier (Le Genest St Isle, France). Animal care was in accordance with the Committee on the Care and Use of Laboratory Animals (Directive 2010/63/EU of the European Parliament) and the corresponding national legislation.

2.3 Co-immunoprecipitation and immunoblotting

Immunoprecipitation and immunoblot analyses were performed as described previously. Briefly, cells and left ventricles were lysed in ice-cold NP40 buffer supplemented with phosphatase and protease inhibitors. Dobutamine stimulation of wild-type hearts was achieved by intravenous infusion of dobutamine via the vena cava, CA, USA. Nuclei were counterstained with hematoxylin.

2.4 Analysis of protein synthesis

Protein synthesis rates in NRCMs were measured in the presence of compounds as indicated, and were determined by [3H]isoleucine incorporation.

2.5 Fluorescence microscopy

COS7 cells and NRCMs were transfected with the indicated cDNAs, stimulated, and fixed. Nuclei were stained with DAPI. Confocal images were taken with a TCS SP5 confocal microscope (Leica Microsystems, Wetzlar, Germany). YFP was excited at 514 nm and DAPI at 405 nm, and fluorescence images were taken at 550–620 and 425–460 nm, respectively. Confocal images were recorded using the sequential mode option and analyzed with ImageJ software. For actin staining, NRCMs were fixed after stimulation with isoproterenol (5 μM, 24 h) and stained with Alexa Fluor®488-labelled phalloidin.

2.6 Osmotic minipumps

Osmotic minipumps (Alzet, model 1002) were implanted subcutaneously in male 8-week-old wild-type (FVB/N background) and transgenic Erk2T188T and Erk2T188S mice. For anesthesia during surgical implantation of the minipumps, ketamine (150 mg/kg body weight) plus xylazine (0.5 mg/kg) were injected intraperitoneally. The disappearance of the toe-pinch reflex indicated adequacy of anesthesia. The implanted pumps continuously released isoproterenol for 14 days (30 μg/g/day).

2.7 Echocardiography

Transthoracic echocardiograms were obtained in a blinded manner using the Vevo2100 high-resolution imaging system (VisualSonics, Toronto, Canada) and a 30 MHz probe. Mice were anaesthetized intraperitoneally with pentobarbital (35 mg/kg). Adequacy of anaesthesia was indicated by disappearance of the righting reflex. Values for end-diastolic septal and posterior wall thicknesses and for internal diameters were obtained from two-dimensional M-mode images in the short axis view at the proximal level of the papillary muscles.

2.8 Histological analyses

Mouse hearts were fixed and embedded in paraffin. Sections were stained with Sirius Red or hematoxylin and eosin (H&E), and fibrosis and cross-sectional areas were quantified as previously described.

2.9 Immunohistochemistry

Histological sections were microwaved in citric acid, blocked with serum, and incubated with anti-phospho-Elk1 antibodies. Primary antibodies were detected using the Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA, USA). Nuclei were counterstained with hematoxylin.

2.10 Real-time PCR

RNA was extracted from left ventricles using RNeasy® (Qiagen, Hilden, Germany). Total RNA was reverse transcribed using Superscript II reverse transcriptase (Invitrogen/Life Technologies, Darmstadt, Germany). QuantiTect RT-PCR kit (Qiagen, Hilden, Germany). YFP was excited at 514 nm and DAPI at 405 nm, and fluorescence images were taken at 550–620 and 425–460 nm, respectively. Confocal images were recorded using the sequential mode option and analyzed with ImageJ software. For actin staining, NRCMs were fixed after stimulation with isoproterenol (5 μM, 24 h) and stained with Alexa Fluor®488-labelled phalloidin.

2.11 Statistical analyses

One-way ANOVA followed by a Bonferroni test was used for statistical analyses. Differences with P < 0.05 were considered significant. All data are shown as means ± SEM. Confocal, histological, and echocardiographic analyses were performed in a blinded fashion.

3. Results

3.1 β-Adrenergic receptor activation leads to ErkThr188 phosphorylation

First, we studied whether autophosphorylation of Erk1/2 at Thr188 might occur in response to β-adrenergic receptor stimulation. We analyzed phosphorylation of overexpressed Erk2 and endogenous
Erk1/2 in response to β-adrenergic stimuli in HEK293 cells (co-transfected with β1ARs), in NRCMs, in adult mouse cardiomyocytes, in wild-type mice, and in mice with cardiac overexpression of wild-type Erk2. Interestingly, short- as well as long-term β-adrenergic stimulation with isoproterenol (Iso) or dobutamine led to a marked increase of Erk autophosphorylation at Thr188 at 14 days (30 μg/g body weight/day). This may be due to the rather oscillating nature of canonical Erk1/2 phosphorylation.12 The extent of β-adrenergically induced ErkThr188 phosphorylation was similar to that of other hypertrophic stimuli that activate Gq-coupled receptors and thereby induce ErkThr188 phosphorylation, such as phenylephrine (Figure 1A) and angiotensin II (Figure 1B).12,16–18 Taken together, these data show that activation of β-adrenergic receptors induces phosphorylation of Erk1/2 at Thr188.

### 3.2 Isoproterenol-induced cardiomyocyte hypertrophy is mediated by ErkThr188 phosphorylation

To confirm that Erk1/2 are involved in Iso-induced cardiomyocyte hypertrophy,19 we used compound PD98059, which blocks Erk1/2 activity by inhibition of canonical Erk phosphorylation [pErk(TEY)] via the upstream kinases of Erk1/2.12 Of note, autophosphorylation of Erk1/2 at Thr188 (ErkThr188) was also efficiently blocked by PD98059 (Figure 2A). PD98059 treatment of NRCMs abolished the hypertrophic effect of Iso, as measured by incorporation of tritiated [3H]isoleucine into newly synthesized protein (Figure 2B). To investigate whether the hypertrophic effect of Iso indeed requires ErkThr188 phosphorylation, we used two mutants of Erk2 that are phosphorylation-deficient for ErkThr188 phosphorylation. In these mutants, Thr188 is exchanged to either alanine (ErkT188A) or serine (ErkT188S). Interestingly, both of these mutants significantly reduced the β-adrenergic-receptor-mediated hypertrophic response in NRCMs compared with control cells expressing similar levels of wild-type Erk2 (designated ErkT188T) (Figure 2C and D). Of note, ErkT188T-overexpressing NRCMs showed hypertrophic responses similar to cells infected with a control virus (Supplementary material online, Figure S2A and S2B). This was shown with the [3H]isoleucine incorporation assay (Figure 2C) as well as by quantification of cell size (Figure 2D). In addition, confocal images of phallidin-stained NRCMs showed that Iso-induced cytoskeletal reorganization was less pronounced in NRCMs overexpressing phosphorylation-deficient mutants, ErkT188A or ErkT188S, than in ErkT188T-overexpressing cells (Supplementary material online, Figure S2C). Together, these data show that β-adrenergically induced cardiomyocyte hypertrophy involves Erk1/2 and that ErkThr188 phosphorylation is essential in this signaling pathway.

### 3.3 Gs (Gα and Gβγ) activation is necessary for ErkThr188 phosphorylation

To exclude the involvement of G proteins other than Gs, we then analyzed whether Iso-induced Erk1/2 activation and cardiomyocyte hypertrophy is indeed induced by Gs. To inhibit Gs signaling, we overexpressed RGS2 (regulator of G protein signaling 2), which is an efficient Gs inhibitor.20 While RGS2 suppressed Gs-mediated
hypertrophic signals in response to phenylephrine (Figure 3A), it did not affect Iso-induced cardiomyocyte hypertrophy (Figure 3B). Furthermore, RGS2 overexpression did not attenuate Erk1/2 activity (Figure 3C and Supplementary material online, Figure S3A and S3B). Participation of G\(_i\) in adenrenergically mediated Erk1/2 activity was excluded by treatment of the cells with pertussis toxin (PTX) (Figure 3D and Supplementary material online, Figure S3C and S3D).\(^{21}\) To examine the involvement of G\(_i\) in \(\beta\)-adrenergically induced hypertrophy, we analyzed the effects of inhibition and activation of the G\(_i\) effector AC. Inhibition of AC with KH7 reduced the cardiomyocyte hypertrophic response to isoproterenol (Figure 3E and Supplementary material online, Figure S3E).

In contrast, forskolin, a direct activator of AC,\(^{22,23}\) did not induce cardiomyocyte hypertrophy at concentrations up to 30 \(\mu\)M (Figure 3F and data not shown), even though Erk1/2 activation in response to Iso or forskolin treatment was comparable (Supplementary material online, Figure S3F).

These results suggest that G\(_i\) induced AC activation is not sufficient to induce cardiomyocyte hypertrophy upon \(\beta\)-adrenergic receptor activation, indicating that G\(_{\beta\gamma}\) might be involved. Co-immunoprecipitation assays of G\(_{\beta\gamma}\) subunits with Flag-tagged Erk2 in HEK293 cells (Figure 3G) and in NRCMs (Figure 3H), as well as endogenous Erk1/2 in NRCMs (Figure 3I), showed that Iso stimulation led to a significant increase in co-immunoprecipitated G\(_{\beta\gamma}\).
Figure 3  

Gs activation is necessary for Erk\textsuperscript{Thr188} phosphorylation. (A and B) \[^{3}H\]isoleucine incorporation and (C) quantification of phospho-Erk1/2(TEY) in NRCMs infected with adenoviruses encoding either GFP (Con) or RGS2. Cells were treated with phenylephrine (Phe; 2 \(\mu\)M, 24 h) or isoproterenol [Iso, 5 \(\mu\)M, 24 h (B) or 10 min (C)]. \(n = 6–14\) experiments, *, \(P < 0.05\) vs. all other conditions (A) or vs. unstimulated controls (B and C); n.s.: vs. unstimulated controls (A). (D) Quantification of phospho-Erk1/2(TEY) in NRCMs. Cells were treated with pertussis toxin (PTX; 0.1 \(\mu\)g/mL, 20 h) and stimulated with Iso (5 \(\mu\)M, 10 min). \(n = 7\) experiments; *, \(P < 0.001\) vs. unstimulated controls. (E and F) \[^{3}H\]isoleucine incorporation in NRCMs pretreated with KH7 (3 \(\mu\)M, 30 min) and stimulated with Iso (5 \(\mu\)M, 24 h) or forskolin (Forsk; 30 \(\mu\)M, 24 h). \(n = 5–10\) experiments; *, \(P < 0.05\) (E) or \(P < 0.001\) (F) vs. all other conditions; n.s.: vs. unstimulated controls. (G–I) Immunoprecipitation of (G and H) Flag-Erk2 or (I) endogenous Erk1/2 from (G) HEK293 cells overexpressing \(\beta\)1-adrenergic receptors or (H and I) from NRCMs after stimulation with isoproterenol [5 \(\mu\)M, 10 min (G) or 24 h (H, I)] or phenylephrine (2 \(\mu\)M, 24 h). Co-precipitated G\(\beta\)\(\gamma\) subunits were detected by immunoblot analysis with antibodies directed against G\(\beta\). (J) Quantification of Erk1/2 phosphorylation at Thr188 and at the TEY-motif in NRCMs treated with Iso (5 \(\mu\)M) or Forsk (30 \(\mu\)M) for 10 min. \(n = 4–6\) experiments; *, \(P < 0.05\) vs. all other conditions (upper graph) or vs. unstimulated controls (lower graph).
(Figure 3G–I). The detected association of Gβγ and Erk in response to Iso implies an involvement of Gβγ in the hypertrophic signalling pathway of Erk1/2. In line with this hypothesis, direct activation of AC by forskolin was not sufficient to induce the Gβγ-mediated and hypertrophic ErkThr188 phosphorylation (Figure 3J). Experiments with HEK293 cells overexpressing Gβγ inhibitory C-terminus of the β-adrenergic receptor kinase (βARK–ct) further delineated the importance of the co-occurrence of Gs and Gβγ activation for the induction of ErkThr188 phosphorylation. While βARK–ct did not affect Erk2 activation, it clearly inhibited ErkThr188 phosphorylation (Supplementary material online, Figure S3G). Taken together, our results suggest that Gs-derived Gβγ subunits interact directly with Erk1/2, activated by signals downstream of AC, to cause ErkThr188 phosphorylation, which is required for cardiac hypertrophy. In contrast, activation of the cAMP pathway alone (by forskolin) lacks the Gβγ-dependent effect on Erk1/2 and is not sufficient to induce cardiac hypertrophy.

### 3.4 Loss of ErkThr188 phosphorylation decreases cardiac hypertrophy in vivo

We then investigated the role of ErkThr188 phosphorylation in β-adrenergically mediated hypertrophy in vivo. For this study, we used wild-type FVB/N mice and transgenic mice overexpressing wild-type Erk2 (T188T) or Erk2T188S (T188S). Erk2 overexpression levels are shown in Supplementary material online, Figure S4. Since we had not observed significant differences between Erk2T188A and Erk2T188S in the in vitro studies (see Figure 2C and D), we used only Erk2T188S transgenic mice for this in vivo analysis. For β-adrenergic stimulation, mice were subjected to isoproterenol applied by osmotic mini-pumps for 14 days. Echocardiographic analyses and histology were performed to phenotype mice before and after isoproterenol treatment. In the absence of isoproterenol there were no differences in morphology or heart function between different genotypes (Figure 4 and Supplementary material online, Figures S5 and S6). Furthermore, consistent with earlier reports, cardiac function was not impaired at this time-point (Supplementary material online, Figure SS–E).24–26

In FVB/N wild-type (WT) mice and in mice overexpressing Erk2 (Erk2T188T), isoproterenol significantly increased left ventricular hypertrophy to a comparable extent, as shown by increases in septal and posterior wall thickness (Figure 4A and Supplementary material online, Figure S5A), left ventricular weight (Figure 4B and Supplementary material online, Figure S5F), and cardiomyocyte size (Figure 4C and Supplementary material online, Figure S6A). In line with previous reports, wild-type Erk2 had no effect on the development of cardiac hypertrophy.12,27 In contrast, phosphorylation-deficient Erk2T188S significantly attenuated the hypertrophic response to Iso for all parameters (Figure 4A–C and Supplementary material online, Figures S5 and 6A). Further, adult myocytes isolated from Erk2T188S transgenic mice developed significantly less Iso-induced hypertrophy than cells isolated from WT mice (Supplementary material online, Figure S6B and 6C). Iso-induced hypertrophy was accompanied by interstitial fibrosis (quantified in histological sections by Sirius Red staining; Supplementary material online, Figure S6D and SE) and elevated heart-failure markers [collagenase 3 and atrial natriuretic factor (ANF)]28,29 in WT and Erk2T188T transgenic mice. All these pathological changes were markedly less pronounced in Erk2T188S transgenic mice (Figure 4D–F). Taken together, our data show that mutation of Thr188 in Erk2 inhibits cardiac hypertrophy and remodelling in vivo, and thus reveal a key role of ErkThr188 phosphorylation in β-adrenergic-receptor-mediated cardiac hypertrophy.

### 3.5 Thr188-phosphorylated Erk accumulates in the nucleus

To investigate the downstream effects of ErkThr188 phosphorylation in β-adrenergic signalling, we analyzed phosphorylation of Elk1, a nuclear Erk1/2 target involved in cardiac hypertrophy.30,31 Western blot analysis revealed a significant increase of Elk1 phosphorylation in hearts from mice overexpressing Erk2T188T after 14 days of Iso treatment. This increase in Elk1 phosphorylation was almost absent in Erk2T188S transgenic mice (Figure 5A), suggesting that ErkThr188 phosphorylation is required for phosphorylation of this nuclear Erk target. Phospho-Elk1 was also detected in response to Iso treatment in the cardiomyocyte nuclei of heart sections from Erk2T188T, but not from Erk2T188S, transgenic mice (Figure 5B). Confocal microscopy analysis of the subcellular distributions of YFP-tagged Erk2T188B and Erk2T188A in COS7 cells (Supplementary material online, Figure S7A and S7B), in NRCMs (Figure 5C), or isolated adult cardiomyocytes (Figure 2E) further showed that phosphorylation-deficient Erk2T188A—in contrast to WT Erk2—did not accumulate in the nucleus in response to Iso stimulation. In line with these findings, YFP-tagged Erk2T188T also did not accumulate in the nucleus after activation by forskolin, which does not induce ErkThr188 phosphorylation (Figure 5D). These experiments suggest that nuclear accumulation of Erk is impaired in the absence of ErkThr188 phosphorylation and that retention of Erk in the cytosol may inhibit phosphorylation of nuclear targets known to mediate hypertrophy.

### 4. Discussion

Transgenic animal studies with constitutive overexpression of β-adrenergic receptors as well as studies of reverse remodelling using β-blocking agents have revealed that the β1-adrenergic receptor subtype is the major mediator of pathological hypertrophy and that it is associated with a marked increase in interstitial fibrosis and heart failure.4,5 However, signalling pathways involved in β-adrenergically induced hypertrophy are not well understood.

As mentioned above, classic β-adrenergic signalling involves activation of Gs, followed by stimulation of ACs, increases in cAMP, and enhanced activity of PKA. However, distinct phenotypes obtained in mice with cardiac overexpression of the different components of the β-adrenergic signalling pathway suggest that hypertrophy is not (or not alone) mediated via this classic pathway. In line with these reports, our experiments showed that direct stimulation of AC with forskolin does not foster the induction of cardiomyocyte hypertrophy (Figure 3F). Taken together, these observations give rise to the hypothesis that an additional pathway branches off at the level of Gs, and that this is important for the induction of cardiac hypertrophy.

In our study, we show that direct interaction of Gα-derived Gβγ subunits with Erk1/2 may represent this additional pathway (Figure 6). Erk1/2 are classically activated by receptor tyrosine kinases, such as growth factor receptors, but also by G protein coupled receptors, and play key roles in cardiac hypertrophy and remodelling.15,29,31–34 Activation of Erk1/2 is initiated by activation of the three-tier mitogen-activated protein kinase (MAPK) module
Mek1/2 phosphorylate Erk1/2 at the conserved TEY motif (amino acids 183–185 in mouse Erk2) and thereby activate Erk1/2.15,35 However, some cardio-hypertrophic stimuli (e.g., angiotensin II and transverse aortic constriction) have been shown to induce an additional autophosphorylation of Erk2 at Thr188 (Thr208 in Erk1). Under these conditions, Erk Thr188 phosphorylation was essential for Erk-mediated cardiac hypertrophy.12,36 Erk Thr188 phosphorylation was shown to be triggered by the association of Gα-derived Gβγ subunits with activated Erk1/2. In addition, dimerization of Erk was shown to be a prerequisite for Gβγ binding to the activated kinases. Erk phosphorylated at Thr188 accumulated in the nucleus and ultimately resulted in Erk-mediated hypertrophy.12,36

Our data show that β-adrenergic receptor activation indeed induces direct interaction of Erk1/2 with Gα-derived Gβγ subunits (Figure 3G–I) as well as Erk Thr188 phosphorylation (Figure 1 and Supplementary material online, Figure S1). Using phosphorylation-deficient mutants of Erk2 (Erk2 T188A and Erk2 T188S), we demonstrated the impact of Erk Thr188 phosphorylation on cardiomyocyte hypertrophy in vitro and in vivo in response to β-adrenergic receptor activation.
activation (Figures 2 and 4). These phosphorylation-deficient mutants seem to display a dominant-negative effect on cardiac hypertrophy by sequestering activated Erk within the cytosol. The dominant-negative effect can be mediated via sequestration of activated Erk1 and/or Erk2, since Erk2 can form dimers, (homodimers, but also heterodimers with Erk1) (data not shown). In line with this hypothesis, our localization studies in COS7 cells (Supplementary material online, Figure S7) and in NRCMs (Figure 5C and D) show that the phosphorylation-deficient mutants are retained in the cytosol. The finding that forskolin did not cause nuclear localization of Erk2 even suggests that Erk2T188S phosphorylation may be mandatory for nuclear Erk localization (Figure 3D). Sequestration of Erk is most likely mediated via dimerization, as it has been shown that Erk2T188S can efficiently dimerize with activated WT Erk2 in HEK293 cells. Analysis of the activation of Elk1 (a prototypical nuclear target of Erk) in our mouse experiments showed that activation of Elk1 is indeed attenuated in the presence of phosphorylation-deficient Erk2T188S (Figure 5A and B). However, the mechanisms of nuclear accumulation of Erk after ErkThr188 phosphorylation are still unclear. These may involve preferential transport of ErkThr188-phosphorylated Erk into the nucleus, as has been shown for TEY-phosphorylated Erk; in contrast, phosphorylation at the TEY motif does not always correlate well with nuclear accumulation of Erk, raising the possibility of additional determinants. Taken together, these
activation of AC resulted in Erk activation but not in Erk Thr188 phosphorylation. Several lines of evidence suggest that the convergence of two β-adrenergically signalling components (i.e. the release of Gβγ and the activation of downstream signalling of Gs) is needed to induce Erk Thr188 phosphorylation and thus hypertrophy: (i) Inhibition of AC with KH7 efficiently inhibited Iso-mediated Erk1/2 activation and cardiomyocyte hypertrophy (Figure 3E and Supplementary material online, Figure S3E); however, direct activation of AC resulted in efficient activation of Erk but did not result in cardiomyocyte hypertrophy (Figure 3F and Supplementary material online, Figure S3F), thus downstream signals of Gs seem to be an important component for the induction of β-adrenergically induced hypertrophy but they are not sufficient to induce hypertrophy. (ii) Treatment of cells with the Mek inhibitor PD98059 showed that Erk activity is critically involved in β-adrenergically induced hypertrophy (Figure 2B) and is also important for Erk Thr188 phosphorylation (Figure 2A). (iii) Direct activation of AC resulted in Erk activation but not in Erk Thr188 phosphorylation. Thus, Erk activation alone is not sufficient to induce Erk Thr188 phosphorylation (Figure 3J). (iv) Overexpression of the Gβγ inhibitor βARK-ct inhibited Erk Thr188 phosphorylation but not Erk activation at the canonical TEY motif (Supplementary material online, Figure S3G). Thus, Gβγ subunits seem to be needed for the induction of Erk Thr188 phosphorylation but not for activation of Erk [pErk(TEY)].

Our experiments thus suggest that induction of hypertrophy via β-adrenergic receptors depends on two pathways: canonical Erk1/2 activation (most likely by activation of signals downstream of AC), and Gβγ release from Gs. These pathways combine to produce Erk Thr188 phosphorylation as the decisive output to hypertrophic gene expression (Figure 6). The elucidated mechanism of β-adrenergically induced hypertrophy helps to explain the apparent differences in the hypertrophic phenotypes of transgenic mice overexpressing different components of the β-adrenergic signalling pathway.

It remains to be elucidated whether Erk Thr188 phosphorylation also mediates β-adrenergic cardiac hypertrophy by regulating other pathways that have been implicated in β-adrenergically mediated cardiomyocyte hypertrophy. Since we have shown here that Erk Thr188 phosphorylation induces Elk1 phosphorylation, and since we have also previously shown that mitogen- and stress-activated protein kinase-1 (MSK1) and c-Myc become hyperphosphorylated, these effects of Thr188-phosphorylated Erk on transcription factors may promote an increase of the expression of exchange factors activated by cAMP (Epac) and Ca2+/calmodulin dependent protein kinase II (CamK II). The latter are two examples of proteins with upregulated expression levels in heart failure and with known involvement in β-adrenergically induced cardiac hypertrophy.

Cardiac hypertrophy is an important trigger for arrhythmias and heart failure. The mechanism described here specifically elucidates the hypertrophic mechanism and accompanied remodelling as part of the pathological signalling of β-adrenergic receptors. Since functional deterioration of the heart was not observed in our experimental model, in line with similar observations by others, it could not be assessed whether blockage of Erk Thr188 phosphorylation can rescue heart failure induced by chronic stimulation of β-adrenergic receptors. However, the observed reduction of interstitial fibrosis and ANF expression support the hypothesis that reduction of cardiac hypertrophy by overexpression of phosphorylation-deficient Erk Thr188 will be protective for the heart. Interference with Erk Thr188 phosphorylation may even be of particular therapeutic interest in β-adrenergically induced hypertrophy, since studies that targeted Erk1/2 more unspecifically (by inhibiting Erk1/2 activity or by genetic deletion) showed enhanced pathological remodelling.

Future studies will be needed to evaluate the impact of Erk Thr188 phosphorylation on β-adrenergically induced heart failure. Taken together, our results show that Erk Thr188 phosphorylation strongly participates in cardiac hypertrophy mediated by β-adrenergic receptor activation and that this effect depends on a complex activation mechanism involving activation of both Erk1/2 and G proteins.

Supplementary material Supplementary material is available at Cardiovascular Research online.

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Disclosure The University of Würzburg holds a patent on ERK Thr188 antibodies.

References


