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Ablation of p21-Activated Kinase-1 in Mice Promotes Isoproterenol-Induced Cardiac Hypertrophy in Association with Activation of Erk1/2 and Inhibition of Protein Phosphatase 2A

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Disclosures

None.

Abstract

Rationale—Earlier investigations in our lab indicated an anti-adrenergic effect induced by activation of p21-activated kinase (Pak-1) and protein phosphatase 2A (PP2A).

Objective—Our objective was to test the hypothesis that Pak-1/PP2A is a signaling cascade controlling stress-induced cardiac growth. We determined the effects of ablation of the Pak-1 gene on the response of the myocardium to chronic stress of isoproterenol (ISO) administration.

Methods and Results—Wild-type (WT) and Pak-1-knockout (Pak-1-KO) mice were randomized into six groups to receive either ISO, saline (CTRL), or ISO and FR180204, a selective inhibitor of Erk1/2. Echocardiography revealed that hearts of the Pak-1-KO/ISO group had increased LV fractional shortening, reduced LV chamber volume in diastole and systole, increased cardiac hypertrophy, and enhanced transmitral early filling deceleration time, compared to all other groups. The changes were associated with an increase in relative Erk1/2 activation in Pak-1-KO/ISO mice versus all other groups. ISO-induced cardiac hypertrophy and Erk1/2 activation in Pak-1-KO/ISO were attenuated when the selective Erk1/2 inhibitor FR180204 was administered. Immunoprecipitation showed an association between Pak-1, PP2A, and Erk1/2. Cardiac myocytes infected with an adenoviral vector expressing constitutively active Pak-1 showed a repression of Erk1/2 activation. p38 MAPK phosphorylation was decreased in Pak-1-KO/ISO and Pak-1-KO/CTRL mice compared to WT. Levels of phosphorylated PP2A were increased in ISO-treated Pak-1-KO mice, indicating reduced phosphatase activity. Maximum Ca^{2+} -activated tension in detergent-extracted bundles of papillary fibers from ISO-treated Pak-1-KO mice was higher than in all other groups. Analysis of cTnI phosphorylation indicated that compared to WT, ISO-induced phosphorylation of cTnI was blunted in Pak-1-KO mice.

Conclusions—Active Pak-1 is a natural inhibitor of Erk1/2 and a novel anti-hypertrophic signaling molecule upstream of PP2A.

Keywords

Mitogen-Activated Protein Kinases; Myocardial hypertrophy; p38 MAPK; Pak-1; Troponin I

1. Introduction

Recent and emerging data indicate that novel signaling cascades in the heart involve p21-activated kinase-1 (Pak-1), a serine/threonine kinase targeted by the small GTP-binding proteins Cdc42 and Rac1. Pak-1 signaling appears to be of significance in both short-term control of cardiac function [1, 2], as well as long term function and remodeling. Sinoatrial node cells expressing constitutively active (CA)-Pak-1 demonstrated an inhibition of isoproterenol (ISO)-induced increases in heart rate and currents through both L-type Ca^{2+} and K_{ACH} channels [1, 2]. Adult cardiac ventricular myocytes expressing CA-Pak-1 also demonstrate enhanced myofilament Ca-sensitivity associated with dephosphorylation of cardiac troponin I (cTnI) and myosin-binding protein C (MyBP-C), as well as a reduced response of Ca^{2+} -transients and spark amplitude to ISO [1-3]. Moreover, bradykinin receptor-mediated activation of Pak-1 also induced effects similar to those obtained with CA-Pak-1 expression [4]. The mechanism for these anti-adrenergic effects of Pak-1 involves activation of protein phosphatase 2A (PP2A) [5]. Pak-1 forms a *trans*-inhibited dimer that complexes with PP2A holoenzyme in cardiomyocytes and sinoatrial nodal cells [1, 2, 4].

Although not systematically investigated in heart, there are data indicating that activation of Pak-1 may be significant in long-term adaptation of the heart to stressors. In non-cardiac cells, Pak-1 activates cell survival and metabolic pathways, including Akt and mitogen-activated protein kinases (MAPKs), namely Erk1/2, p38 MAPK, and JNK1/2/3. The Ras/Raf/MEK/Erk signaling pathway is typically associated with modulation of the hypertrophic

response of the heart to neurohumoral stimuli and pressure overload [6]. Hunter et al. have demonstrated that the expression of a CA Ras (H-Ras-V12) in mouse heart leads to LV and cardiomyocyte hypertrophy with no associated fibrosis [7]. Patients affected with Noonan and LEOPARD syndromes, two diseases caused by mutations leading to activation of the Ras/Raf/MEK/Erk signaling pathway, exhibit symptomatic hypertrophic cardiomyopathy [8]. In addition, patients treated with a left ventricular assist device demonstrate reversal of cardiac remodeling, reduction in myocyte hypertrophy, decreased Erk activity and activation of Sprouty-1, an endogenous inhibitor of the Erks [9]. Overexpression of MEK1 has also shown similarities with constitutive activation of Ras, whereas dominant negative Raf attenuated hypertrophy and fetal gene induction in response to pressure overload [10]. These findings indicate a potential but unexplored linkage between Pak-1 and Erk and suggest therapeutic strategies that directly interfere with the Pak-1/Erk1/2 signaling pathway, which could provide a powerful therapy in cardiac hypertrophy.

In experiments reported here, we tested the hypothesis that Pak-1/PP2A/Erk1/2 represents an important signaling cascade controlling β -adrenergic stress-induced cardiac growth. β -adrenergic receptor stimulation by ISO leads to an increase in Pak-1 activation [11], and β -adrenergic agonists promote cardiomyocyte hypertrophy via indirect interaction between Erk and beta-arrestin [12]. We report the first evidence of association of Pak-1 and PP2A to Erk1/2 in the heart, as well as reduced PP2A and enhanced Erk1/2 activation in Pak-1-KO hearts. Our data indicate an important role of Pak-1 as an inhibitor of the Erks and as a novel anti-hypertrophic signaling enzyme with a role in modulation of β -adrenergic signaling, suggesting that Pak-1 plays a significant contribution in the mechanism of adaptive control of cardiac contractility.

2. Methods

An expanded Methods section is available in the Supplement Material online.

2.1 Pak-1-KO mouse model

Mice with Pak-1 gene disruption at both alleles were created as described previously [13]. Echocardiography for assessment of ventricular size and function was performed in all mice.

2.2 Mouse model of LV concentric cardiac hypertrophy

To study the role of Pak-1 in signaling of LV myocardial hypertrophy, we employed an ISO-induced mouse model of LV concentric cardiac hypertrophy. Twenty-four age-matched, three- to four-month-old, FVB male and female mice (12 WT and 12 Pak-1-KO) were randomized in four groups of six mice each to receive for one week: i) continuous subcutaneous administration of 25 μ g/g/day of ISO, or ii) an equivalent volume of 0.91% w/v NaCl (CTRL). Additionally, eleven age-matched, three- to four-month-old, FVB male and female mice (5 WT and 6 Pak-1-KO) were randomized in four groups of two or three mice each to receive for one week: iii) continuous subcutaneous administration of 25 μ g/g/day of ISO, or iv) continuous subcutaneous administration of 25 μ g/g/day of ISO and FR180204 (Tocris Bioscience, MO, USA), a selective inhibitor of Erk1/2, at the dose of 100 mg/kg, administered intraperitoneally [14]. Six groups (WT/CTRL; WT/ISO; Pak-1-KO/CTRL; Pak-1-KO/ISO; WT/ISO + FR180204; Pak-1-KO/ISO + FR180204) were obtained and hearts were used for assessment of morphometric, biochemical, cytochemical, and hemodynamic analysis.

2.3 Histological findings of WT and PAK-1-KO hearts

Hearts were removed from mice and embedded in paraffin. Paraffin-embedded sections were stained with hematoxylin-eosin stain.

2.4 Western immunoblotting and co-immunoprecipitation

Generation of protein samples from tissue, as well as Western immunoblotting, co-immunoprecipitation and chemifluorescent detection have been described previously [1, 2].

2.5 Studies in isolated cardiomyocytes

Adult ventricular myocytes were isolated as described previously [15]. AdPak-1 construction, viral amplification, plaque assays as well as adenoviral infection of adult rat cardiac myocytes have been described previously [3].

2.6 Preparation of skinned fibers

Left ventricular papillary muscles were dissected and detergent-extracted fiber bundles were prepared as previously described [16]. The sarcomere length was set to 2.2 μm using He-Ne laser diffraction.

2.7 Quantification of myofilament phosphorylation by 2-D Difference In Gel Electrophoresis (2-D DIGE)

Generation of protein samples from tissue, labeling, separation of myofilament proteins by 2-D DIGE, image acquisition and analysis have been described previously [17].

2.8 Statistics

Data were statistically analyzed using either Student's t-test or one-way ANOVA followed by Holm-Sidak test where appropriate, with $P < 0.05$ as the criterion for significance. Data are reported as means $\pm$ SEM.

3. Results

3.1 Validation of the Pak-1-KO mouse model

To confirm the deletion of the Pak-1 gene in this Pak-1-KO mouse model, we genotyped the mice by PCR (Supplemental Figure 1A) and determined the levels of protein expression of Pak-1, Pak-2 and Pak-3 (two less abundant protein isoforms of Pak-1) in the heart (Supplemental Figures 1B through 1E). Pak-1 gene was successfully deleted and Pak-1 protein levels were undetectable by WB in Pak-1-KO mice. Levels of expression of Pak-1, Pak-2 and Pak-3 were unchanged in ISO-treated WT hearts compared to untreated WT mice. In addition, the levels of expression of Pak-2 and Pak-3 were unchanged in Pak-1-KO hearts, in the absence or in the presence of ISO. These data illustrate the successful validation of the Pak-1-KO, FVB mouse model.

3.2 Transthoracic M-mode, and Doppler echocardiography

Transthoracic M-mode and Doppler echocardiography were performed in untreated and ISO-treated WT and Pak-1-KO mice. M-mode measurements and transmitral flow velocities were obtained from parasternal short-axis view (Figure 1) and are reported in Supplemental Table 1. We found that ablation of Pak-1 promotes increased ISO-induced cardiac hypertrophy, compared to ISO-treated WT mice. Also, hearts of Pak-1-KO mice stimulated with ISO responded with enhanced LV contractile performance as indicated by reduced LV chamber volume in systole, compared to ISO-treated WT mice. These findings were accompanied by significantly reduced LV chamber volume in diastole in ISO-treated Pak-1-KO mice compared to ISO-treated WT mice. ISO-treated Pak-1-KO mice developed a significantly faster transmitral early filling deceleration time (EDT) suggestive of increased LV operating stiffness, when compared to ISO-treated WT mice. Atrial dimensions did not change significantly among groups (data not shown). Our data demonstrate that chronic

stimulation of the $\beta_{1/2}$ -adrenergic receptors with ISO results in increased susceptibility to LV myocardial hypertrophy with associated enhanced LV systolic function, yet impaired LV diastolic relaxation in Pak-1-KO mice.

3.3 Gross and histological findings in WT and Pak-1-KO hearts

The external examination of WT and PAK-1-KO hearts subjected to treatment with ISO or control saline confirmed the results obtained by echocardiography. Pak-1-KO hearts subjected to ISO treatment for a week were characterized by increased myocardial and myocyte hypertrophy compared to ISO-treated WT hearts or control hearts (Figures 2A through 2C).

3.4 Analysis of Akt, Erk 1/2, JNK, and p38 MAPK phosphorylation in LV cardiac tissue

In view of evidence that Pak-1 may signal via Akt, we determined Akt phosphorylation at Thr308 (Figure 3A) and Ser473 (results not shown). Akt phosphorylation was not significantly different between WT and Pak-1-KO mice, in the presence or in the absence of ISO. We also investigated Erk1 phosphorylation at residues T202 and Y204 by WB to assess Erk-1 activation in WT and Pak-1-KO LV tissue, in the presence and absence of ISO (Figures 3B and 3C). Results showed maximal Erk1 activation in Pak-1-KO/ISO mice vs. all other groups. The ratio of phospho-Erk1/total Erk1 was significantly increased in Pak-1-KO/ISO ($94.44\% \pm 1.49$) compared to WT/CTRL ($3.37\% \pm 1.44$), WT/ISO ($27.24\% \pm 7.11$), and Pak-1-KO/CTRL ($58.73\% \pm 7.14$) ($p < 0.05$ for all groups). We also assessed Erk2 phosphorylation at residue T188 (Figures 3B and 3D). The ratio of phospho-Erk2/total Erk2 was significantly increased in Pak-1-KO/ISO ($9.27\% \pm 1.21$) compared to WT/CTRL ($1.81\% \pm 0.35$), WT/ISO ($2.27\% \pm 0.33$), and Pak-1-KO/CTRL ($5.31\% \pm 0.32$) ($p < 0.05$ for all groups). We then investigated phosphorylation of JNK1/2/3 and p38 MAPK (Figures 3E through 3G). Phosphorylation of JNK1/2/3 was not significantly different among all groups, while p38 MAPK phosphorylation was reduced in Pak-1-KO mice, either in the presence or in the absence of ISO, compared to WT mice. The ratio of phospho-p38 MAPK to total p38 MAPK was significantly higher in WT/CTRL ($91.89\% \pm 4.05$) compared to Pak-1-KO/ISO ($32.79\% \pm 1.79$) and Pak-1-KO ($35.54\% \pm 3.38$) ($p < 0.05$ for both groups). Therefore, since Erk1/2 activation was maximal in ISO-treated Pak-1-KO mice, we hypothesized that Pak-1 could play an inhibitory role on Erk1/2 activation *in vivo*.

3.5 Co-immunoprecipitation and immunofluorescence

We demonstrated that Pak-1, Erk1/2, and PP2A establish a protein-protein interaction in LV cardiac tissue, as assessed by co-immunoprecipitation in whole lysates obtained from WT and Pak-1-KO hearts (Supplemental Figures 2A and 2B). In addition, Erk1/2 and PP2A also associate in the absence of Pak-1 (Supplemental Figure 2C). Pak-1 and Erk1/2 co-localize in the adult mouse cardiac myocyte as demonstrated by immunofluorescence (Supplemental Figure 2D).

3.6 Analysis of Erk phosphorylation in adult rat cardiac myocytes infected with CA Pak-1

To test whether Pak-1 could play an inhibitory role on Erk-1/2 activation, we analyzed Erk-1/2 phosphorylation in lysates obtained from adenovirally-infected adult rat cardiomyocytes that express CA-Pak-1, both in the presence and absence of ISO (Supplemental Figures 2E and 2F). Adenoviral infection of adult rat cardiac myocytes with CA-Pak-1, in the presence and in the absence of ISO resulted in a significant reduction in Erk1/2 phosphorylation compared to untreated and ISO-treated lacZ control ($p < 0.05$). These results confirmed that Pak-1 plays an inhibitory role on Erk1/2.

3.7 Analysis of PP2A phosphorylation in LV cardiac tissue

Earlier evidence indicated that a significant element in the anti-adrenergic actions of Pak-1 is an activation of PP2A [5]. Phosphorylation of Y307 is a reporter of the relative activity of PP2A, as activation is associated with dephosphorylation at this residue. We therefore investigated PP2A phosphorylation at residue Y307 to assess PP2A activation in WT and Pak-1-KO LV tissue, in the presence and in the absence of ISO (Figures 3H and 3I). Results showed that PP2A phosphorylation was maximal in Pak-1-KO/ISO mice compared to all other groups. The ratio of phospho-PP2A/total PP2A significantly increased in Pak-1-KO/ISO ($83.86\% \pm 2.913$) compared to WT/CTRL ($7.06\% \pm 0.28$), WT/ISO ($10.43\% \pm 2.22$), and Pak-1-KO/CTRL ($58.90\% \pm 7.83$) ($p < 0.05$ for all groups). No significant differences were observed between the WT/ISO and WT/CTRL groups.

3.8 Analysis of pharmacological inhibition of Erk1/2 by FR180204 on ISO-treated WT and Pak-1-KO hearts

In order to understand whether Erk1/2 activation is a major kinase leading to increased susceptibility to LV myocardial hypertrophy in hearts that are deficient of Pak-1, we administered the pharmacological selective Erk1/2 inhibitor FR180204 to ISO-treated WT and Pak-1-KO mice for one week. Echocardiographic M-mode measurements and transmitral flow velocities were obtained from parasternal short-axis view (Figure 1) and are reported in Supplemental Table 2. Following administration of FR180204, progression towards LV myocardial hypertrophy and LV stiffness was reduced in ISO-treated WT and Pak-1-KO mice. In addition, co-administration of FR180204 in ISO-treated WT and Pak-1-KO hearts resulted in attenuated development of myocardial and myocyte hypertrophy, compared to WT and Pak-1-KO hearts that received ISO only (Figures 2A through 2C). Finally, WB analysis demonstrated a reduction in Erk1/2 phosphorylation in WT and Pak-1-KO mice that received ISO and FR180204, compared to WT and Pak-1-KO mice treated with ISO alone (Supplemental Figure 4). These findings support the initial observation that Erk1/2 is a major kinase driving ISO-induced myocardial hypertrophy in the absence of Pak-1.

3.9 Myofilament response to Ca^{2+} measured in detergent-extracted (skinned) fiber bundles

The enhanced contractility reflected in the reduced ESV and EDV observed during echocardiography in the ISO-treated Pak-1-KO mice suggested a possible change in myofilament Ca^{2+} sensitivity. Therefore, we determined myofilament Ca^{2+} responsiveness of skinned fiber bundles of LV papillary muscles isolated from WT and Pak-1-KO mice ($N=3$ mice/group) and hypertrophied papillary muscles from ISO-treated WT and Pak-1-KO mice ($N=5$ and 7 , respectively). One fiber bundle preparation per mouse was analyzed. As shown in Figures 4A and 4B, maximum Ca^{2+} activated isometric tension (expressed in mN/mm^2) was greater in papillary fibers obtained from the ISO-treated Pak-1-KO mice (51.31 ± 1.87) compared to fibers obtained from WT mice (35.44 ± 1.4), ISO-treated WT mice (41.0 ± 2.15), or Pak-1-KO mice (36.77 ± 3.0) ($p < 0.05$ for all groups). The Ca^{2+} concentration to elicit 50% of P_0 (pCa_{50}) was not significantly different among all groups (WT/CTRL: 6.04 ± 0.03 ; Pak-1-KO/CTRL: 5.97 ± 0.07 ; WT/ISO: 6.00 ± 0.04 ; Pak-1-KO/ISO: 5.90 ± 0.03 ; $p = 0.16$). The Hill coefficient was not significantly different among all groups (WT/CTRL: 2.78 ± 0.54 ; Pak-1-KO/CTRL: 3.00 ± 0.66 ; WT/ISO: 2.94 ± 0.78 ; Pak-1-KO/ISO: 2.83 ± 0.60 ; $p = 0.99$).

3.10 Quantification of myofilament phosphorylation with 2-D DIGE

It is known that the response of cardiac muscle to cardiac hypertrophy includes changes in the expression of numerous signaling proteins as well as post-translational modifications (phosphorylation) of myofilament proteins. Therefore, we assessed phosphorylation of

sarcomeric proteins employing 2-D DIGE. Labeled proteins were identified with mass spectrometry and were consistent with identifications made previously. There were no significant differences in expression or phosphorylation of cardiac troponin T (TnT), tropomyosin (Tm), myosin binding protein C (MyBP-C) or myosin light chain 2 (MLC2) between fiber bundles obtained from ISO-treated and untreated WT and Pak-1-KO mice (data not shown). However, we did find alterations in levels of phosphorylation of cTnI (Figures 5A and 5B). We assessed phosphorylation of cTnI in WT and Pak-1-KO preparations (control and ISO treated) (N = 4/group). We have previously identified with specially configured DIGE gels unphosphorylated (U) and phosphorylated (P1, P2, P3, P4) spots in cTnI [17]. Results (Figures 5A and 5C) show increases in total phosphorylation of cTnI in WT sarcomeres after treatment with ISO, compared to untreated WT cTnI ($80.4\% \pm 0.8$ vs. $90.4\% \pm 2.2$, $p < 0.05$). However, no change in total cTnI phosphorylation was observed in Pak-1-KO mice ($82.8\% \pm 4.7$ vs. $87.7\% \pm 3.0$, $p = 0.413$). Individual analysis of phosphorylation of spot P1 (calculated as ratio of spot P1 to total cTnI) (Figures 5B and 5D) revealed a decrease in phosphorylation in Pak-1-KO mice treated with ISO vs. Pak-1-KO mice that received no treatment ($30.1\% \pm 3$ vs. $20.7\% \pm 2.1$, $p < 0.05$). In addition, analysis of phosphorylation of spot P4 (calculated as ratio of spot P4 to total cTnI) (Figures 5B and 5E) revealed an increase of phosphorylation in Pak-1-KO mice that received ISO vs. untreated control mice ($6.4\% \pm 0.6$ vs. $11.8\% \pm 1.0$, $p < 0.05$). Individual analysis of spots P2 and P3 did not show any statistically significant difference among groups. However, although the data shown in Figure 5 indicates differences, the exact source of the charge changes is not certain with regard to site-specific phosphorylations. We therefore employed site specific anti-phospho-peptide antibodies to further investigate these differences.

3.11 Analysis of cTnI phosphorylation in LV cardiac tissue

In view of the demonstrated effects of Pak and ISO on cTnI phosphorylation and myofilament response to Ca^{2+} , we investigated cTnI phosphorylation at residues S23/24 and S150 in WT and Pak-1-KO LV tissue, in the presence and in the absence of ISO (Supplemental Figures 3A through 3C). There was a significant increase of the ratio of phospho-(S23/24)-cTnI/total cTnI in WT/ISO ($83\% \pm 3.46$), in Pak-1-KO/CTRL ($94.67\% \pm 2$) and in Pak-1-KO/ISO ($93\% \pm 2.51$) vs. WT/CTRL ($68.67\% \pm 4.66$) ($p < 0.05$ for all groups). The ratio of phospho-(S150)-cTnI/total cTnI was increased in WT/ISO group ($95.33\% \pm 2.18$) vs. WT/CTRL ($56.67\% \pm 3.18$), Pak-1-KO/CTRL ($53.67\% \pm 1.76$) and in Pak-1-KO/ISO ($49.67\% \pm 1.45$) ($p < 0.05$ for all groups).

4. Discussion

Data reported here are the first to demonstrate direct association of Pak-1 to Erk1/2 in WT hearts. The involvement of Pak-1 and its upstream signals Cdc42 and Rac1 in cell remodeling had indicated a potential role in cardiac hypertrophy and dilatation [18, 19]. However, the molecular mechanisms underlying the effects produced by Pak-1 were uncertain. Based on our evidence that there is enhanced activity of Erk1/2 in the Pak-1-KO mouse heart, we propose that a significant role of Pak-1 is to normally suppress Erk1/2 activity. The mechanism is likely to involve a regulation of the β -adrenergic signaling, through a mechanism mediated by Pak-1, which, in turn, regulates PP2A activation. Enhanced activation of Erk1/2 and reduced activation of PP2A in ISO-treated Pak-1-KO mouse hearts support this mechanistic interpretation. Additionally, administration of FR180204, an Erk inhibitor, reduced the increased LV hypertrophy in ISO-treated Pak-1-KO mice. Overall, our findings indicate that in the absence of Pak-1, there is an exacerbation of the stress response of the myocardium to isoproterenol, which supports the idea that Pak-1 is an anti-hypertrophic signaling kinase and may serve the role as a natural modulator of the β -adrenergic signaling cascade and the Erks.

Previous studies support our hypothesis that a significant element in the Pak-1 signaling cascade is activation of PP2A and suppression of effects of β -adrenergic stimulation. Pak-1 has been shown previously to activate PP2A [1], which in turn dephosphorylates cTnI [1], myosin-binding protein C [1], phospholamban [20], inwardly rectifier potassium channels [21], and L-type Ca^{2+} channels [22]. In the current study, we demonstrated association of Pak-1, PP2A, and Erk1/2 *in vivo*. Previous studies have found β -adrenergic stimulation in cardiomyocytes [23], HEK293 cells [24], COS-7 cells [25], and Chinese hamster ovary cells [26] results in increased Erk phosphorylation. Our results demonstrate increased Erk1 phosphorylation at T202 and Y204 residues, increased Erk2 phosphorylation at T188, and increased phosphorylation of PP2A at Y307 in Pak-1-KO mice treated with ISO compared to all other groups studied (Pak-1-KO/CTRL, WT/CTRL, WT/ISO). This demonstrates that β -adrenergic stimulation triggers enhanced Erk phosphorylation due to a suppression of PP2A activation in the absence of Pak-1, promoting Erk-induced LV cardiac hypertrophy. This is in agreement with previous studies, which reported Erk1/2 to be involved in the development of cardiac hypertrophy [27]. Thus, Pak-1 may have a role in regulating the progression to LV cardiac hypertrophy during β -adrenergic stimulation through regulation of Erks. Previously, Pak-1 has been reported to counter-regulate adrenergic stimulation by mediation of PP2A [11], and a role for PP2A has been shown in regulating MEK and Erk in a receptor-independent manner [28]. In the present study, intracellular phosphorylation and activation of Erk1/2 during β -adrenergic stimulation may be maximal in the Pak-1-KO mice because the loss of Pak-1 coupling to PP2A leads to the consequent loss of an association between PP2A and Erk1/2. Figure 6 illustrates this proposed mechanism of LV myocardial hypertrophy.

Although our data indicated that Akt phosphorylation was not significantly different among any of the groups studied, some studies report Pak-1 activation of Akt [29, 30]. Mao et al. reported that endogenous Pak-1 is physically associated with Akt in cardiac cells and may act as a potential phosphoinositide-dependent protein kinase-2 essential for regulation of Akt phosphorylation [29]. However, others report Akt regulation of Pak [31], and yet others report that these two proteins may possibly regulate each other [30]. An interdependence of Pak and Akt may explain why we did not detect a change in Akt phosphorylation in hearts of the Pak-1-KO mouse model.

Our studies employing a KO mouse model provided a unique advantage in investigating the effects of Pak-1 on p38 MAPK and JNK1/2/3. Despite an extensive number of studies of Pak-1 effects on MAPK in various cell types, the role of Pak-1 in cardiac tissue remained uncertain. In the particular model investigated here our experiments demonstrated that phosphorylation of MAPK JNK1/2/3 was similar in all the models previously investigated. However, there was a significant depression of phosphorylation of p38 MAPK in Pak-1-KO mice in the presence and in the absence of ISO, compared to ISO-treated and untreated WT mice. This result agrees with previous studies demonstrating that CA Pak mutants activate p38 MAPK. We speculate that the reduction in phosphorylation of p38 we observed in the Pak-1-KO hearts is the consequence of the lack of Pak-1, a well-known activator of p38 [32]. Yet our data contrast with studies that reported activation of JNK in COS-7 cells [33]. Pak-1 and Pak-2 in HEK 293 cells induced activity of p38 MAPK but also JNK/SAPK [34]. CA Pak-3 was also reported to activate JNK1 in COS1 cells [35]. In that study, activated Cdc42 stimulated the activity of p38 MAPK, but was a less effective activator of Erk2 [35]. Others found that expression of the constitutive human Pak isoform, hPak-1, a kinase in COS7 mammalian cells, led to specific activation of the JNK1 MAPK pathway, but not the Erk MAP kinase pathway [33]. These varied results may arise from different Pak isoforms being studied in different cell types isolated from different species.

An unexpected and novel finding in our studies was the demonstration of a significant increase in tension generated by skinned fiber preparations from Pak-1-KO/ISO hearts compared to all other groups. This increase in tension generating capability indicates that in addition to the increase in cardiac mass in the Pak-1-KO/ISO hearts compared to controls, the relative increase in maximum tension was likely to contribute to the enhanced contractility reflected in a reduced ESV and EDV. To determine the mechanism for this increase in tension, we performed an analysis of protein phosphorylation in myofilament preparations from the experimental groups. Previous studies had indicated the potential for complex Pak-1 related mechanisms controlling phosphorylation of myofilament proteins. *In vitro* studies demonstrated direct phosphorylation of cTnI, cTnT, and desmin by Pak-3 [36] and phosphorylation of cTnI by Pak-1 [1]. However, we also reported that activation of PP2A by Pak-1 induces dephosphorylation of cTnI and myosin binding protein C [1]. The analysis in the present paper showed no changes of phosphorylation of myofilament proteins except for cTnI. In the case of cTnI it is seemed likely, and indeed our data demonstrated, that S23/S24, well known PKA-sites, would be phosphorylated in WT/ISO compared to controls. On the other hand S23/S24 residues in the Pak-1-KO hearts were nearly fully phosphorylated, and thus there was little further increase in Pak-1-KO/ISO hearts. In view of evidence that cTnI S150 is site phosphorylated by Pak-1 [36, 37], we assessed modifications in this residue. With ISO treatment in WT hearts, phosphorylation of S150 increased significantly. However there was no difference between in cTnI-S150 phosphorylation between WT controls and Pak-1-KO with or without ISO treatment. These data either further support the evidence of direct phosphorylation of S150 by Pak-1 or indicates discoordinate dephosphorylation of cTnI by Pak-1-PP2a. Phosphorylation at S23/S24 is known to depress [38], whereas phosphorylation at S150 of cTnI is known to enhance myofilament Ca-sensitivity [36]. This may account for the lack of differences in pCa-tension relations between preparations from WT controls and WT/ISO. We have no clear interpretation of the mechanism of the enhancement of maximum tension in the Pak-1 KO/ISO group. This appears to represent a novel and previously unknown state of cTnI, which may involve as yet undetermined modifications as noted in our 2-D DIGE analysis (Figure 5).

Yet, the major implications of our data remain with regard to our demonstration of the significant role of Pak-1 as a determinant of growth signaling and sarcomeric function in the myocardium. We conclude that an important role of Pak-1 is its function as a natural inhibitor of the Erks and a novel anti-hypertrophic signaling enzyme with a role in modulation of β -adrenergic signaling. Thus, Pak-1 plays a significant contribution in the mechanism of adaptive control of cardiac contractility.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Non-standard Abbreviations and Acronyms

2-D DIGE	2-D Difference In Gel Electrophoresis
CA	Constitutively active
cTnI	Cardiac troponin I
cTnT	Cardiac troponin T
CTRL	Control
EDT	Transmitral early filling deceleration time
EDV	End-diastolic volume
Erk	Extracellular signal-regulated kinase
ESV	End-systolic volume
FS	Fractional shortening
FVB	Friend Virus B-Type
HE	Heterozygous
ISO	Isoproterenol
JNK	Jun N-terminal Kinase
KO	Knockout lacZ: Lac operon Z
LV	Left ventricular/ventricle
LVAWD	Left ventricular diastolic anterior wall thickness
LVPWD	Left ventricular diastolic posterior wall thickness
MAPK	Mitogen activated protein kinase
MyBPC	Myosin binding protein C
P38 MAPK	P38 mitogen-activated protein kinase
Pak-1	P21-activated kinase
PP2A	Protein phosphatase 2A
Tm	Tropomyosin
WB	Western immunoblotting
WT	Wild type

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Highlights

- ISO-treated Pak-1-KO hearts are highly susceptible to cardiac hypertrophy
- Pak-1/PP2A and Erk1/2 bind to carry out their biological function in the heart;
- Erk1/2 activation is increased in ISO-treated Pak-1-KO hearts
- Pak-1 has a inhibitory role on Erk1/2 activation via PP2A
- FR180204, a selective inhibitor of Erk1/2, attenuates myocardial hypertrophy in ISO-treated Pak-1-KO mice.

Figure 1. Cardiac hemodynamics

M-mode echocardiography obtained from the parasternal short axis view at the mid-papillary level (*top*), and mitral pulsed-wave Doppler recordings showing early (E) and late (*atrial-A*) ventricular filling velocity (*bottom*), in WT and Pak-1-KO mice, in the absence (CTRL) and presence of ISO, or during administration of ISO and FR180204. Ablation of Pak-1 in mice promotes ISO-induced cardiac hypertrophy, compared to ISO-treated WT mice, which is prevented when the selective Erk1/2 inhibitor FR180204 is administered.

Figure 2. Gross anatomy and histology of WT and PAK-1-KO hearts

A, Gross anatomy of WT and Pak-1-KO hearts illustrating the increased cardiac mass in ISO-treated Pak-1-KO hearts (middle, right) compared to WT and Pak-1-KO hearts that received ISO or CTRL. ISO-treated Pak-1-KO hearts that received FR180204, a selective inhibitor of Erk1/2, were characterized by LV total mass comparable to WT and Pak-1-KO hearts that received CTRL saline. **B**, Cross-section at the mid-papillary level of hearts in (A). **C**, Hematoxylin and eosin stain of myocardial LV tissue shows myocyte hypertrophy in ISO-treated hearts, and attenuated myocyte growth in mice that received FR180204. Data are representative of three separate experiments. Magnification is 40X for all images.

Figure 3. Western immunoblotting analysis of AKT, MAPKs, and PP2A indicate that cardiac hypertrophy is due to enhanced Erk1/2 activation in Pak-1-KO mice

A, AKT phosphorylation at T308 was not significantly different among any of the experimental groups. **B-D**, Western immunoblotting of Erk1/2 phosphorylation in whole cardiac tissue shows maximal activation in Pak-1-KO/ISO mice vs. all other groups. Erk1 phosphorylation at residues T202 and Y204, and Erk2 phosphorylation at T188 indicate that Erks activation is dependent on ISO stimulation and Pak-1 expression. **E**, Phosphorylation of JNK1/2/3 MAPK at residues Y185 + Y185 + Y223 was not significantly different among all experimental groups. **F-G**, Phosphorylation of p38 MAPK is reduced in Pak-1-KO mice, in the presence and in the absence of ISO, compared to ISO-treated and untreated WT mice. **H-I**, Phosphorylation of PP2A at residue Y307 was increased in treated and untreated Pak-1-KO mice, compared to WT groups. * indicates $p < 0.05$.

Figure 4. Ca²⁺-dependent isometric tension development of detergent-extracted cardiac fibers
A-B, ISO-treated Pak-1-KO hearts develop a statistically significantly higher maximal Ca²⁺ activated tension compared to all other experimental groups. * indicates p < 0.05.

Figure 5. Analysis of cTnI by 2-D DIGE

A-B, Two-dimensional map of the cTnI in WT and Pak-1-KO mice. **C**, Total phosphorylation of cTnI increased in WT mice that received ISO vs. untreated WT control. No change in total cTnI phosphorylation was observed in Pak-1-KO mice after ISO treatment. **D**, Individual analysis of phosphorylation of spot P1 revealed a decrease in phosphorylation in Pak-1-KO mice treated with ISO vs. untreated Pak-1-KO mice. **E**, Individual analysis of phosphorylation of spot P4 revealed an increase of phosphorylation in Pak-1-KO mice that received ISO vs. untreated Pak-1-KO mice. * indicates $p < 0.05$.

Figure 6. Proposed mechanism of LV myocardial hypertrophy in a Pak-1-KO mouse model
A, In the presence of Pak-1, ISO stimulation of the β -adrenergic receptor activates protein kinase A (PKA) signaling via activation of heterotrimeric G protein (α , β , and γ) and adenylate cyclase (AC). Also, ISO stimulation of the β -adrenergic receptor activates Erk1/2 signaling pathway via β -arrestin-mediated transactivation of the Epidermal Growth Factor receptor (EGFr). Active PKA activates Pak-1 by promoting Pak-1 autophosphorylation; in turn, active Pak-1 mediates PP2A conformational change and autodephosphorylation leading to PP2A activation. The now active PP2A induces dephosphorylation and reduced activation of Erk1/2, resulting in attenuation of cardiac hypertrophy. **B,** In the absence of Pak-1, Pak-1/PP2A-mediated inhibition of Erk-1/2 is lost (//), resulting in enhanced, Erk1/2-mediated cardiac hypertrophy. The dotted line proposes a potential PKA-mediated mechanism through which PP2A phosphorylation and inactivation may occur when Pak-1 is absent.