Knockout of p21-activated kinase-1 attenuates exercise-induced cardiac remodelling through altered calcineurin signalling

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Aims

Despite its known cardiovascular benefits, the intracellular signalling mechanisms underlying physiological cardiac growth remain poorly understood. Therefore, the purpose of this study was to investigate a novel role of p21-activated kinase-1 (Pak1) in the regulation of exercise-induced cardiac hypertrophy.

Methods and results

Wild-type (WT) and Pak1 KO mice were subjected to 6 weeks of treadmill endurance exercise training (ex-training). Cardiac function was assessed via echocardiography, in situ haemodynamics, and the pCa–force relations in skinned fibre preparations at baseline and at the end of the training regimen. Post-translational modifications to the sarcomeric proteins and expression levels of calcium-regulating proteins were also assessed following ex-training. Heart weight/tibia length and echocardiography data revealed that there was marked hypertrophy following ex-training in the WT mice, which was not evident in the KO mice. Additionally, following ex-training, WT mice demonstrated an increase in cardiac contractility, myofilament calcium sensitivity, and phosphorylation of cardiac myosin-binding protein C, cardiac TnT, and tropomyosin compared with KO mice. With ex-training in WT mice, there were also increased protein levels of calcineurin and increased phosphorylation of phospholamban.

Conclusions

Our data suggest that Pak1 is essential for adaptive physiological cardiac remodelling and support previous evidence that demonstrates Pak1 signalling is important for cardiac growth and survival.

Keywords

Physiological hypertrophy • Cardiac function • Exercise • Treadmill running

1. Introduction

Exercise training-induced cardiac remodelling is an important physiological adaptation that has been demonstrated to improve quality of life and health, as well as to reduce morbidity and mortality. In contrast to pathological cardiac remodelling, physiological cardiac hypertrophy is associated with numerous multifactorial benefits including improvements in myocardial perfusion capacity, contractile function, and calcium handling. These positive alterations ultimately lead to the improvement in myocardial work capacity at submaximal and maximal exercise intensities. Despite the known cardiovascular benefits, the cellular, molecular, and integrative mechanisms underlying exercise-induced cardiac growth remain poorly understood.

Studies in our laboratory indicate that a potential mechanism for physiological hypertrophy in exercise involves signalling via p21-activated kinase 1 (Pak1). Pak1 belongs to a highly conserved family of serine–threonine protein kinases that are regulated by the Ras-related small G proteins, Cdc42 and Rac1. Over the past decade, there has been a growing interest in understanding the role of Pak1 in a diverse range of biological processes, such as control of cell growth, motility, excitation–contraction (E–C) coupling, survival, and death. Emerging evidence indicates that Pak1 is a multifunctional protein that has anti-hypertrophic effects in hearts stressed with pathological stimuli. Nevertheless, little is known about the role of Pak1 signalling that plays in mediating changes in cardiac E–C coupling and contractility following exercise training (ex-training). Previous studies
exploring the relations between Pak1 activity and Akt, an established regulator of myocardial growth following exercise training.\textsuperscript{15,16} Emphasis on the importance of addressing the hypothesis that Pak1 signalling is important in exercise. It has been demonstrated in vitro that Pak1 is essential for Akt activation by hypertrophic stimuli. Furthermore, previous work from our laboratory has demonstrated that Pak1 is an important modulator of β-adrenergic signalling.\textsuperscript{17} However, to date, the role of Pak1 in physiological hypertrophy in vivo has yet to be investigated. In the present study, to elucidate the role of Pak1 in physiological remodelling, we compared responses to ex-training in wild-type (WT) mice and in a mouse model with Pak1 gene deletion (Pak1 KO). The results of our investigation indicate a novel molecular player involved with endurance exercise and physiological cardiac hypertrophy and substantiate the fundamental importance of Pak1 signalling in the heart.

2. Methods

All protocols were approved by the Animal Care and Use Committee of the University of Illinois at Chicago and conform to the 'Guide for the Care and Use of Laboratory Animals' published by the US National Institutes of Health (NIH Publication No. 85-23, revised 2011).

2.1 Animals

WT and global Pak1 KO mice, described previously,\textsuperscript{17,18} were randomized into four groups: WT sedentary (32.2 g), WT ex-trained (29.8 g), Pak1 KO sedentary (31.5 g), and Pak1 KO ex-trained (31.2 g). Adult 2-month-old male mice were used for the acquisition of functional and biochemical data. Male age-matched friend virus B mice purchased from Charles River Laboratories were used as WT controls.

2.2 Exercise training protocol

WT and Pak1 KO mice were sedentary controls or mice subjected to regular aerobic endurance ex-training on a rodent treadmill. Mice were first subjected to 5 days of treadmill familiarization (5 m/min, 0% incline, 30 min/day) followed by 6 weeks of treadmill endurance exercise (55–60% MAV, 15% incline, 60 min/day, 5 days/week). Before the commencement and at the end of the 6-week training regimen, all mice were assessed for maximal aerobic velocity (MAV). Briefly, after a warm-up period, treadmill speed was increased (6–45 m/min) by 3 m/min every 3 min at 0% grade until exhaustion and fatigue of the mouse was achieved. Fatigue was defined as the inability to maintain normal running position despite mechanical prodding, mild electrical stimulus, or an unwillingness to run. Distance was recorded and the speed at which the mice became exhausted and fatigued was recorded as the MAV.

2.3 Histology

Excised hearts from sedentary and ex-trained WT and Pak1 KO mice were cannulated via the aorta and the coronary arteries retrogradely perfused with saline solution followed by formalin. The hearts were then transversely sliced into four pieces and each piece placed into a cassette. Haematoxylin and eosin (H&E), Mason’s trichrome, and Picrosirius Red staining were conducted on 5 μm sections.

2.4 Echocardiography

WT and Pak1 KO mice were anaesthetized with isoflurane and echocardiography was performed using a Vevo 770 High-Resolution In Vivo Imaging System and RMVTM 707B scan head with a centre frequency of 30 MHz (VisualSonics, Toronto, ON, Canada) as previously described.\textsuperscript{19} Pulsed Doppler was performed with the apical four-chamber view. The mitral inflow was recorded with the Doppler sample volume at the tip of the mitral valve leaflets at the centre of the mitral valve orifice. To measure time intervals, the Doppler sample volume was moved towards the left ventricular outflow track, and both the mitral inflow and left ventricle (LV) outflow were obtained in the same recording. All measurements and calculations were averaged from three consecutive cycles and performed according to the American Society of Echocardiography guidelines. Echocardiography was performed in each animal at baseline before the acclimatization phase and following 6 weeks of endurance ex-training. Data analysis was performed with the Vevo 770 software.

2.5 In situ haemodynamics

In situ pressure—volume measurements were performed in sedentary and exercise-trained mice as previously described.\textsuperscript{19} Mice were anaesthetized with 1% isoflurane in 100% oxygen by face mask and then placed on a thermally controlled table that allowed for the maintenance of body temperature at 37 °C. A tracheotomy was performed and a steel intubation cannula (1.2 mm diameter; Hugo Sachs Electronic-Harvard, March, Germany) was inserted into the airway and secured with a suture. The right common carotid artery was then isolated, the distal end tied off with a 6-0 suture, and the artery cannulated with an ultra-miniature pressure—volume (P—V) catheter (1.0F PVR1045, Millar Instruments, Houston, TX, USA). The transducer was advanced down the right carotid artery, into the aorta, through the aortic valve, and into the LV. To record P—V loops in different loading conditions, an abdominal access was obtained to allow transitory veno cava occlusion right below the diaphragm. Data analysis was performed using the LabChart Software.

2.6 pCa–force relation in skinned fibre preparations

Measurements of pCa–force relations were performed in detergent-extracted bundles of ventricular myocytes (skinned fibres) isolated from hearts of sedentary or exercise-trained mice as previously described.\textsuperscript{19} Mice were anaesthetized by an intraperitoneal injection of pentobarbitral sodium (50 mg/kg). Hearts were quickly excised and LV papillary muscles dissected into fibre bundles (4–5 mm long and 150–250 μm in diameter) in cold, high-relaxing (HR) buffer (10 mM of EGTA, 6.5 mM of MgCl₂, 42 mM of KCl, 6.2 mM of ATP, 10 mM of creatine phosphate, 100 mM of N,N,N,N-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid, pH 7.0, and ionic strength 180 mM) plus protease inhibitors (2.5 mg/L of pepstatin A, 1 mg/L of leupeptin, and 30 mM of phenylmethylsulfonyl fluoride). Fibre bundles were skinned in HR solution containing 1% Triton X-100 for 30 min and mounted between a force transducer and a micro-manipulator. Resting sarcomere length was set at 2.2 μm using laser diffraction and the cross-sectional area of the fibre bundle was calculated as previously described.\textsuperscript{17} The fibres were then sequentially bathed in a series of solutions containing increasing Ca²⁺ concentrations (pCa 8–4.5). Isometric tension was recorded on a chart recorder. Isometric tension measurements were plotted as a function of pCa and fit by a nonlinear least-squares regression analysis to the Hill equation using Graph Prism 6. From this fitted curve, we derived the pCa50 (pCa required to produce 50% of the maximal tension obtained), maximal tension, and the Hill coefficient.

2.7 Assessment of myofilament phosphorylation by Pro-Q Diamond stain

Hearts from mice were immediately flash frozen in liquid nitrogen upon harvest. Myofibrils were purified from ~25 mg of liquid nitrogen frozen mouse tissue to be analysed with Pro-Q Diamond (Invitrogen, Eugene, OR, USA) stain as previously described with minor modifications.\textsuperscript{20} The tissue was homogenized twice in standard relax buffer (10 mM imidazole pH 7.2, 75 mM KCl, 2 mM MgCl₂, 2 mM EDTA, and 1 mM NaN₃) with 1% (v/v) Triton X-100. Myofibrils were centrifuged, the supernatant fraction was removed, and the pellets washed once within standard relax buffer to remove the Triton X-100. The standard relax buffers contained both the protease
2.8 Western blots
Frozen tissue (10–20 mg) was pulverized using a pre-chilled tissue pulverizer and solubilized in Laemml buffer containing protease and phosphatase inhibitors using a Dounce homogenizer. Total heart lysates were separated on SDS–PAGE gels and transferred to PVDF membranes that were blocked and then blotted with antibodies recognizing CaMKII (4346S, Cell Signaling Technology, Danvers, MA, USA), phospho-CaMKII Thr²⁸⁶ (3361S, Cell Signaling Technology), phospholamban (PLN; 05-205, Millipore, Billerica, MA, USA), phospho-PLN Ser¹⁶ (07-052, Millipore), phospho-PLN Thr¹⁷ (A010-13AP, Badrilla, Leeds, UK), SERCA2a (3361S, Cell Signaling Technology), and calcineurin (PA5-17446, Thermo Scientific). Blots were incubated overnight and stained for 90 min with Pro-Q Diamond (Invitrogen) then overnight, and stained for 90 min with Pro-Q Diamond (Invitrogen) and ImageLab 3.0. (for Coomassie)

2.9 Citrate synthase activity
Hindlimb skeletal muscles (20 mg) were homogenized on ice in 1 mL of 0.1 M Tris buffer containing 0.1% Triton X-100, pH 8.35. Citrate synthase (CS) activity was determined spectrophotometrically according to the method of Srere. The homogenate was frozen under liquid nitrogen and thawed four times to disrupt the mitochondria to expose the CS. The assay system was contained in a total volume of 200 μL of 0.1 M Tris buffer containing 0.1% Triton X-100, pH 8.35. Citrate synthase activity was determined spectrophotometrically according to the method of Srere. The homogenate was frozen under liquid nitrogen and thawed four times to disrupt the mitochondria to expose the CS. The assay system was contained in a total volume of 200 μL of 0.1 M Tris buffer containing 0.1% Triton X-100, pH 8.35. Citrate synthase activity was determined spectrophotometrically according to the method of Srere. The homogenate was frozen under liquid nitrogen and thawed four times to disrupt the mitochondria to expose the CS. The assay system was contained in a total volume of 200 μL of 0.1 M Tris buffer containing 0.1% Triton X-100, pH 8.35.

2.10 Statistics
All data were analysed using a two-way ANOVA and Tukey’s post hoc analysis using GraphPad Prism 6. Data are expressed as mean ± SE. Differences were considered significant when P < 0.05.

3. Results
3.1 Cardiac morphology, histology, and endurance capacity
Endurance ex-training induced an adaptive response in maximal running velocity and endurance capacity (Figure 1A and B). Moreover, there was a significant increase in hindlimb skeletal muscle CS activity (Figure 1C) and heart weight-to-tibia length (HW/TL) in WT ex-trained mice (Figure 1D). Sedentary Pak1 KO mice did demonstrate a diminished skeletal muscle CS activity; however, this was significantly improved with regular ex-training, such that it was comparable to WT ex-trained mice. Following 6 weeks of treadmill running, HW/TL ratios in significantly increased WT mice, but not in the Pak1 KO mice (Figure 1D). Histological studies did not reveal marked myocardial disarray, fibrosis, or the presence of collagen deposition in the extracellular matrix following training in WT or Pak1 KO mice (Figure 1E).

3.2 Global cardiac function
High-resolution echocardiography demonstrated that WT mouse hearts undergo significant chamber remodelling following 6 weeks of endurance ex-training (Figure 2 and Table 1). These adaptations were not observed in the trained Pak1 KO mice (Table 1). WT endurance exercise-trained mouse hearts had larger left atria, left ventricular inner diameter during systole (LVIDs), and LV mass compared with their sedentary counterparts (Table 1). Ex-trained Pak1 KO mice had smaller relative wall thickness (RWTh) and higher peak early to late diastolic filling velocity ratios compared with WT ex-trained mice. Overall, we did not observe any changes in morphology or cardiac function following ex-training in Pak1 KO mice when assessed by echocardiography. We also used a P—V conductance catheter to perform in situ haemodynamic studies in these mice (Figure 3). Following ex-training, only WT mice displayed significantly elevated +dP/dt, preload recruitable stroke work (PRSW), and end-systolic pressure–volume relation, a load-independent parameter of contractility. Figure 3 (top panel) shows representative P—V loops during occlusion of the inferior vena cava in ex-trained and sedentary WT and Pak1 KO mice. We observed a decreased tau (τ) in ex-training WT mice, suggesting improved active relaxation following endurance ex-training (Figure 3F). In accordance with other reports,22,23 ex-training did not alter LV stiffness in WT mice. However, similar to echocardiography results, we did not observe any improvements in systolic or diastolic cardiac function in the Pak1 KO mice following ex-training. Pak1 KO ex-trained mice did demonstrate a reduction in left ventricular developed pressure compared with WT ex-trained mice (see Supplementary material online, Table S1).

3.3 Myofilament responsiveness to Ca²⁺
To further elucidate the mechanism(s) for the functional alterations observed following ex-training, we focused on the level of the sarcomere and studied Ca²⁺-dependent tension development of skinned fibre bundles (Figure 4). We observed a 0.05 shift (leftward) in the pCa50 in WT mice, whereas Pak1 KO mice showed no changes in Ca²⁺ response following endurance training. This was accompanied by a significant increase in maximum tension (see Supplementary material online, Table S2). Conversely, we found that there was no change in pCa50 in Pak1 KO ex-trained mice compared with sedentary Pak1 KO mice (Figure 48). In fact, sedentary and ex-trained Pak1 KO mice displayed significantly lower pCa50 compared with ex-trained WT mice. We

(Sigma #P-8340) and phosphatase (Calbiochem #524624) inhibitors at a 1:100 dilution. The pellet was then solubilized in 8 mol/L of urea, 2 mol/L of thiourea, and 4% CHAPS (UTC buffer), and the protein concentration of the samples was determined with an RCD assay kit (Bio-Rad, Hercules, CA, USA). A 12% SDS–PAGE gel was loaded with 8 μg of total protein per lane and run at 200 V for 75 min. The gel was placed directly into 120 mL of fix solution (50% methanol and 10% acetic acid) for 30 min, then overnight, and stained for 90 min with Pro-Q Diamond (Invitrogen) gel stain. The gel was imaged on a Typhoon 9410 imager with a CY3 filter set. To visualize total protein, the gel was Coomassie blue-stained, and imaged on a ChemiDoc XRS+ (Bio-Rad), using the Coomassie blue filter set. Band densities from gels were determined using ImageQuant TL (for ProQ) and ImageLab 3.0. (for Coomassie)
Figure 1  Maximal running velocity, endurance capacity, CS activity, and morphology and histology of WT and Pak1 KO sedentary and ex-trained mice. MAV (A), endurance exercise capacity (B), hindlimb skeletal muscle CS activity (C) HW/TL ratio (D), and 40x magnification of heart sections stained with H&E (line 1), Masson’s Trichrome (line 2), and Picrosirius Red (PSR, line 3) (E) in sedentary and ex-trained mice. Data are presented as mean ± SE.

*significantly different from WT sedentary, †significantly different from WT ex-trained, and ‡significantly different from Pak1 KO sedentary, P < 0.05. n = 6–7 per group.
did not observe any changes in the co-operativity (Hill coefficient) of the pCa–force relation in skinned fibres from these mice (Figure 4C).

3.4 Myofilament protein status
Post-translation modifications to sarcomeric proteins are central to cardiac function and are a critical element that occurs with myocardial remodelling. Therefore, we assessed phosphorylation status of the myofilament proteins (Figure 5). There were no differences in the levels of phosphorylation of cardiac troponin I or myosin light chain 2 among any of the groups. However, myofilaments of WT ex-trained mouse hearts showed an increase in total phosphorylation of myosin-binding protein C (MyBP-C) and cardiac troponin T (cTnT) compared with WT sedentary controls (Figure 5A and B). Sedentary Pak1 KO mice also displayed elevated levels of phosphorylation of cTnT (Figure 5B). Pak1 KO ex-trained mice have lower tropomyosin phosphorylation compared with WT ex-trained mice (Figure 5C).

3.5 Ca$^{2+}$ regulating proteins
We also assessed protein content of the Ca$^{2+}$ regulating proteins PLN and SERCA2A using western blotting. Interestingly, we also found an increase in total PLN in the WT mice following ex-training (Figure 6A). Phosphorylation of PLN in WT ex-trained mice was selectively elevated at Ser-16, whereas Thr-17 remained unchanged compared with sedentary mice (Figure 6B). Additionally, WT ex-trained mice had significantly higher levels of SERCA2a expression compared with Pak1 KO mice (Figure 6C). We also observed an increased expression of calcineurin in WT mice following exercise training, which was not evident in Pak1 KO mice (Figure 7A).

Moreover, hearts from endurance-trained WT mice displayed a trend ($P = 0.098$) for increased phosphorylation at Thr-287 of CAMKII$\beta$, which was not seen in the Pak1 KO mice (Figure 7C).

4. Discussion
This is the first investigation to analyse the role of Pak1 in exercise-induced cardiac hypertrophy and alterations in cardiac function. Data presented here demonstrate that Pak1 signalling in the heart is absolutely required for the adaptive cardiac growth and improvement in cardiac function observed with regular ex-training in mice. The present study is novel in demonstrating that Pak1 activity in the heart is essential for the phosphorylation of MyBP-C, TnT, and TM in response to regular aerobic endurance exercise. Another important finding of this study was that the increased levels of calcineurin and phosphorylation of Ser-16 PLN observed with ex-training are

![Figure 2](https://example.com/figure2.png)

**Figure 2** Evaluation of cardiac morphology by M-mode of high-resolution echocardiography. Representative view of M-mode echocardiography from sedentary and ex-trained WT and Pak1 KO mice (A), left ventricular inner diameter during diastole (B), and LVIDs (C) in WT and Pak1 KO sedentary and ex-trained mice. *significantly different from WT sedentary, $P < 0.05$, $n = 8–10$ per group.
Table 1  High-resolution echocardiography data from sedentary and ex-trained WT and Pak1 KO mice

<table>
<thead>
<tr>
<th>Parameter</th>
<th>WT sed</th>
<th>Pak1 KO sed</th>
<th>WT ex-trained</th>
<th>Pak1 KO ex-trained</th>
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<tr>
<td>Animals</td>
<td>8</td>
<td>8</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Heart rate (b.p.m.)</td>
<td>562.75 ± 24.23</td>
<td>512.63 ± 17.04</td>
<td>500.75 ± 21.51</td>
<td>479.50 ± 11.40*</td>
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<tr>
<td>LA (mm)</td>
<td>1.56 ± 0.07</td>
<td>1.76 ± 0.03</td>
<td>1.87 ± 0.03*</td>
<td>1.77 ± 0.08</td>
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<tr>
<td>LVIDd (mm)</td>
<td>3.91 ± 0.06</td>
<td>4.26 ± 0.07*</td>
<td>4.18 ± 0.09</td>
<td>4.00 ± 0.06</td>
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<td>LVIDs (mm)</td>
<td>2.41 ± 0.10</td>
<td>2.57 ± 0.08</td>
<td>2.80 ± 0.12*</td>
<td>2.64 ± 0.06</td>
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<td>LVAVd (mm)</td>
<td>0.60 ± 0.02</td>
<td>0.62 ± 0.05</td>
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<td>LVPWd (mm)</td>
<td>0.68 ± 0.01</td>
<td>0.60 ± 0.04</td>
<td>0.72 ± 0.02</td>
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<td>RWTi (mm)</td>
<td>0.32 ± 0.01</td>
<td>0.29 ± 0.02b</td>
<td>0.39 ± 0.02</td>
<td>0.31 ± 0.01b</td>
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<tr>
<td>Lvmass (mg)</td>
<td>70.79 ± 2.60</td>
<td>69.75 ± 4.57</td>
<td>89.01 ± 6.21**</td>
<td>72.14 ± 3.65</td>
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<td>FS (%)</td>
<td>37.51 ± 2.06</td>
<td>38.56 ± 1.51</td>
<td>40.92 ± 1.73</td>
<td>36.4 ± 1.77</td>
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<tr>
<td>EF (%)</td>
<td>66.98 ± 2.43</td>
<td>69.0 ± 1.90</td>
<td>72.4 ± 1.49</td>
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<tr>
<td>SV (μL)</td>
<td>48.18 ± 0.82a</td>
<td>56.31 ± 2.08</td>
<td>49.4 ± 1.84c</td>
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<td>CO (mL/min)</td>
<td>27.02 ± 0.67</td>
<td>28.04 ± 1.07</td>
<td>25.64 ± 0.91</td>
<td>25.4 ± 0.90</td>
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<td>E/A (mm/s)</td>
<td>1.30 ± 0.04</td>
<td>1.54 ± 0.05</td>
<td>1.22 ± 0.05</td>
<td>1.57 ± 0.14b</td>
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<tr>
<td>IVRT (ms)</td>
<td>10.94 ± 0.41</td>
<td>10.32 ± 0.73b</td>
<td>12.8 ± 0.41</td>
<td>12.0 ± 0.49</td>
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<tr>
<td>E-wave DT (mm/s²)</td>
<td>22.03 ± 1.54</td>
<td>26.9 ± 2.32b</td>
<td>20.13 ± 1.73</td>
<td>22.43 ± 1.21</td>
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<tr>
<td>E/Em (mm/s)</td>
<td>29.08 ± 1.52</td>
<td>32.91 ± 1.77b</td>
<td>23.40 ± 1.30</td>
<td>29.39 ± 1.33</td>
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<td>Vcf (circ/s)</td>
<td>8.13 ± 0.42</td>
<td>7.37 ± 0.42</td>
<td>9.30 ± 0.72</td>
<td>7.7 ± 0.32</td>
</tr>
</tbody>
</table>

*a vs. WT sed.  
*b vs. WT ex-trained.  
*c vs. Pak1 KO sed.  
LVIDd, left ventricular inner diameter during diastole; LVIDs, left ventricular inner diameter during systole; RWTi, relative wall thickness; E/A, ratio of peak early to late diastolic filling velocity; IVRT, isovolumetric relaxation time; E/Em, ratio of early transmittal flow velocity to the early diastolic tissue velocity; Vcf, velocity of circumferential shortening.

dependent on Pak1 activity. Collectively, the results from this study demonstrate that Pak1 signalling in the heart is indispensable for the physiological remodelling that occurs with regular aerobic endurance ex-training.

### 4.1 The role of Pak1 signalling in the exercise-induced improvement in whole heart function

Numerous factors may potentially influence myocardial adaptation to exercise, such as age, gender, modality, animal strain, and exercise intensity. The treadmill protocol used in this study was able to elicit a significant increase in the HW/TL ratio in WT mice after ex-training, whereas Pak1 KO mice were insensitive to exercise stress. Accordingly, only results of echocardiography measurements in WT mice revealed enlargement of the cardiac chambers following ex-training. Our results expand previous findings that have reported an increase in LV dimensions with exercise in humans and animals. We did not observe any significant basal differences in cardiac structure or function in sedentary WT or Pak1 KO mice. This discovery extends previous findings from our group and others. However, with ageing, it appears that Pak1 KO mice demonstrate smaller heart growth rates compared to age-matched WT mice (data not shown). Thus, we speculate that Pak1 signalling is important for the initiation of cardiac hypertrophy in response to physiological stress, such as with regular ex-training.

The majority of the previous work that has examined the effects of exercise on cardiac contractility has done so by using in vitro studies on isolated rodent hearts, papillary muscles, and isolated cardiomyocytes. We are aware of only one other investigation to measure pressure–volume relations to determine ventricular function with ex-training. However, Radovits et al. employed swimming exercise in rats. Our P—V data are in agreement with Radovits et al. in that we also found a significant improvement in systolic cardiac function following ex-training. Interestingly, Pak1 KO mice did not demonstrate any improvement in systolic cardiac function. Athlete’s heart is known to be the structural and functional remodelling that occurs with regular ex-training and there have been numerous ongoing investigations into the structural/morphological changes, and the cellular/molecular remodelling that occurs with exercise training. However, there is little known about the functional effects of exercise-induced cardiac hypertrophy. Therefore, using in situ haemodynamics, we demonstrate that systolic and diastolic cardiac function is improved in WT mice following regular ex-training, and our data suggest that Pak1 plays an important role in this adaptive remodelling process.

### 4.2 Exercise-induced increase in myofilament responsiveness to Ca²⁺ and phosphorylation of MyBP-C is Pak1-dependent

Alterations in the myofilament responsiveness to Ca²⁺ provide one potential mechanism for the improved contractile function in the heart. It has been well established that there is an increase in Ca²⁺ sensitivity of the myofilament following ex-training. In the current study, we observed a 0.05 shift (leftward) in the pCa50 in WT mice, whereas Pak1 KO mice showed no changes in Ca²⁺ response following endurance training. Another mechanism that has been previously demonstrated to cause an increase in myofilament Ca²⁺ sensitivity following...
Figure 3  In situ cardiac function following 6 weeks of aerobic endurance exercise in WT and Pak1 KO mice. Ejection fraction (A), the maximal rate of contraction (+dP/dt) (B), PRSW (C), end-systolic pressure–volume relation slope (ESPVR) (D), the maximal rate of relaxation (−dP/dt) (E), relaxation time constant calculate by Weiss method (τ) (F), and end-diastolic pressure–volume relation (EDPVR) (G) in sedentary and ex-trained WT and Pak1 KO mice. Data are presented as mean ± SE. *significantly different from WT sedentary, †significantly different from WT ex-trained, P < 0.05. n = 5 per group.
ex-training is the improvement in the regulation of intracellular pH; however, this was not examined in the current study. To date, there have been a limited number of studies that have examined the effects of ex-training on modifications in Ca\(^{2+}\) sensitivity in heart failure (HF). Interestingly, endurance ex-training was able to normalize Ca\(^{2+}\) sensitivity to control levels post-myocardial infarction (MI) regardless whether MI resulted in an increase or decrease in Ca\(^{2+}\) sensitivity. Our data suggest that Pak1 signalling is required for the adaptation of cardiac Ca\(^{2+}\) sensitivity with exercise; therefore, we think that Pak1 signalling will also be requisite for the alteration in myofilament Ca\(^{2+}\) with ex-training in HF. However, this remains to be investigated.

Although the biological mechanisms are not fully understood, we and others have demonstrated that modification of the sarcomeric proteins is one potential mechanism for the changes in cardiac myofilament Ca\(^{2+}\) sensitivity. However, few studies have examined the modifications of the sarcomere proteins and Ca\(^{2+}\) sensitivity following ex-training. Our findings extend the results of previous reports, which found an increase in myosin-binding protein-C (MyBP-C) phosphorylation following ex-training. MyBP-C is generally thought to control the kinetics of myosin head interactions with the thin filament. Furthermore, it has been previously demonstrated that phosphorylation of MyBP-C occurs via beta-adrenergic stimulation, which leads to activation of PKA and CaMKII, the latter was shown to be upregulated in the WT-exercised mice in the current study. Indeed, Kemi et al. demonstrated that there is an exercise-induced chronic phosphorylation of CaMKII that may account for the increase in myofilament Ca\(^{2+}\) sensitivity. MyBP-C has been demonstrated to have diverse roles in regulating cardiac function and known to have important implications in altering Ca\(^{2+}\) homeostasis. In this regard, Sadayappan et al. demonstrated the importance of basal phosphorylation of MyBP-C and its effects on Ca\(^{2+}\) sensitivity. Additionally, it has been demonstrated that phosphorylation of MyBP-C is cardioprotective and is decreased following ischaemia/reperfusion (IR) injury. Here, we provide the first evidence that the exercise-induced phosphorylation of MyBP-C is Pak1-dependent and can result in an increase in the myofilament responsiveness to Ca\(^{2+}\).

4.3 The role of Pak1 in the improvement in Ca\(^{2+}\)-handling proteins and calcineurin signalling following ex-training

Adaptations in the regulation of Ca\(^{2+}\) homeostasis are another potential mechanism for the observed improvement in cardiac function in WT mice following ex-training. There have been several previous studies that have provided evidence that endurance ex-training increases the level of SERCA2a and NCX. In agreement with others, we also found an increase in phosphorylation of PLN and PLB levels. Importantly, ex-training also induced chronic activation of CaMKII and, consequently, a hyperphosphorylation of PLB at the serine-16 residue. These results extend previous reports that found an increase in PLB Ser-16 residue phosphorylation in mice subjected to 8 weeks of treadmill running which had HF. It has been previously demonstrated that ex-training induces phosphorylation of PLN (Thr-17), as well as normalizes PLB levels in a disease model. Recently, the activation of serum response factor (SRF) by Pak1 signalling was implicated in control of SERCA2a expression. Interestingly, it has also been demonstrated that SRF...
Figure 5  Phosphorylation of myofilament proteins as detected by ProQ. Representative ProQ gel demonstrating changes in phosphorylation of various myofilament proteins in WT and Pak1 KO sedentary and ex-trained mice (top panel). Actin was used as a loading control from Coomassie-stained gel. MyBP-C, myosin-binding protein C (A); TnT, troponin T (B); TM, tropomyosin (C). Data are presented as mean ± SE. *significantly different from WT sedentary. †significantly different from WT ex-trained, \( P < 0.05 \). \( n = 6 \) per group.

Figure 6  Expression of Ca\(^{2+}\)-handling proteins. Expression of PLN (A), phosphorylated-PLN at site Ser16 (B), and SERCA2a (C) in sedentary and ex-trained WT and Pak1 KO mice. Data from PLN monomer and pentamer were pooled. Actin was used as a loading control. *significantly different from WT sedentary, \( P < 0.05 \). \( n = 6–10 \) per group.
phosphorylation is increased following ex-training in human subjects. Therefore, it seems plausible that the exercise-induced improvement in SERCA2a activity may be dependent on SRF. However, future studies are required to test this hypothesis.

Although calcineurin is known to be a critical player in the development of pathological hypertrophy, its involvement in physiological cardiac hypertrophy has been controversial and limited studies exist. Our data demonstrate that calcineurin protein levels are increased following regular ex-training in WT mice. These data are in agreement with previous studies that have established the importance of calcineurin in physiological cardiac remodelling. It has been previously demonstrated that the activation of calcineurin is required for ERK and Akt activity in response to physiological stress, and it is generally accepted that calcineurin activity is important for the initiation of cardiac hypertrophy (i.e. adaptive). In this regard, standard Pak1 KO mice live a normal life span without notable cardiac problems. However, cardiac function under stress conditions may be compromised. Given that we did not observe an increase in calcineurin in Pak1 KO mice, which are completely insensitive to exercise stress, our data reveal Pak1 signalling as a potentially important mediator of the initiation of cardiac hypertrophy, as well as the development of athlete’s heart.

4.4 Pak1 signalling as a modulator of β-adrenergic signalling and pharmacological target in a disease setting

Our understanding of Pak1 signalling has increased substantially within the last decade. We and others have established that Pak1 signalling can...
be anti-hypertrophic in the face of pathological stimuli, however, the role of Pak1 in physiological hypertrophy is not well understood. Previous work from our laboratory has demonstrated that Pak1 may serve as a natural modulator of β-adrenergic signalling cascade via activation of PP2A and Erk1/2. This previous work indicated that β-adrenergic stimulation triggered enhanced Erk phosphorylation due to a suppression of PP2A activation in the absence of Pak1, which promoted Erk-induced LV cardiac hypertrophy. Thus, indicating Pak1 may play a role in the cardiac hypertrophy during β-adrenergic stimulation, via isoproterenol (ISO) infusion. However, in the current investigation, we did not observe any differences in PP2A or ERK expression and activity (data not shown) between any of the groups. The exact mechanism for the differential effects of regular ex-training and ISO on β-adrenergic stimulation is unclear, although it has been previously demonstrated that chronic ISO infusion can lead to cardiac oxidative stress and extracellular matrix biosynthesis, leading to the alteration of wall stiffness and altered cardiac function, which can be reversed by regular ex-training. Thus, exercise training appears to be a better model to examine the beneficial effects of β-adrenergic signalling. Nevertheless, we can conclude that Pak1 plays an important role in the modulation of β-adrenergic signalling and the adaptive control of cardiac growth.

In the current study, Pak1 KO mice were resistant to the beneficial adaptations to endurance ex-training. Moreover, previous data suggest that Pak1 signalling is cardioprotective, a known benefit of endurance exercise. The mechanisms presented here demonstrate the fundamental importance of Pak1 signalling in exercise-induced cardiac hypertrophy and also highlight how manipulation of this cascade may be beneficial in a clinical setting. In this regard, Wang et al. recently reported that Pak1 activation (via pak activating peptide-a bioactive peptide) was able to reverse ANG-II-induced cardiac hypertrophy and ventricular arrhythmias. FTY720 has also been reported to reverse established hypertrophy in association with activation of Pak1. Thus, Pak1 signalling appears to be a novel cardioprotective signalling cascade that can be targeted in disease models. For example, in HF models, exercise reverses or prevents depression in myofilament response to Ca++. It is apparent from our studies that Pak1 activation may play a role in this protection. It is also important to note that exercise induces oxidative stress related to work-load and release of catecholamines. While determination of the role of Pak1 in redox signalling in exercise awaits future studies, we have reported that active Pak1 is a negative regulator of NADPH oxidase. In general, further studies are needed to gain better insights into the mechanism(s) or the role of Pak1 in ex-training and its effects in diseased states.

4.5 Important considerations

Much to our surprise, we did not observe any significant alterations in the cardiac function following ex-training when assessed by non-invasive echocardiography. There have been other previous reports in the literature that demonstrate echocardiography analysis of the heart following exercise training is less sensitive in discerning changes than postmortem measurements. One potential reason for the inability to detect cardiac adaptations that may accompany ex-training is an insufficient axial resolution. Furthermore, recent reports have implicated Pak1 as an important element in glucose transporter 4 (GLUT4) recruitment involved in insulin-stimulated glucose clearance/uptake in mouse skeletal muscle, which could potentially contribute to the lack of exercise capacity in Pak1 KO mice. Although skeletal muscle GLUT4 was not examined in the current investigation, we did not observe any differences at baseline in MAV or endurance capacity between groups. However, we did observe a significant reduction in CS activity of the hind limb skeletal musculature, although both WT and Pak1 KO mice demonstrated hypertrophy of the skeletal muscles (data not shown) and improvements in CS activity with ex-training. Therefore, at present, we cannot formally disregard the possibility that Pak1 KO mice have diminished skeletal muscle function that may be a contributor to our findings.

In conclusion, knowledge gained from this research provides useful biological insights into the cellular and molecular responses required for adaptive physiological growth and improvement in cardiac function with regular ex-training. In addition, these studies shed light on how Pak1 may be a novel molecular target for therapeutic approaches aimed at restoring cardiovascular health.

Supplementary material

Supplementary material is available at Cardiovascular Research online.

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References


77. DeSantiago J, Bare DJ, Xiao L, Ke Y, Solaro RJ, Banach K. P21-activated kinase1 (Pak1) is a negative regulator of NADPH-oxidase 2 in ventricular myocytes. J Mol Cell Cardiol 2014;67:77–85.
