Cytoskeletal role in protection of the failing heart by β-adrenergic blockade

Guangmao Cheng, Harinath Kasiganesan, Catalin F. Baicu, J. Grace Wallenborn, Dhandapani Kuppuswamy, and George Cooper 4th

Gazes Cardiac Research Institute, Cardiology Division, Department of Medicine, Medical University of South Carolina and the Department of Veterans Affairs Medical Center, Charleston, South Carolina

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IN 1993 A UNIQUE CYTOSKELETAL alteration was reported in the hypertrophying heart (51, 52). It was discovered that in myocardium hypertrophying in response to pathological pressure overloading, but not in response to an equivalent degree and duration of physiological volume overloading, there is a persistent increase in microtubule network density that causes contractile dysfunction. This cytoskeletal alteration becomes more pronounced during the deterioration of initially compensatory right ventricular (RV) or left ventricular (LV) pressure overload hypertrophy into the congestive heart failure state, both in animal models of human disease (47, 48) and in human disease itself (58). In addition to contributing to the systolic and diastolic contractile dysfunction that is characteristic of pathological hypertrophy (11), the extensive decoration of cardiomyocyte microtubules with myocardial microtubule-associated protein (MAP4) that was found to cause this microtubule network densification by stabilizing the microtubules (43) was also found to inhibit the kinesin-based transport of mRNA along microtubules that is required to support the translation of myofibrillar proteins (44). Thus this dense, heavily MAP4-decorated microtubule network appears not only to contribute to the progressive contractile dysfunction that is characteristic of pathological hypertrophy but also to undermine the very basis for the compensatory hypertrophic growth response itself.

A search for the etiology of this cytoskeletal alteration led to the discovery, again in pathological pressure overloading but not in physiological volume overloading, of site-specific dephosphorylation of MAP4 that drives its microtubule binding and stabilizing function (10); that is, dephosphorylation of the serine in the KXGS motif of the first of four basic pseudorepeats within the MAP4 microtubule binding domain was both found in pathological hypertrophy and phenocopied each of the major features of the pathological microtubule network when introduced genetically into normal cardiomyocytes. It was then found that this MAP4 dephosphorylation is, in turn, driven by persistent activation of type 1 and type 2 phosphatases, and especially that of protein phosphatase 2A (PP2A) in pathological hypertrophy (10). Furthermore, this phosphatase activation is apparently driven by ongoing activation of the upstream stress-related kinase p21-activated kinase-1, or Pak1. Genetic induction of increased activity of upstream Pak1 or downstream PP2A or PPI in normal cardiomyocytes and/or myocardium reproduced each of the major features of the pathological microtubule network (7).

This continuous activation of a signaling cascade leading to MAP4 dephosphorylation becomes quite interesting in the context of another progressive change, i.e., in the β-adrenergic system, that is characteristic of the specific pathological setting of decompensated cardiac hypertrophy and failure, especially because increased cardiac microtubules have been seen as a rather general feature of end-stage clinical heart disease (2, 24, 31, 58). Thus, a hallmark of deteriorating intrinsic myocardial function, even well before the onset of overt heart failure, is the increased circulating norepinephrine levels and depleted myocardial norepinephrine content that result from continuous activation of the sympathetic nervous system (9), as well as cardiomyocyte β1-adrenergic receptor downregulation (5), including that caused by MAP4 decoration of microtubules (6). This common pathophysiological setting for both microtubule network densification and increased circulating norepinephr...
rine, coupled with the recent observation of \( \beta \)-agonist activation of cardiac Pak1 (33), led to the hypothesis tested in the present study. This hypothesis is that persistent \( \beta \)-adrenergic overdrive is the root cause of the microtubule network abnormality and its maladaptive effects and that \( \beta \)-adrenergic blockade would prevent or reverse its occurrence. The data in the present study, generated in our animal model of severe RV pressure overloading wherein persistent microtubule network densification (51, 52) and persistent \( \beta \)-adrenergic overdrive (14) have been well documented, support this hypothesis.

**METHODS**

**RV pressure overload hypertrophy.** This was induced as before (15, 47) by placing a 3.2 mm internal diameter band around the proximal pulmonary artery of adult cats of either sex weighing 3.0–3.8 kg after the induction of anesthesia with ketamine HCl (10 mg/kg im) and meperidine (2.2 mg/kg im). This produced severe RV pressure overloading, with an approximate tripling of RV systolic pressure (Table 1). The LV including the interventricular septum of these pulmonary artery band (PAB) cats served as a same-animal normally loaded control, and the control cats were sham-operated by transiently passing a band around the proximal pulmonary artery. Under the same anesthesia at terminal study 4 wk later, the cats were characterized in terms of the measures given in Table 1. All operative procedures were done under full surgical anesthesia; all procedures and the care of the cats were in accordance with institutional guidelines, which met or exceeded those of the American Physiological Society and the American Association for Accreditation of Laboratory Animal Care and were approved by the Institutional Animal Care and Use Committee.

**Cardiac \( \beta \)-adrenoceptor blockade.** Because increases in both RV mass and RV cardiomyocyte microtubule network density stabilize by 2 wk after PAB placement (49), for the period of 2 wk through 4 wk after surgery there was either no further treatment in half of the PAB cats or treatment by pilling with the nonselective \( \beta \)-adrenergic blocker propranolol HCl extended release (40 mg po b.i.d.) in the other half of the PAB cats. Given recent data suggesting that both the \( \beta_1 \)- and \( \beta_2 \)-adrenergic receptor subtypes contribute to nonlocalized cAMP signaling in the failing heart (37), it was necessary to use a nonselective agent. The following protocol allowed us to determine whether our \( \beta \)-adrenergic blockade regimen provided cardiac \( \beta \)-blockade throughout the treatment cycle. As described before in this setting (14), the adequacy of \( \beta \)-adrenergic blockade was assessed by maximally challenging the cats with a \( \beta \)-adrenoceptor agonist (continuous infusion of isoproterenol, 1.5 mg·kg\(^{-1} \)·min\(^{-1} \)) iv) during ketamine anesthesia (10 mg/kg im) at terminal study 12 h after the last dose of propranolol and immediately before the full anesthesia used for the terminal studies noted above. The percent heart rate increase from baseline in the control group during isoproterenol infusion was 75 ± 13%; it was 72 ± 11% in the untreated PAB cats, and it was 26 ± 7% in the chronically \( \beta \)-blocked PAB cats.

**Cardiomyocyte isolation.** Feline cardiomyocytes were isolated separately from the RV and LV of cats as described before (32). After completion of the hemodynamic studies, the cats were heparinized (1,000 units iv) and placed on oxygen. A left thoracotomy was performed, the pericardium was opened, and the heart was rapidly removed, placed in cold buffer solution, and weighed. The aorta was then cannulated, and the coronary arteries were perfused retrograde for 10 min first with a recirculating buffer solution of the composition of (in mmol/l) 130.0 NaCl, 4.8 KCl, 1.2 MgSO\(_4\), 1.2 NaH\(_2\)PO\(_4\), 4.0 NaHCO\(_3\), 0.5 CaCl\(_2\), 10.0 HEPES, and 12.5 glucose; second with a nonrecirculating buffer of the same composition but without supplemental calcium; and third with a recirculating calcium-free buffer supplemented with type II collagenase (155 units/ml). Buffer perfusion was terminated when the heart was flaccid. The heart was removed from the cannula, the RV was carefully dissected from the heart, and the RV and remaining myocardium were weighed separately. The LV free wall then was dissected from the heart and weighed; the remaining myocardium was discarded. Cardiocytes from the RV and LV free walls were then isolated and used as described previously (47, 51) or they were maintained for 1 h at 37°C and pH 7.4 in collagenase-free 2.5 mmol/l Ca\(^{2+} \)-buffer before defining contractile function.

**Cardiomyocyte adenosine infection.** Freshly isolated and plated adult feline cardiomyocytes were adenosinized in Pipers medium (2 ml/35-mm plate) at a multiplicity of infection (MOI) of 1 for 24 h. The cells were then rinsed in serum-free medium and incubated for a further 48 h to permit transgene expression. Parallel control cultures were infected at the same MOI with Ad-\( \beta \)-Gal. Over a MOI range of 1–100 plaque forming units/cell of Ad-\( \beta \)-Gal, there was after 48 h 90% cardiomyocyte infection as determined by immunochemistry. The feline cardiomyocyte studies here, cells were infected 48 h earlier at an MOI of 1 either with Ad-\( \beta \)-Gal, an adenovirus encoding bacterial \( \beta \)-galactosidase as a control or with AdKDPak1, an adenovirus encoding kinase-dead Pak1-K299R as a competitive inhibitor of Pak1 activity. AdKDPak1 was a generous gift from Q. Liang (33).

**Total, free, and polymerized tubulin.** Myocardial protein fractions were prepared as before (50). For the total tubulin fraction, the myocardium was homogenized in 1% SDS buffer containing (in mM) 10 Tris-HCl (pH 7.4), 0.5 DTT, and 1 Na\(_3\)VO\(_4\), boiled for 5 min, and centrifuged at 16,000 \( g \), 4°C for 10 min; this supernatant was saved as the total protein fraction. For immunoblotting, an equal amount of protein as determined by bicinchoninic acid (BCA) assay (Pierce Biotechnology) was loaded in each lane. For the free tubulin heterodimer and polymerized tubulin (microtubule) fractions, the myocardium was homogenized in a microtubule stabilization buffer containing 50% glycerol, 5% DMSO, 10 mM Na\(_2\)HPO\(_4\), 0.5 mM EGTA, and 0.5 mM MgSO\(_4\) and centrifuged at 100,000 \( g \), 25°C for 20 min. The supernatant was saved as the tubulin heterodimer fraction. The pellet was resuspended in 1% SDS buffer 4°C, boiled for 10 min, and centrifuged at 16,000 \( g \) for 10 min. This supernatant was saved as the tubulin microtubule fraction. For immunoblotting, to compare the amount of tubulin in the heterodimer versus microtubule fraction of a given sample, an equal proportion of the two fractions from the same sample was applied. For between-sample comparisons, an equal BCA-based amount of protein from each was applied. The blots were incubated for 1 h with the primary antibody. After blots were incubated with peroxidase-labeled secondary antibody, specific protein bands were detected by using horseradish peroxidase in conjunction with enhanced chemiluminescence (Perkin-Elmer). To derive semiquantitative data from these blots, National Institutes of Health Image J software was used to provide background-corrected integrated optical density values from scanned images of lightly exposed films.

**Total, non-phospho-ser-924 MAP4, and non-phospho-ser-1056 MAP4.** For MAP4, myocardial samples were homogenized in a high-salt buffer of 100 mM Tris-HCl (pH 7.4), 10 mM EGTA, and 0.35 M NaCl containing a protease inhibitor cocktail (No. P8340; Sigma), phosphatase inhibitor cocktails 1 (No. P2850; Sigma) and 2 (No. P5726; Sigma), and 1 mM DTT; immediately put on ice for 20 min; boiled; and centrifuged at 16,000 \( g \), 4°C for 30 min. SDS-PAGE was carried out on the supernatants with equal BCA-based protein loading for each sample using a precast 3–8% gradient Tris-acetate gel (NuPAGE; Invitrogen). Separated proteins were transferred to polyvinylidene difluoride membranes (Immobilon; Millipore); incubated overnight with a 1:1,000 dilution of our antibodies to MAP4, by 10.220.33.1 on April 4, 2017 http://ajpheart.physiology.org/ Downloaded from
well as antibody specificity in each case (Fig. 4, C and D, in Ref. 10), were given previously (10).

PP1 activity. To examine this, we took advantage of the fact that autophosphorylation of Pak1 at Threonine-423, a conserved threonine in the activation loop, induces and is essential for Pak1 activity (28, 45). Furthermore, it is known that multiple hypertrophic agents and growth factors activate myocardial Pak1 as determined by phosphorylation of Pak1 at Threonine-423 (27). Therefore, we examined both total and active Pak1 via cellular or myocardial immunoblots prepared with an antibody to total Pak1 (anti-Pak1-sc-881; Santa Cruz Biotechnology), an antibody to total Pak1 (No. 2602S; Cell Signalling), and an antibody to active Pak1 (anti-pThr423 Pak1-No. 2601S; Cell Signalling). Note that the epitope that the total Pak1 antibody recognizes is at a different location than the phospho-threonine antibody; therefore the total Pak1 blot includes all Pak1 regardless of phosphorylation status. For tissue immunoblots, samples from control and PAB cats were extracted with lysis buffer of 50 mM Tris-HCl (pH 7.4), 150 mM NaCl containing 1% protease inhibitor cocktail (No. P8340; Sigma); phosphate inhibitor cocktails 1 (No. 2850; Sigma) and 2 (No. 5726; Sigma), and 1 mM DTT; 20 mg of the protein fraction was used for Pak1 and p-Pak1 immunoblotting. For cellular immunoblots, protein was collected using lysis buffer of 100 mM Tris-HCl, 10 mM EGTA, 2 mM NaCl, 1 mM Na3VO4, and 2% Triton X-100 (pH 7.4) containing a protease inhibitor cocktail (Catalog No. P8340; Sigma) and phosphate and protease inhibitor cocktails 1 (Catalog No. P2850; Sigma) and 2 (Catalog No. P5726; Sigma), as well as 2 mM of calpain inhibitor E-64. Cells were scraped, homogenized, and centrifuged at 10,000 rpm for 10 min at 4°C. The supernatants were transferred into separate tubes containing SDS sample buffer, after which they were vortexed and boiled for 3 min. Equal amounts of protein were loaded into each lane, and SDS-PAGE was carried out using a precast 10% tris-acetate gel (NuPAGE; Invitrogen). A Fisher recombinant protein ladder was used as a protein size standard (BP3603; Fisher). Separated proteins were transferred onto a polyvinylidene difluoride membrane (Invitrogen), blocked in 2% BSA, and incubated overnight with a 1:200 dilution of a rabbit polyclonal anti-pThr423Pak1 antibody (Catalog No. 2601; Cell Signalling Technology), a 1:1,000 dilution of a rabbit polyclonal anti-Pak1 antibody (Catalog No. 2602; Cell Signalling Technology), or a 1:20,000 dilution of a mouse monoclonal anti-GAPDH antibody (Catalog No. 10R-G109a; Fitzgerald) as a loading control. After this, blots were incubated with a 1:10,000 dilution of either anti-rabbit IgG (Catalog No. WB401B; Promega) or anti-mouse IgG (Catalog No. WB402B; Promega), both conjugated to horseradish peroxidase, and visualized via enhanced chemiluminescence.

PP2A activity. This was determined as reported elsewhere (22) using a PP2A immunoprecipitation phosphatase assay (No. 17-313; Upstate Biotech) that measures free phosphate with a malachite green substrate (750 mM; KRP, TIRR). Following incubation for 10 min at 30°C in a shaking incubator, the reaction mixture was centrifuged briefly and the supernatant was transferred to a 96-well microtiter plate. The reaction was terminated by the addition of malachite green phosphate detection solution for 10–15 min at room temperature, and free phosphate was quantified by measuring the absorbance of the mixture at 650 nm using a microplate reader. This was determined as reported elsewhere (41) using the PSP Assay System (No. P0780S; New England Biolabs). Total protein from the RVs and LVs of control and PAB cats was extracted with lysis buffer of 50 mM Tris-HCl, 1% Triton X-100, and 150 mM NaCl (pH 7.4) containing a 1% protease inhibitor cocktail (No. P8340; Sigma); 10 ml of protein fraction was used for the phosphatase assay. The assays were performed in a 50-ml buffer of 50 mM Tris-HCl, 0.1 mM Na3EDTA, 5 mM DTT, 5 mM caffeine, and 0.01% Brij 35 (pH 7.0) at 30°C. The PP1 activity was measured with 32P-labeled myelin basic protein as a substrate in the presence of either 4 nM okadaic acid to block PP2A activity or 1 mM okadaic acid to block both PP1 and PP2A activity (7, 57). Thus PP1 activity was calculated from okadaic acid-sensitive protein phosphatase activity by deducting phosphatase activity with 1 mM okadaic acid from activity with 4 nM okadaic acid. The reaction was initiated by adding 10 ml of 32P-labeled myelin basic protein as a substrate and incubated at 30°C for 10 min. The reaction was terminated by the addition of 200 ml of 20% trichloroacetic acid, cooled on ice for 5 min, and centrifuged; 200 ml of the supernatant was used to count the released 32P in the assay.

Immunofluorescence/confocal microscopy. The cells were incubated at 4°C overnight in a 1:200 dilution of primary antibody in 2% normal donkey serum in PBS. After being washed three times with PBS, the cells were incubated at room temperature for 2 h in a 1:200 dilution of fluorescent-conjugated secondary antibody in 2% normal donkey serum in PBS. Optical sections (0.1 mm) were acquired using a Zeiss LSM510 META confocal microscope equipped with a 30 W Argon laser (458, 477, 488, and 514 nm), a 1 mW Helium-Neon laser (543 nm), a 5 mW Helium-Neon laser (633 nm), and a Plan-Apochromat 63X/1.4 objective to obtain high resolution images. Adobe Photoshop 7.0 software was used for superimposing the laser channels and for cropping and rotating images. To derive semiquantitative data from confocal microscope graphs, the channel of interest was converted to grayscale, the “Lasso” tool in Adobe Photoshop 7.0 software was then used to outline the cell boundary, and the mean pixel intensity within this boundary was found using the “Histogram” tool.

Sarcomere mechanics. Sarcomere motion in isolated cardiomyocytes was studied using the IonOptix system as used before (26) (IonOptix, Milton, MA). Freshly isolated cardiomyocytes in 2.5 mM Ca2+ buffer were placed in a custom-made double-jacketed temperature-controlled study chamber that was positioned on an inverted Olympus IX70 microscope (Olympus America, Melville, NY). Temperature was maintained constant at 37 ± 0.1°C by a M3 Lauda water bath (Lauda Wobser, Germany). Two opposing platinum electrodes were mounted in the study chamber to deliver 0.25-Hz, 5-ms DC pulses of alternating polarity at 20% greater than threshold. The cardiomyocyte image that was collected by a 40X objective lens (Olympus) was diverted to a video charge-coupled device MyoCam camera (IonOptix). The charge-coupled device camera was adapted to acquire images at 240 Hz with a sarcomere length measurement time resolution of 4.2 ms. Calibration of the system was performed before each experiment using a standard micrometer slide having a 2 mm line spacing. Sarcomere dynamics were recorded by specialized data acquisition and analysis software (SoftEdge and IonWizard; IonOptix). The signal-to-noise ratio was significantly improved by averaging 10 sequential contraction runs. Only cardiomyocytes with the following characteristics were recorded: single rod-shaped cells that were unattached to any adjacent cell that contracted in response to each electrical stimulus and that were quiescent between stimuli. Standard

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video edge-detection techniques and Fast Fourier Transform spectral analysis were then used to isolate a well-defined sarcomere pattern in the cell of interest. When after 5 to 10 contractions the extent of shortening was stable, 10 contractions were sampled and averaged to improve signal-to-noise ratios and to yield a final profile of sarcomere length versus time during one cell contraction. Absolute twitch amplitude was measured as the difference between systolic sarcomere length and resting sarcomere length. Shortening velocities of sarcomere length transients were determined by numerical time differentiation.

Data analysis. Data were expressed as means ± SE; any statistical comparisons are specified in the legends of the table and of the individual figures.

RESULTS

Effects of systemic β-adrenergic receptor blockade during severe pressure overload hypertrophy on components of the cardiac microtubule array. As seen in Table 1, a band of the diameter used here reliably produces within 4 wk severe RV hypertrophy (47) but not RV failure. Overt myocardial failure with pleural effusion and ascites, as well as abnormally elevated RV end-diastolic pressure and arteriovenous O2 difference, are only seen with tighter banding (47). However, microtubule network densification and cellular contractile dysfunction are characteristic of the level and duration of RVH (47, 51, 52) seen in this study. Indeed, at 2 wk after PAB, when β-blockade was initiated in the PAB + β-blockade group, the amount of RVH (49) and the extent of microtubule changes are already at a new steady state that continues for the subsequent 2 wk (51). Note that throughout this study, including here, propranolol treatment initiated at the midpoint of the 4-wk PAB period, when the hypertrophic growth is complete and the abnormal microtubule phenotype is well established, returns the cytoskeletal changes toward but not to baseline. However, the residual difference is generally not statistically different. In simplest terms, this residual difference may well exist because a competitive β-antagonist is being used and perhaps also because factors other than adrenergic input have a role in this pathophysiology.

For the two cats whose data are shown as an example in Fig. 1A, the RV systolic pressure was increased from our normal value of 25 ± 2 mmHg to 60 mmHg for the untreated PAB cat and to 65 mmHg for the β-blocked PAB cat treated with propranolol, a nonselective β-adrenergic blocker. In both cats, PAB produced a robust hypertrophic response: our RV/body weight value for normal cats is 0.58 ± 0.02 g/kg; for the untreated PAB cat this value was 1.3 g/kg, and for the β-blocked PAB cat this value was 1.2 g/kg. The most obvious differences in Fig. 1A between the non-β-blocked and β-blocked cats are major reductions in the hypertrophy-induced increases in RV polymerized tubulin and MAP4 in the latter. These conclusions are born out in Fig. 1, B–D, by summary data from these and additional cats whose characteristics are given in Table 1. Despite similar RV pressure overload and a similar hypertrophic response in the two groups of cats, β-blockade attenuated the hypertrophy-induced increase in free tubulin and especially in polymerized tubulin and MAP4.

Linkage of β-adrenergic stimulation to Pak1 activation in vitro. Figure 2A shows that the nonselective β-adrenergic agonist isoproterenol causes increased phosphorylation of Pak1 at Thr889-423, an activation loop threonine whose phosphorylation induces and is required for Pak1 activity (28, 45). Because we are postulating here a linkage between β-adrenergic activation of Pak1 and excess cardiomyocyte microtubules, it is important to note that this effect is present both acutely at 30 min and at the much later time point of 48 h, such that persistent cardiomyocyte β-adrenergic receptor stimulation leads to persistent Pak1 activation. This persistence of Pak1 activation is especially pertinent in the context of our previous in vivo data, which showed ongoing Pak1 activation in hypertrophied myocardium at the latest time point examined, i.e., 10 wk after severe pressure overloading (Fig. 1 in Ref. 7). This isoproterenol effect is blocked by propranolol, which, in turn, has no independent effect on Pak1 activation. Finally, as would be expected (Fig. 1B in Ref. 33), there is no β-agonist or β-antagonist effect on total Pak1.

Note further that Fig. 2A shows that exposure of these cells to forskolin, which increases intracellular cAMP and thus protein kinase A via β-adrenoceptor-independent direct adenyl cyclase activation, also causes an increase in active, phos- 
pho-Thr-423 Pak1. This would suggest that Gs-dependent adrenergic signaling may be at least as important to Pak1 activation as the currently postulated primacy of Gi-dependent adrenergic Pak1 signaling (27). This distinction is important, since the in vivo portion of the present study is predicated on the idea that any beneficial effects of β-blockade on Pak1 activation and downstream cytoskeletal changes are based on blockade of the primarily β1-adrenergic, Gi-dependent effects of the excessive circulating norepinephrine found in heart failure (5, 9). We therefore did the experiment in isolated cardiomyocytes shown in Fig. 2B in an effort to clarify this situation. Figure 2B shows that isoproterenol and forskolin cause a similar increase in activated, phos- 
pho-Thr-423 Pak1. Pretreatment with the β1-selective antagonist ICI-118,551 did not affect the isoproterenol-induced increase in phos- 
pho-Thr-423 Pak1, but pretreatment with the β1-selective antagonist CGP 20712 reduced the isoproterenol-induced increase in phos- 
pho-Thr-423 Pak1. Pretreatment with pertussis toxin, which impairs Gi protein interaction with β-adrenoceptors, did not affect the isoproterenol-induced increase in phos- 
pho-Thr-423 Pak1, suggesting that activation is via the β1-adrenorecep-

Table 1. Characteristics of the pulmonary artery band models

<table>
<thead>
<tr>
<th>Pulmonary Artery Band</th>
<th>Control</th>
<th>No Treatment</th>
<th>β-Blockade</th>
</tr>
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<tbody>
<tr>
<td>n</td>
<td>7</td>
<td>11</td>
<td>9</td>
</tr>
<tr>
<td>RV systolic pressure, mmHg</td>
<td>24.9 ± 1.7</td>
<td>80.4 ± 4.9*</td>
<td>72.4 ± 7.3*</td>
</tr>
<tr>
<td>Body weight, kg</td>
<td>3.5 ± 0.2</td>
<td>3.4 ± 0.1</td>
<td>3.4 ± 0.1</td>
</tr>
<tr>
<td>Arteriovenous O2 difference, ml/l</td>
<td>36.4 ± 4.0</td>
<td>41.1 ± 2.3</td>
<td>39.6 ± 2.6</td>
</tr>
<tr>
<td>RV weight/body weight, g/kg</td>
<td>0.58 ± 0.02</td>
<td>1.26 ± 0.03*</td>
<td>1.19 ± 0.04*</td>
</tr>
<tr>
<td>Left ventricle weight/body length, g/cm</td>
<td>0.17 ± 0.01</td>
<td>0.33 ± 0.01*</td>
<td>0.34 ± 0.01*</td>
</tr>
<tr>
<td>Liver weight/body weight, g/kg</td>
<td>8.6 ± 1.3</td>
<td>31.5 ± 1.3</td>
<td>27.7 ± 1.1</td>
</tr>
<tr>
<td>Heart rate, beats/min</td>
<td>191 ± 7</td>
<td>195 ± 6</td>
<td>155 ± 3°*</td>
</tr>
</tbody>
</table>

Values are means ± SE. After a one-sample Kolmogorov-Smirnov test showed that the data comprising each of these values were normally distributed, parametric statistical comparisons were made via one-way ANOVA followed by Scheffé’s S procedure. RV, right ventricle. *P < 0.01 for difference from control; †P < 0.01 for difference from No Treatment.
tor. Figure 2C shows that when a subsaturating concentration of isoproterenol was used, pertussis toxin pretreatment greatly enhanced Pak1 phosphorylation at Threonine-423, indicating that Pak1 is activated through α1-adrenoceptors. In summary, these findings in quiescent adult cardiomyocytes from a large mammal show that the nonselective β-adrenergic agonist isoproterenol causes phosphorylation and thus activation of Pak1 primarily through the α1-adrenoceptor and its downstream protein kinase A activity rather than through the β2-adrenoceptor and its downstream phosphoinositide-3-kinase activity (23).

Specificity of β-adrenergic-Pak1 linkage to tubulin polymerization in vitro and effect of systemic β-adrenergic receptor blockade during severe pressure overload hypertrophy. A: example immunoblots from the right ventricles (RVs) and left ventricles (LVs) of a pair of cats with 4 wk of pulmonary artery band (PAB)-induced RV hypertrophy; 1 animal was β-blocked with propranolol for the final 2 wk, and the other was not β-blocked. A monoclonal anti-β-tubulin antibody (clone DM-1B; Abcam) was used for the tubulin blots, our anti-myocardial microtubule-associated protein (MAP)4 antibody (43) was used for the MAP4 blot, and a monoclonal anti-GAPDH antibody (clone 6C5; Upstate Biotech) was used for the background-corrected integrated optical density (I.O.D.) ratio of RV to LV in immunoblots from the indicated numbers of normal control cats, PAB cats without β-blockade, and PAB cats with β-blockade. *P < 0.05 for difference from control; †P < 0.05 for difference from PAB by 1-way ANOVA with Bonferroni post hoc analysis. For the LVs alone, there was no within-group difference for any of these 3 variables by 1-way ANOVA with Bonferroni post hoc analysis (P = 0.69 for LV free tubulin; P = 0.43 for LV polymerized tubulin; P = 0.29 for LV MAP4; each P value is the minimum value for the 3 comparisons within that group).

Fig. 1. The effects of β1- and β2-adrenergic receptor blockade on components of the cardiac microtubule network during pressure overload hypertrophy. A: example immunoblots from the right ventricles (RVs) and left ventricles (LVs) of a pair of cats with 4 wk of pulmonary artery band (PAB)-induced RV hypertrophy; 1 animal was β-blocked with propranolol for the final 2 wk, and the other was not β-blocked. A monoclonal anti-β-tubulin antibody (clone DM-1B; Abcam) was used for the tubulin blots, our anti-myocardial microtubule-associated protein (MAP)4 antibody (43) was used for the MAP4 blot, and a monoclonal anti-GAPDH antibody (clone 6C5; Upstate Biotech) was used for the background-corrected integrated optical density (I.O.D.) ratio of RV to LV in immunoblots from the indicated numbers of normal control cats, PAB cats without β-blockade, and PAB cats with β-blockade. *P < 0.05 for difference from control; †P < 0.05 for difference from PAB by 1-way ANOVA with Bonferroni post hoc analysis. For the LVs alone, there was no within-group difference for any of these 3 variables by 1-way ANOVA with Bonferroni post hoc analysis (P = 0.69 for LV free tubulin; P = 0.43 for LV polymerized tubulin; P = 0.29 for LV MAP4; each P value is the minimum value for the 3 comparisons within that group).

Turning from in vitro to in vivo data, and starting upstream at Pak1, the immunoblots in Fig. 3C show that in the pressure-overloaded RV versus the normally loaded same-animal control LV from a PAB cat there is a modest increase over the control cat in the quantity of Pak1 but a greater increase in Pak1 activity as estimated from phosphorylation of Pak1 at Thr423. The right two lanes of these immunoblots show that in a β-blocked PAB cat each of these increases is greatly attenuated by β-adrenergic blockade with propranolol. Thus, these in vivo data are consistent with the in vitro data shown in Fig. 2, A and B.
Effects of systemic β-adrenergic receptor blockade during severe pressure overload hypertrophy on events downstream from Pak1 activation. Looking next at downstream type 1 and type 2A phosphatase activity, the data in Fig. 4 show that there is a doubling of the activity of both PP2A and PP1 at 4 wk after PAB when comparing the pressure-overloaded RVs with the normally loaded same-animal control LVs. These data are similar to those we reported recently for this model of pressure overload hypertrophy.
overload hypertrophy (Fig. 5 in Ref. 7). However, what is uniquely shown here is that each of these increases in phosphatase activity is greatly attenuated by β-adrenergic blockade with propranolol.

In our study using mass spectrometry to characterize site-specific MAP4 phosphorylation status in hypertrophied myocardium (10), we found MAP4 dephosphorylation at Ser-924 and Ser-1056 in the assembly-promoting region of the COOH-

Fig. 3. Pak1 specificity of β-adrenergic signaling through active Pak1 to increased microtubules in vitro and β-adrenergic blockade of this effect in vivo. A and B: AdKDPak1 infection of normal feline cardiomyocytes, which doubled the amount of total Pak1 in these cells, prevented isoproterenol induction of both Pak1 activation (A) and tubulin polymerization (B). Data similar to these were gathered in additional experiments. C: these immunoblots showing total and active Pak1 were prepared from myocardial homogenates from the RVs and LVs of 4-wk PAB cats with and without β-adrenergic blockade with propranolol for the final 2 wk. For 3 experiments such as that shown in this panel, the denstometric ratio of RV to LV Pak1 was 1.83 ± 0.06 for the untreated PAB cats and 1.33 ± 0.03 for the β-blocked PAB cats. For the ratio of RV to LV p-Pak1, the values were 1.81 ± 0.37 for the untreated PAB cats and 1.26 ± 0.14 for the β-blocked PAB cats. For the 3 panels in this figure, a polyclonal anti-Pak1 antibody (No. 2602S; Cell Signaling) was used for the Pak1 blots, a polyclonal anti-pThr-423Pak1 antibody (No. 2601S; Cell Signaling) was used for the p-Pak1 blots, a monoclonal anti-β-tubulin antibody (clone DM-1B; Abcam) was used for the tubulin blots, and a monoclonal anti-GAPDH antibody (No. 10R-G109a; Fitzgerald) was used for the loading control blots.

Fig. 4. Myocardial activity of protein phosphatase 2A (PP2A) and protein phosphatase 1 (PP1). Myocardial homogenates for these assays were prepared from the same animals as those used to prepare the data in Fig. 1. PP2A activity was measured using an immunoprecipitation assay kit (No. 17-313; Upstate Biotech) as described in the METHODS. PP1 activity was determined using the PSP Assay System (No. P0780S; New England Biolabs), as is also described in the METHODS. *P < 0.05 for difference from control; †P < 0.05 for difference from PAB by 1-way ANOVA with Bonferroni post hoc analysis.
terminal microtubule binding domain. Ser-924 dephosphoryla-
tion and to a lesser extent Ser-1056 dephosphorylation in-
creased MAP4 binding to microtubules (Fig. 7 in Ref. 10). We 
therefore looked here as shown in Fig. 5 yet further down-
stream from the data shown in Fig. 4 at MAP4 Ser-924 and 
Ser-1056 dephosphorylation, since this appears to be the major 
determinant of MAP4-microtubule affinity and thus microtu-
bule network stability and density (10). It is clear from the data 
in Fig. 5 that pathological hypertrophy-associated MAP4 de-
phosphorylation at Ser-924 and Ser-1056 is largely prevented 
by β-adrenergic blockade with propranolol.

Effects of systemic β-adrenergic receptor blockade during 
severe pressure overload hypertrophy on cardiomyocyte mi-
crotubule network density and contractile function. The con-
focal micrographs in Fig. 6 show that the hypertrophy-associ-
ated increase in cardiomyocyte microtubule network density 
following RV pressure overloading is largely prevented by 
concurrent β-adrenergic receptor blockade. Likewise, although 
the PAB cats did not yet exhibit overt right heart myocardial 
failure, the sarcomere mechanics data in Fig. 7 show that 
although the contractile function of normally loaded LV car-
diomyocytes is unaffected by β-adrenergic receptor blockade, 
the striking decrease in RV cardiomyocyte contractile function 
seen after severe RV pressure overloading is prevented by 
concurrent β-adrenergic receptor blockade. Thus, not only the 
structural hypertrophy-associated microtubule network densi-
fication but also its functional consequence of cellular contrac-
tile dysfunction are prevented by β-adrenergic receptor block-
ade.

DISCUSSION

Why was it important to do this study? The impetus for this 
study was to discover the root cause of hypertrophy-dependent 
microtubule network densification and the means for its pre-
vention or reversal. This was not simply because of its intrinsic 
scientific interest. Instead, and as a matter having major trans-
lational potential, apart from microtubule depolymerization we 
are unaware of anything other than acute pharmacological 
inotropic stimulation that has anything as dramatic an effect in 
reversing the contractile dysfunction of pressure-hypertrophied 
myocardium in vivo or of its constituent cardiomyocytes in 
vitro as that shown in Figs. 3–5 of our earlier study of 
microtubule depolymerization in canine aortic stenosis (30), a 
chronically progressive, clinically highly relevant model of its 
human analog (58).

Cause(s) of microtubule network densification-STAT3? An 
attractively simple candidate etiology for this cytoskeletal 
abnormality is signal transducer and activator of transcription 
3 (STAT3) inhibition of the microtubule destabilizing protein 
stathmin, where STAT3 stabilizes microtubules by binding to 
the carboxy-terminal tubulin-interacting domain of stathmin, 
thereby antagonizing its microtubule destabilizing activity 
(35). Indeed, there is transient STAT3 tyrosine phosphoryla-
tion after acute pressure overloading of the LV in the rat (39), 
rabbit (34), and mouse (56), as well as more persistent STAT3 
phosphorylation in association with a stabilized microtubule 
network in murine LV hypertrophy and failure (36). However, 
in the same feline RV pressure overload model used in our 
earliest work on the cardiac cytoskeleton (51), in much of our

![Fig. 5. Non-phospho-Ser-924 and non-phos-
pho-Ser-1056 MAP4. Example immunoblot 
above and summary immunoblot data below 
from the RVs and LVs of control cats and of 
cats with 4 wk of PAB-induced RV hyper-
trophy are shown; the animals were either 
β-blocked with propranolol for the final 2 wk 
or they were not β-blocked. A: example im-
munoblot and summary data prepared using 
our anti-non-phospho-Ser-924 MAP4 anti-
body (10). B: example immunoblot and sum-
mary data prepared using our anti-non-phos-
pho-Ser-1056 MAP4 antibody (10). For the 2 
immunoblots, Coomassie Blue staining of 
corresponding gels was used to verify equal 
protein loading. *P < 0.05 for difference 
from control; †P < 0.05 for difference from 
PAB by 1-way ANOVA with Bonferroni 
post hoc analysis.](http://ajpheart.physiology.org/)

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subsequent work in this area (11), and here, although there is transient STAT3 recruitment to the cytoskeleton at 2 days after pressure overloading (54), this is no longer significant by 1 wk after pressure overloading when the characteristic indefinitely persistent dense microtubule network is just beginning to form (49). Thus it is not clear that STAT3 has a causal role in producing and more especially in maintaining the hypertrophy-related dense cardiac microtubule network.

Cause(s) of microtubule network densification—MAP4 binding to microtubules? Apart from this specific problem with a STAT3-dependent mechanism, a more general and crucial problem is that any unifying explanation for the hypertrophy-dependent microtubule phenotype must account not only for increased αβ-tubulin heterodimers and microtubules (51, 52) but also for increased MAP4 and MAP4 decoration of microtubules (43). In thinking about this, especially given the prevalent control of microtubule stability and thus network density in interphase cells by the family of structural MAPs (16), the microtubule-MAP abnormality seen in Alzheimer’s disease provided an important insight, since microtubule network density and stability, and its regulation, are just the opposite of what we see in pathological cardiac hypertrophy. Thus, in Alzheimer’s disease a neuronal MAP, Tau, dissociates from microtubules and forms characteristic neurofibrillary tangles of paired helical filaments (25). This is caused by hyperphosphorylation of Tau, especially at sites within the microtubule-binding domain, which reduces its affinity for microtubules (53). Specifically, phosphorylation of the single human Tau Ser-262 residue within the KXGS motif of the first microtubule binding domain repeat essentially abolishes microtubule binding of Tau (4), thus destabilizing the microtubule network. In direct contrast, when we used mass spectrometry to examine site-specific phosphorylation of native MAP4 from hypertrophied myocardium, we found striking dephosphorylation of feline MAP4 Ser-924 (10). This serine residue, which is within the KXGS motif of the first microtubule binding domain repeat of MAP4, is homologous to human Tau Ser-262. In an etiologically critical experiment, when we expressed a dephosphomimetic Ser-924 → Ala MAP4 mutant in normal cardiomyocytes, all of the features of the microtubule network seen in pathological pressure overload hypertrophy were reproduced (10).

Does site-specific MAP4 dephosphorylation produce a dense, stable microtubule network? Because this persistent site-specific MAP dephosphorylation in hypertrophied myocardium increases MAP4-microtubule affinity, this finding could explain both the extensive MAP4 decoration of microtubules and their stabilization (43). Furthermore, as we have discussed elsewhere (10), the increased synthesis of the tubulins and MAP4 that we observe (43, 49) could well be caused by the loss of negative feedback control when the hyperstabilized microtubules and their associated MAP4 are effectively isolated from their ordinarily dynamic intracellular pools. The question then becomes that of what is controlling MAP4 Ser-924 phosphorylation status. Turning again to the analogy with regulation of microtubule stability in Alzheimer’s disease, decreased activity of PP2A appears to be primarily responsible for increased phosphorylation of human Tau Ser-262 (17, 21) in that setting. We therefore asked whether a directionally opposite phosphatase dysregulation from that seen in Alzheimer’s disease could be responsible for the site-specific MAP4 dephosphorylation that appears to be responsible for microtubule stabilization in pathological cardiac hypertrophy. Our recent data indicate that such is the case, since increased activity of the predominant myocardial protein phosphatases, both that of PP1 and especially that of PP2A, is a striking and persistent feature of severe pressure overload hypertrophy (7). Finally, in asking what is responsible for persistent activation of PP2A in this setting, the observation...
that activity of an upstream multifunctional enzyme, Pak1, is increased in early cardiac hypertrophy (33) provided another important insight, since in our hands Pak1 activity is persistently increased in severe pressure overload hypertrophy and overexpression of Pak1 in normal cardiomyocytes reproduces the hypertrophic microtubule phenotype (7).

Is activated Pak1 etiologically upstream of increased phosphatase activity? Of interest in this context, it would appear that Pak1 activation is protective against short-term toxic effects of doxorubicin on isolated neonatal cardiomyocytes (33). Although the following potential etiology for this Pak1 protective effect was not investigated in that study, doxorubicin has striking microtubule disrupting effects in isolated neonatal cardiomyocytes (40), and this microtubule disruption may be acting through effects on MAP4 (3, 20). Thus, in a setting where a toxin is disrupting microtubules, the microtubule stabilizing pathway initiated by Pak1 activation of phosphatases may be protective, whereas in a hypertrophied cardiomyocyte with contractile and transport abnormalities, Pak1 induction of microtubules in excess of a normal baseline is deleterious.

Does increased β-adrenergic activity reproduce the signaling to an endpoint of a dense microtubule network? The fact that increased β-adrenergic activity is regularly seen in pathological cardiac hypertrophy, including this model of severe RV pressure overloading (14), together with the recent observation that β-adrenergic agonists cause Pak1 activation (33), and that adenosine inhibits the cardiac hypertrophy-dependent microtubule changes (19), likely through its antiadrenergic effects (12), led to the present study; that is, in the setting of this knowledge, we hypothesized and then showed here that chronic β-adrenergic blockade would prevent the abnormal microtubule network from forming during pathological cardiac hypertrophy.

Can β-adrenergic blockade prevent this? The data in the present study, which was framed in terms of the schema shown in Fig. 8, strongly support this idea. Thus chronic β-adrenergic blockade prevents the increases in free tubulin, microtubules, and MAP4 that otherwise occur quite reliably and predictably in the feline RV pressure overload model that we employed here. Furthermore, our in vitro data in Fig. 2 show that β-adrenergic stimulation activates Pak1 and that active Pak1 causes microtubule polymerization, whereas the balance of the
in vivo data in this study shows that β-adrenergic blockade suppresses the ongoing activity of each member of the signaling cascade that we have identified (7) as causing and maintaining via increased phosphatase activity site-specific MAP4 dephosphorylation and consequent microtubule network MAP4 binding, as well as network stabilization and densification (10). Indeed, by establishing in this and the two preceding studies (7, 10) a signaling cascade originating in the β1-adrenergic overdrive characteristic of pathological cardiac hypertrophy that leads to microtubule network densification, we have delineated a basic mechanism that helps to explain the counterintuitive observation that β-adrenergic blockade is beneficial to the failing heart even when characterized by a reduced inotropic state.

Is this an exclusive mechanism? It should be clear that the data in this study do not constitute a general mechanism explaining the entirety of the well-established beneficial effects of chronic β-adrenergic blockade in pathological myocardial states (38), which are likely based to some extent on preventing the more general cytotoxic cardiomyocyte effects such as we initially observed with sustained β-adrenergic stimulation (32). As an example, β-blockade-mediated reductions in phosphatase activity could increase Ca2+ cycling protein phosphorylation, thus enhancing inotropism via effects on the rate and amplitude of the Ca2+ transient (41). Instead, the data reported here are but one specific, concrete example of the more general improvement in biological function of the cardiac myocyte by β-blockade predicted some time ago (18). Furthermore, and as an explicit example of settings to which these findings do not apply, it is known that there is myocardial microtubule depolymerization in the immediate postischemic period (42), such that prevention of microtubule polymerization by β-blockade cannot explain its beneficial effects in this setting (1). It is nonetheless true that a significant portion of chronic clinical heart disease eventuates as high wall stress pressure overload hypertrophy, and in our hands both in patients (58) and in multiple animal models (12) of this entity the pathological microtubule phenotype is quite characteristic. Furthermore, apart from this rather specific hemodynamic setting that we have studied, other investigators have identified increased microtubules more generally in advanced clinical heart disease (2, 24, 31).

**Conclusion**

In this context, given the deleterious effects of a dense, MAP4-decorated microtubule network on myocardial contractile and transport function (12), might the prevention of these functional abnormalities favorably impact the natural history of those cardiac diseases in which they would be expected to occur? In terms of practical implications framed within the outlined shown in Fig. 8, where β1- but not β2-adrenergic input initiates Pak1-driven microtubule network densification, this is the most interesting question raised by this entire series of studies of the cardiac cytoskeleton originating in 1993 (51), since it is entirely possible that chronic β-adrenergic blockade instituted early might delay or prevent the appearance of the pathological microtubule phenotype.

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**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the author(s).

**AUTHOR CONTRIBUTIONS**


**Fig. 8.** Signaling pathways mediating β-adrenergic effects on the cardiac microtubule network. The nonselective β-agonist isoproterenol, which is structurally similar to adrenaline, activates both β1- and β2-adrenergic receptors (AR), but as shown by the data in Fig. 2 the predominant effects on Pak1 and then phosphatase activation are mediated through the β1-adrenoceptor-Gi pathway. The nonselective competitive β-antagonist propranolol blocks or reverses the activation of this same pathway. Feline MAP4 Serine-924 is homologous to human MAP4 Serine-914. PI3K, phosphatidylinositol-3-kinase.
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REFERENCES


