

**P21-Activated Kinase-1 Signaling in Myocardial Hypertrophy,
Ischemic Heart Disease and Remodeling**

BY

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THESIS

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To Michelle

To my Mother, Father, and Sister

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LIST OF ABBREVIATIONS

1D	One-Dimensional
2-D DIGE	2-D Difference In Gel Electrophoresis
ANOVA	Analysis of Variance
ATP	Adenosine Triphosphate
AT-II	Angiotensin II
Ca ²⁺	Calcium
CA	Constitutively active
CAMKII	Calmodulin-dependent Protein Kinase II
CO	Cardiac Output
cTnC	Cardiac Troponin C
cTnI	Cardiac Troponin I
cTnT	Cardiac Troponin T
CTRL	Control
ddH ₂ O	Double Distilled Water
DIGE	Difference in Gel Electrophoresis
EDT	Transmitral early filling deceleration time
EDV	End-Diastolic Volume
EF	Ejection Fraction
Erk	Extracellular signal-regulated kinase
ESV	End-Systolic Volume
FS	Fractional Shortening

LIST OF ABBREVIATIONS (continued)

FVB	Friend Virus B-Type
HE	Heterozygous
HF	Heart Failure
HR	Heart Rate
HR	High Relaxing Solution
ISO	Isoproterenol
IVRT	Isovolumic Relaxation Time
IVS	Inter-Ventricular Septum
JNK	Jun N-terminal Kinase
KO	Knockout
LA	Left Atrium
lacZ	Lac operon Z
LV	Left ventricular/ventricle
LVAWD	Left ventricular diastolic anterior wall thickness
LVIDd	End-diastolic LV Internal Dimension
LVIDs	End-systolic LV Internal Dimension
LVPWD	Left ventricular diastolic posterior wall thickness
MAPK	Mitogen activated protein kinase
MyBPC	Myosin binding protein C
MyBPC	Myosin binding protein C
NaN ₃	Sodium Azide

LIST OF ABBREVIATIONS (continued)

n_H	Hill coefficient
P38 MAPK	P38 mitogen-activated protein kinase
Pak-1	P21-activated kinase
pCa ₅₀	Negative Log of Molar Ca ²⁺ Concentration at Half Maximal Activation
pH	Negative Log of the Hydrogen Ion Concentration
pI	Isoelectric Point
PKA	c-AMP-dependent Protein Kinase
PKC	Protein Kinase C
PLB	Phospholamban
PMSF	Phenylmethylsulfonyl Fluoride
PP2A	Protein phosphatase 2A
PWT	Posterior Wall Thickness
RV	Right Ventricle
SDS	Sodium Dodecyl Sulfate
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
SEM	Standard Error of the Mean
Ser	Serine
SERCA	Sarcoplasmic Reticulum Ca ²⁺ -ATPase
SL	Sarcomere Length

LIST OF ABBREVIATIONS (continued)

TBST	Tris-Buffered Saline and 0.1% Tween
TG	Transgenic
Thr	Threonine
TM	Tropomyosin
Tn	Troponin
WB	Western immunoblotting
WT	Wild type

SUMMARY

The main aim of this doctoral dissertation is to understand the functional role of p21-activated kinase-1 (Pak-1) and its role in the development of cardiomyopathies. Recent and emerging data indicate that novel signaling cascades in the heart involve 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, as well as long term function and remodeling. 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.

Earlier investigations in our lab indicated an anti-adrenergic effect induced by activation of Pak-1 and an associated activation of protein phosphatase 2A (PP2A). The activation of PP2A by Pak-1 results in the dephosphorylation of myofilament proteins and increased myofilament Ca^{2+} sensitivity. Also, emerging evidence indirectly indicates a role for Pak-1 in ischemia/reperfusion (I/R) injury, but direct evidence is lacking.

Objective of the Studies

The overall governing hypothesis is that activation of p21-activated kinase-1 is beneficial and protective against development of hypertrophy,

SUMMARY (continued)

ischemia/reperfusion injury, and potentially in post-ischemic cardiac remodeling.

In this thesis, the specific aims address the following questions:

1. Is Pak-1/PP2A a signaling cascade which controls stress-induced cardiac growth?
2. Does Pak-1/PP2A/Erk1/2 represent an important signaling cascade controlling β -adrenergic stress-induced cardiac growth?
3. Is activation of the Pak-1 signaling pathway a cardioprotective mechanism that prevents or reverses the detrimental effects of ischemic injury?
4. Is Pak-1 signaling involved in post-ischemic cardiac remodeling?

Pak-1 in 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. 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

SUMMARY (continued)

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 Ca²⁺-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. In conclusion, our data indicate that active Pak-1 is a natural inhibitor of Erk1/2 and a novel anti-hypertrophic signaling molecule upstream of PP2A.

Pak-1 in Ischemia/Reperfusion Injury and Post-Ischemic Remodeling

We subjected *ex vivo* hearts from WT and Pak-1-KO mice to 20 min of global cardiac ischemia followed by 30 min of reperfusion. In the absence of Pak-1, there was an exacerbation of the increased end diastolic pressure and reduced left ventricular developed pressure occurring after I/R injury. ProQ analysis revealed an increase in TnT phosphorylation at baseline in Pak-1-KO hearts compared to WT. Significantly decreased myosin light chain 2 (MLC2) phosphorylation in Pak-1-KO hearts compared to WT after I/R injury was

SUMMARY (continued)

confirmed by Western immunoblotting. These data indicate that Pak-1-KO hearts have reduced recovery of myocardial performance after global I/R injury concomitant with changes in troponin-T and MLC2 phosphorylation. Finally, a protein-protein association between Pak-1 and MLC2 was determined by co-immunoprecipitation. These data indicate that Pak-1-KO hearts have reduced recovery of myocardial performance after global I/R injury concomitant with changes in troponin-T and MLC2 phosphorylation. In a separate set of experiments, compared to WT controls, *in vivo* hearts from Pak-1-KO mice subjected to ligation of the left anterior descending coronary artery for 10 weeks develop significantly reduced global systolic and diastolic function as assessed by transthoracic echocardiography, as well as significantly reduced compensatory left ventricular mass, indicative of impaired cardiac remodeling. At the end of the 10 weeks, mortality was significantly higher in the Pak-1-null mice group versus WT, as assessed by Kaplan-Meier survival analysis. Thus, results of our study provide a basis for targeting a novel pathway including Pak-1 in the therapies for patients with ischemic events.

Overall Conclusions

Pak-1 signaling is protective in multiple disease models (isoproterenol-induced hypertrophy, acute global I/R injury, and left anterior descending coronary artery ligation). Our studies provide a novel target for future investigations of the molecular signaling during stress.

I. INTRODUCTION

A. Implication of p21-Activated Kinase-1 Signaling in Myocardial Hypertrophy, Ischemic Heart Disease and Remodeling

Pathological cardiac hypertrophy is the response of the heart to stress or disease such as hypertension, myocardial infarction or neurohormones. If untreated, pathological cardiac hypertrophy leads progressively to heart failure. Heart failure affects close to 5 million people in the USA and each year close to 500,000 new cases are diagnosed. In addition, more than 50% of patients seek re-admission within 6 months after treatment and the average duration of hospital stay is 6 days. Costs have been estimated to amount to more than \$35 billion in the United States ^{1, 2}. In 2006 there were 17.6 million cases of coronary heart disease (CHD), of which 8.5 million were cases of myocardial infarction (MI), or heart attack. This resulted in 425,400 deaths due to CHD, of which 141,500 were due to MI, according to the AHA website. In the clinical setting, when patients experience myocardial infarction, reperfusion injury occurs when coronary flow is re-established resulting in reduced myocardial mechanical function. However, despite a vast number of studies, the signaling mechanisms identified so far have contributed little to the improvement of the clinical outcome of these patients. Although extensively studied, the signal transduction mechanisms that underlie cardiac hypertrophy and heart failure remain unclear. Currently, the drug treatments for heart failure focus on inhibiting pathological hypertrophic pathways.

My thesis research focused on the understanding of the role of Pak-1 signaling in the regulation of both pathological and physiological cardiac hypertrophy using genetically engineered mice with altered Pak-1 levels in the heart. A main objective of experiments proposed here is to identify a role for Pak-1 in protection against the development of i) left ventricular (LV) myocardial hypertrophy and ii) myocardial ischemia/reperfusion injury and remodeling. P21-activated kinases (Paks) are a family of serine/threonine protein kinases that are widely expressed in mammalian tissues including the heart. Paks are activated by external stimuli that act through various cell surface receptors including G protein-coupled receptors and receptor tyrosine kinases. Upon activation, Paks modulate the activities of multiple downstream signaling pathways that control cell growth, shape, motility, survival, and death³. Thus, Paks appear to be positioned at a converging point in the intracellular signaling network that integrate signals from cell surface receptors and then transmit them to downstream effectors to achieve distinct functions. However, it is still largely unknown whether Paks play a physiological or pathological role in the heart. Although there is evidence that activation of Pak-1 stimulates PP2A, the specific mechanisms by which activation of the Pak-1/PP2A complex might participate in protection against in vivo LV myocardial hypertrophy, as well as the role of the Pak-1/PP2A complex in myocardial ischemia and reperfusion injury, remain incompletely understood.

We hypothesize that regulation of the Pak-1/PP2A signaling pathway promotes cardioprotective mechanisms that i) prevent or attenuate the development of LV myocardial hypertrophy, and ii) prevent or reverse myocardial dysfunction during ischemic myocardial injury. We will test this hypothesis using the following aims:

Aim #1. To determine the role of Pak-1 in regulating diverse signaling pathways that modulate progression to LV cardiac hypertrophy. This approach involves determination of the effects of Pak-1 regulation on cardiac function and its role in the activation of PI3K/Akt and Mitogen-Activated Protein Kinases (MAPKs) pathways in a Pak-1–KO murine model of LV cardiac hypertrophy. Past and current studies have highlighted that Pak-1 directly activated by Cdc42 and Rac1 is an important signaling molecule potentially involved in major cardiac processes regulating excitation and contraction in healthy and disordered hearts. Cell remodeling produced by Pak-1 and its upstream signals has suggested its potential role in cardiac hypertrophy and dilatation ⁴. However, the intracellular signaling mechanism underlying the cytoskeletal effect leading to pathological cardiac growth produced by Pak-1 remains uncertain.

AIM #2. To elucidate novel, Pak-1-mediated, upstream signaling mechanisms that regulate myocardial contractility after acute I/R injury. Studies in our laboratory have discovered a significant signaling cascade involving Pak-1 and PP2A. Emerging evidence indicates a role for Pak-1 in I/R, but the direct role of Pak-1/PP2A signaling in I/R injury in this process is poorly understood ⁵. The generation of a mouse model without Pak-1 expression provides a powerful tool

to investigate these questions. Understanding the role of Pak-1 activation and its downstream effects on contractility is critical for the development of pharmaceutical interventions for use in the clinic for patients with ischemic insults. Our experiments presented here have advanced the field by determining the effects of Pak-1 regulation on cardiac function and its role in the induction and regulation of several important pathways during acute and chronic myocardial ischemia. Our results demonstrate that Pak-1 is sufficient and necessary to prevent pathological hypertrophy. In addition, Pak-1 protects the heart from pathological remodeling. Our studies help to define the role of Pak signaling pathway in cardiac growth and survival, and suggest Pak-1 as a potential target for pharmacological intervention for treating cardiac hypertrophy and heart failure.

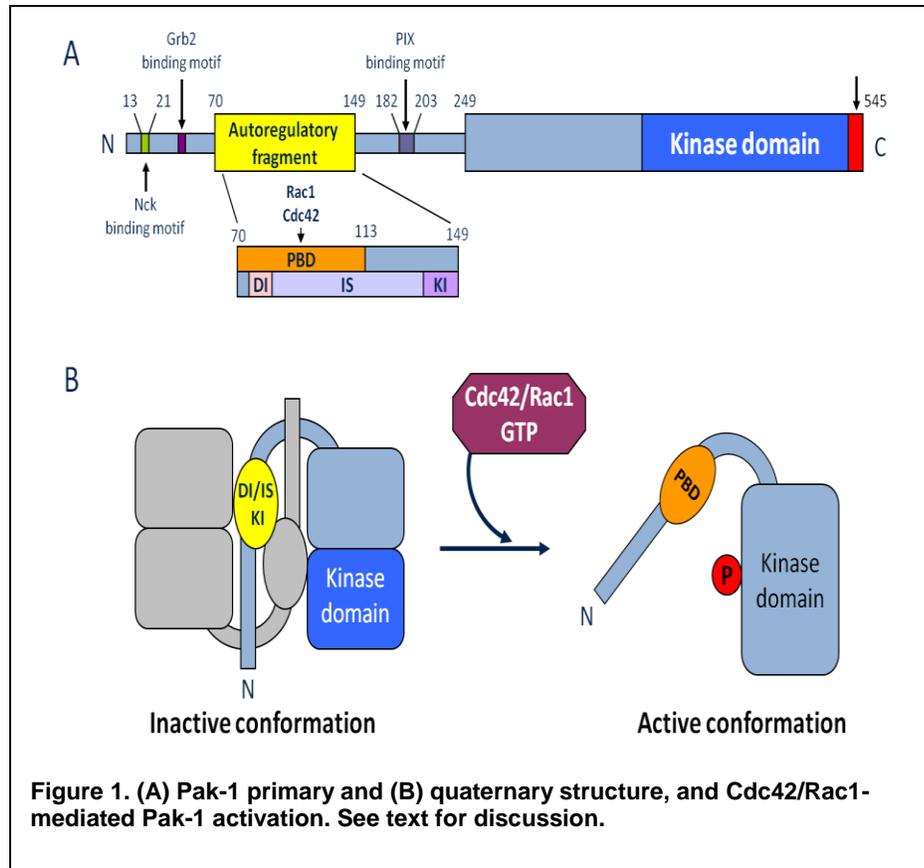
B. P-21 Activated Protein Kinase 1 (Pak-1)

P-21-activated kinase-1 belongs to a family of serine/threonine kinases initially identified as Rho-family GTPase binding partners^{6, 7}. GTP-bound Cdc42 and Rac were found to interact with three proteins of ~68, 65 and 62 kDa⁸. These proteins were subsequently identified as three isoforms of the p21-activated kinases, Pak-1 (α -Pak), Pak-2 (β -Pak) and Pak-3 (γ -Pak). These Group I Pak isoforms were later investigated by a number of studies as targets and effectors of Cdc42 and Rac^{6, 7, 9}. There are three other Pak isoforms, namely Pak-4, Pak-5 and Pak-6. The latter Pak isoforms, which belong to Group II Paks, share less homology in their catalytic domain with the Group I Paks¹⁰. For the

Group I Paks, highest expression of Pak-1 is observed in brain, muscle and spleen; Pak-2 is expressed ubiquitously while Pak-3 is primarily expressed in the brain^{8, 11}. For the less well studied Group II Paks, Pak-4 is expressed predominantly in prostate, testis and colon^{12, 13}; Pak-5 is highly expressed in the brain¹⁴, whereas Pak-6 was found to be expressed in kidney, prostate, brain, testis and placenta^{10, 15}. Investigations in knockout animal models indicate that members of the Pak family have unique biological functions during development. For example, *Pak-2*^{-/-} mice experience embryonic lethality due to multiple developmental abnormalities¹⁶. *Pak-4*^{-/-} mice also are embryonic lethal (they die at day 11.5) due to heart defects and neuronal abnormalities¹⁷. On the other hand, Pak-3 has a role in the regulation of long-lasting synaptic plasticity and cognition; *Pak-3* knockout mice show severe impairment of late-phase hippocampal long-term potentiation, a process that involves regulation of gene expression¹⁸. In contrast, *Pak-5*^{-/-} mice appear to develop normally and are fertile, and Pak-6 knockout mice have yet to be reported. Importantly, the biological role of Pak-1 has received more interest since results obtained from white blood cells showed that while *Pak-1*^{-/-} mice are viable, healthy and fertile, they carry defects in MAPK signaling that cause abnormal responses in immune cells¹⁹.

Among the six identified isoforms, Pak-1 is the most extensively studied member of Pak family. Pak-1 contains an N-terminal regulatory domain and a C-terminal catalytic domain (a.a 255-529), which is highly conserved among Group I Paks²⁰ (Figure 1). At least four known PXXP SH3-binding motifs were identified

in the regulatory region of Pak-1. The first motif is responsible for Pak-1 interaction with adapter protein Nck²¹, and the second motif interacts with adapter protein Grb2²². Interaction with these adaptor proteins suggests that upon growth factor stimulation, Pak-1 may be recruited to the plasma membrane and become active. Indeed, it has been reported that blocking the interaction between Pak-1 and Nck prevented Pak-1 activation²³, whereas targeting Pak-1 to the plasma membrane is sufficient to activate this kinase²⁴. Adjacent to the two proline-rich SH3-binding sites is the p-21 binding domain (PBD), containing elements responsible for interaction with activated Cdc42 and Rac. The PBD overlaps with an autoinhibitory domain (AID) that controls the basal catalytic activity of Pak-1^{20, 25}. In addition, there exists a binding site for the G $\beta\gamma$ subunit complex of heterotrimeric G proteins at the C terminal region²⁶.



Under basal conditions, Pak-1 exists in a trans-inhibited homodimeric conformation, where the N-terminal regulatory domain of one Pak-1 binds and inhibits the C-terminal catalytic domain reciprocally. The binding of activated GTPases such as Cdc42 and Rac induce a conformational change in Pak-1 dimerization, destabilizing the interaction between the AID and the catalytic domain, leading to autophosphorylation, events required for Pak-1 full catalytic activity²⁷. Seven autophosphorylation sites have been identified in activated Pak-1, Ser21, Ser57, Ser144, Ser149, Ser199, Ser204 and Thr423²⁸. Among these phosphorylation sites, the Thr423 residue was shown to be critical in sustaining Pak-1 in the autoinhibition-free conformation²⁷. Pak-1 activation can be triggered in a GTPase-independent manner as well. PDK-1 and Akt/PKB phosphorylate and activate Pak-1^{27, 29}. PDK-1 was shown to phosphorylate Pak-1 within its catalytic domain, at Thr423³⁰, which is important for full catalytic activity of Pak-1. Akt/PKB phosphorylates Pak-1 at Ser21. This modification is associated with reduced binding of Pak-1 to adaptor protein Nck, and an increase in Pak-1 mediated cell migration²⁹. Pak-1 has a host of biological functions including cytoskeletal organization and regulation, cell motility, neurogenesis, angiogenesis and has also been implicated in metastatic neoplasia²⁰. More than 30 Pak-1 substrates have been identified to date. We have reported that Pak-1 physically interacts with PP2a and localizes to the Z-disk, cell membrane, intercalated disc, and nuclear membrane of adult rat heart myocytes³¹. Some of the better characterized substrates include MLCK (myosin light-chain kinase), LIMK (LIM kinase), Bad, Raf and Mek1. MLCK and LIMK both play important

roles in actin polymerization, and actin and myosin interaction, which are events required for lamellipodia and filopodia formation, thus implicating Pak-1 function in the regulation of cytoskeletal dynamics and cell motility.

A prominent Pak-1 downstream target includes myosin light chain 2 (MLC2) Phosphoryl group transfer to MLC2 is controlled by a distinct cardiac myosin light chain kinase (cMLCK), whereas dephosphorylation is regulated by PP2A or a specific myosin phosphatase. Our lab has shown that ablation of MLC2 phosphorylation decreases ventricular power, lengthens the duration of ventricular ejection, and may also modify other sarcomeric proteins (e.g. cardiac troponins) as substrates for kinases and phosphatases³²⁻³⁴. In addition, myofilament calcium sensitivity increases when MLC2 is phosphorylated, a mechanism that may contribute to the improvement of myocardial contractility during ischemia and reperfusion injury. However, even though our knowledge on MLC2 regulation by upstream kinases and phosphatases in normal hearts is progressing, the significance of MLC2 phosphorylation in hearts subjected to ischemia and reperfusion injury has not yet been fully understood.

C. Mitogen-Activated Protein Kinase (MAPK)

Mitogen-activated protein kinases are serine/threonine protein kinases that respond to extracellular stimuli (mitogens, osmotic stress, heat shock and proinflammatory cytokines) and regulate various cellular activities, such as gene expression, mitosis, differentiation, proliferation, and cell survival/apoptosis³⁵. Erk1 and Erk2 were the first of the Erk/MAP kinase subfamily to be cloned. Other

related mammalian enzymes have been detected including: two Erk3 isoforms, Erk4, Jun N-terminal kinases/stress-activated protein kinases (JNK/SAPKs), p38/HOG, and p57 MAP kinases. The four best characterized MAPK subfamilies, Erk1/2, JNK, p38, and Erk5, are the targets of pharmacological and genetic manipulations to uncover their roles in cardiac development, function, and diseases:

1. extracellular signal-regulated kinases (Erk1, Erk2). The Erk1/2 (also known as classical MAP kinases) signaling pathway is preferentially activated in response to growth factors and phorbol ester (a tumor promoter), and regulates cell proliferation and cell differentiation.
2. c-Jun N-terminal kinases (JNKs), (MAPK8, MAPK9, MAPK10) also known as stress-activated protein kinases (SAPKs).
3. p38 isoforms (p38- α (MAPK14), - β (MAPK11), - γ (MAPK12 or Erk6) and - δ (MAPK13 or SAPK4)) Both JNK and p38 signaling pathways are responsive to stress stimuli, such as cytokines, ultraviolet irradiation, heat shock, and osmotic shock, and are involved in cell differentiation and apoptosis.
4. Erk5. Erk5 (MAPK7), which has been found recently, is activated both by growth factors and by stress stimuli, and it participates in cell proliferation.

MAPK pathway is organized in a way that a G-protein works upstream of three tiers of kinases, a MAPK kinase kinase (MAPKKK) which activates a MAPK kinase (MAPKK), that further activates MAPK ^{36, 37}. The MAPK components relevant to signaling are Ras as the G-protein, Raf as the MAPKKK,

mitogen/extracellular-signal regulated kinase kinase (Mek) as the MAPKK and extra cellular-signal regulated kinase (Erk) as the MAPK^{38, 39} Raf and Mek are components of the MAPK signaling pathway, regulating gene expression and promote cell growth and proliferation. It has been reported that Pak-1 phosphorylates Raf-1 on Ser338 and induces its catalytic activity. In addition, Pak-1 enhances the interaction between Raf-1 and its substrate Mek1 by phosphorylating Mek1 on Ser298 suggesting that Pak-1 has an imperative role in regulating cell growth and survival. Also, it is known that β -adrenergic receptor stimulation by isoproterenol (ISO) leads to a profound increase in Pak-1 activation and that β -adrenergic agonists promote cardiomyocyte hypertrophy via indirect interaction between Erk and beta-arrestin⁴⁰.

D. Activation of Mek/Erk

A focus of the studies presented here is the role of Pak-1 activation in signaling cascades modifying the response of the heart to stressors. One of these signaling pathways is the Ras/Raf/Mek/Erk cascade. Activated Raf proteins initiate a cascade of phosphorylation events. Raf activates Meks through the phosphorylation of Ser217/218 on Mek1 and Ser221 on Mek2, and Meks subsequently activate Erks. Meks are 43-46 kDa proteins belonging to a superfamily of dual-specific kinases, that share the ability to phosphorylate Erks on both is threonine and tyrosine residues. Mek1 and Mek2 activate Erk1 and Erk2 through phosphorylation of Thr183 and Tyr185, respectively [94, 103]. Mek1/2 contains a nuclear export signal (NES) sequence in its N terminal domain

that tethers them to the cytoplasm. Via binding interaction with Mek1/2, Erk1/2 is also mostly localized to the cytoplasm in quiescent state [104]. Erk1 and Erk2 are 44- and 42kDa ubiquitously expressed serine threonine kinases, and share 83% amino acid sequence identity with each other. Insulin stimulated phosphorylation and activation of Erk leads to its dissociation from Mek, and translocates to the nucleus. Erk has been shown to translocate into the nucleus by (i) simple passive diffusion [105], (ii) active transport by importin- β family proteins and low molecular weight GTPase Ran [106] or (iii) directly bind with the nuclear pore complex [107]. Subsequently, activated Erk targets over 50 substrates, some of which are key transcription factors, including activator protein 1 (AP-1), nuclear factor kappa-light-chain-enhancer of activated B cells (NFkB), Myc, kinases including ribosomal s6 kinase (RSK), B-cell lymphoma 2 (Bcl-2), and cytoskeletal scaffold proteins such as paxillin [108]. The activities of these Erk substrates then achieve the effects of insulin mediated cell growth and differentiation.

E. Regulation of MAPK Pathway

The three-tiered MAPK pathway can be regulated positively and negatively. Protein phosphatase 1 (PP1) and PP2A are both able to dephosphorylate 14-3-3 binding sites located in the Raf N terminal region, thereby promoting Ras interaction, and subsequent Raf membrane recruitment and activation⁴¹. Other regulators of the MAPK pathway are scaffold proteins that act as docking platforms to colocalize with Erk components to enhance its activation. For example, suppressor of ras-8 (Sur-8) augments Raf activation by

increasing the interaction between Ras and Raf³⁵. On the other hand, kinase suppressor of ras-1 (KSR1) facilitates Mek/Erk activation in a more complicated manner. Without stimulation, KSR1 interacts with 14-3-3, impedes mitogenic signal propagation (IMP), Mek1/2 and PP2A. The interaction with 14-3-3 masks the cysteine-rich C1 domain of KSR1 necessary for membrane targeting, hence sequesters KSR1 from the plasma membrane⁴². Upon IR activation, not only is IMP released from KSR1, C1 domain also becomes exposed, leading to the translocation of KSR1 to the membrane. Membrane-associated Ras then catalyzes the dephosphorylation of 14-3-3 binding sites on KSR1 leading to the dissociation of 14-3-3. This not only exposes the C1 membrane targeting domain of KSR1, but also the docking sites for Erk1/2, colocalizing Mek with Erk as well as other upstream MAPK signaling components, including Ras and Raf. Negative regulators of MAPK pathway operate by interfering with critical interactions between signaling components of the MAPK pathway. For example, Erbin sterically blocks the interaction between Ras and Raf by binding with Ras³⁵. Sprouty and Sprouty-related protein with EVH1 domains (SPRED) both interact with Raf at its catalytic domain, and interferes with the phosphorylation of Raf activating residues. Alternatively, Raf kinase inhibitor protein (RKIP) interacts with the kinase domain of Mek and Raf, and prevents the interaction between Raf and Mek that is required for MAPK activation⁴³.

F. Pak-1-PP2A-Mediated Phosphorylation and Dephosphorylation of Thin Filament Proteins

Our laboratory has been the first to demonstrate that Pak-1 activation has significant functional role in cardiac myocytes³¹. Early data have shown that Pak-1 mediates dephosphorylation of cTnI and myosin binding protein C while enhancing myofilament Ca^{2+} sensitivity. These effects of Pak-1 activation may be attributable to increased activity of phosphatase, most likely PP2A. Previously reported results indicate that the Ca^{2+} -sensitizing effects on myofilament tension occur by a different mechanism mediated by direct in vitro phosphorylation of cTnI by Pak-3. It is noteworthy that Pak-2, a ubiquitously expressed member of the Pak family of proteins, may also be expressed in heart. Pak-3 has been identified to be expressed in rat and pig hearts. cTnI-S150 has since been identified as a substrate for Pak-1⁴⁴. In vitro phosphorylation induces structural changes modifying the interaction of cTnI with cTnC, and an increase in response to Ca^{2+} ⁴⁵. Although there has been progress in understanding the kinases and phosphatases that control phosphorylation of TnI and TnT, signaling cascades controlling these mechanisms remain poorly understood. Moreover, site-specific dephosphorylation, which is likely to be an important mechanism, has been understudied and is poorly elucidated. The major phosphatases regulating thin filament protein phosphorylation are PP1 and PP2A. PP1 and PP2A are located in the proximity of the Z-discs, placing them in close proximity to the A- and I-band regions of the sarcomere and indicating that their localization may be strain-sensitive. Also, our laboratory has demonstrated that

tropomyosin phosphorylation decreases in hearts that express constitutively activated p38 MKK6bE and demonstrated co-localization of this MAPK with α -actinin at the Z-disc, as well as protein phosphatases (PP2 α and PP2 β)⁴⁶. Little information exists with regard to substrate specificities of PP2A and PP1; however, sufficient evidence indicates that PP1 has preference for cTnT over cTnI but with no effect on dephosphorylation of Tm⁴⁷. PP2A is an important mediator of induced cTnI dephosphorylation in preparations previously treated with both PKA and PKC. On the other hand, Ser23 and Ser24 were the preferred substrates for PP1⁴⁷. In the case of cTnT, Thr199 and an unidentified residue were the least favorable for dephosphorylation by PP1⁴⁸. Importantly, these studies taken together demonstrate that dephosphorylation is a site-specific, a concept often neglected when considering the integrated effects of signaling to the thin filaments. Finally, PKC ζ exists in a complex with Pak-1 and PP2A, as confirmed by immunoprecipitation and Western blotting. Our lab has also investigated the effects of constitutively active PKC ζ on myofilaments; our data concluded that a significant decrease in Thr phosphorylation of cTnI and cTnT occurs when PKC ζ T560E is expressed⁴⁹.

II. 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

A. 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^{31, 50}, 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^{31, 50}. 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^{31, 50, 51}. Moreover, bradykinin receptor-mediated activation of Pak-1 also induced effects similar to those obtained with CA-Pak-1 expression⁵². The mechanism for these anti-adrenergic effects of Pak-1 involves activation of protein phosphatase 2A (PP2A)⁵³. Pak-1 forms a *trans*-inhibited dimer that complexes with PP2A holoenzyme in cardiomyocytes and sinoatrial nodal cells^{31, 50, 52}.

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 overload⁵⁴. 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 fibrosis⁵⁵. 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 cardiomyopathy⁵⁶. 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 Erks⁵⁷. 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 overload⁵⁸. 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 activation⁵⁹, and β -adrenergic agonists

promote cardiomyocyte hypertrophy via indirect interaction between Erk and beta-arrestin⁶⁰. 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.

B. Methods

1. Pak-1-KO Mouse Model

All protocols were in accordance with the guidelines of the Animal Care and Use Committee of the University of Illinois at Chicago. Mice with Pak-1 gene disruption at both alleles were created as described previously⁶¹. Mice with Pak-1 gene disruption at both alleles were created in sv129 background^{61, 62} and were bred with WT, FVB mice purchased from Charles River Laboratories. Subsequently, Pak-1-KO mice were rederived in a FVB background in our animal facility at the University of Illinois at Chicago. The heterozygous F₁ mice from the breeding were inbred at the seventh generation of backcrossing to generate Pak-1-homozygous KO (-/-), Pak-1(+/-) and WT for Pak-1 (+/+). Pak-1-KO and the WT littermates were used in our experiments. In some experiments, purchased WT FVB mice were also included as WT controls. Echocardiographic measurements showed no significant differences between the purchased WT FVB mice and those obtained from inbreeding between (+/-) siblings. Mice were

genotyped to confirm the deletion of the Pak-1 gene, and the levels of protein expression of Pak-1, Pak-2 and Pak-3 (two less abundant protein isoforms of Pak in the heart) were measured in the LV cardiac tissue lysate by Western immunoblotting.

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 µg/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 µg/g/day of ISO, or iv) continuous subcutaneous administration of 25 µg/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 ⁶³. 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.

3. Surgical Procedure and Infusion System

Mice were initially anesthetized with 3% isoflurane and 100% oxygen inhaled in a closed anesthesia chamber. A plane of anesthesia for surgery was then regulated by delivery of 1% isoflurane administered through a nose cone with 100% oxygen. ALZET Osmotic Pumps (Cupertino, CA) delivered either ISO or saline subcutaneously after being placed in a small retroauricular subcutaneous pouch and secured with surgical metal clips. At day nine, mice were anesthetized and hearts were rapidly excised, rinsed in cold saline, flash-frozen in liquid nitrogen, weighed, and stored at -80°C until further analysis.

4. Transthoracic Two-Dimensional and M-mode and Pulsed Doppler Echocardiography

Induction and maintenance of anesthesia were performed as indicated above. Mice were placed in the dorsal decubitus position on a warming pad to maintain normothermia. Transthoracic two-dimensional, M-mode and pulsed Doppler images were acquired with a high-resolution echocardiographic system (VeVo 770, Visual Sonics, Toronto, ON, Canada) equipped with a 30-MHz mechanical transducer. Echocardiographic measurements were performed in all groups, before and eight days after miniosmotic pump implantation. All measurements were taken in compliance with the American Society of Echocardiography guidelines⁶⁴. Results were based on the average of at least three cardiac cycles.

5. 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.

6. 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^{31, 50}.

Western immunoblotting (IB) was performed using antibodies recognizing total Pak-1 (Cell Signaling Technology, Inc, anti-Pak-1 antibody, #2602), PP2A (Millipore, anti-PP2A, C subunit, clone 1D6, # 05-421), phospho-PP2A (Upstate biotechnology, anti-phospho-PP2A (Y307), # 05-547), total Erk1 (Abcam Inc., anti-Erk1 antibody [Y72], # ab32537), total Erk2 (Abcam Inc., anti-Erk2 antibody [5E8E1], # ab47701), total Erk1 + Erk2 antibody (Abcam Inc., anti- Erk1 + Erk2, # ab17942), phospho-Erk1 (Abcam Inc., anti-Erk1 (phospho T202 + Y204) antibody [EP197Y], # ab76299), phospho-Erk2 (ECM Biosciences, anti-Erk2 (Thr-188), phospho-specific, # EP4101), total p38 MAPK (Abcam Inc., anti-p38 MAPK antibody [M138], # ab31828), phospho-p38 MAPK (Abcam Inc., anti-p38 MAPK (phospho T180 + Y182) antibody [E229], # ab32557), phospho-JNK1+JNK 2+ JNK3 (Abcam Inc., anti-JNK1+JNK2 + JNK3 (phospho Y185 + Y185 + Y223) antibody [EP1597Y], # ab76572, total JNK (Abcam Inc., anti-JNK1+JNK2 antibody [279Q38], # ab37228)). Incubation with secondary antibodies was performed with peroxidase-conjugated antibody anti-rabbit IgG

(Sigma-Aldrich, # A0545) or peroxidase-conjugated antibody anti-mouse IgG (Sigma-Aldrich, # A2304). Detection occurred by chemiluminescence (Amersham ECL™ Western Blotting System, GE Healthcare).

Total proteins (1 mg) obtained from ISO-stimulated cardiac tissue lysate were incubated overnight with rabbit monoclonal total Pak-1 antibody (Cell Signaling Technology, Inc., anti-Pak-1 antibody, # 2602), or total Erk1/2 antibody (Abcam Inc., anti-Erk1 + Erk2 antibody, # ab17942), followed by incubation with protein G agarose beads (20 µl of 50% bead slurry) with gentle rocking for 3 hours at 4° C. Complexes were then precipitated and analyzed by SDS-PAGE. Brain lysate was used as a positive control for total Pak-1, Erk1/2, and PP2A. As a negative control, we used a cardiac lysate incubated overnight in the absence of primary antibody. Secondary antibody incubation was performed with mouse anti-rabbit IgG mAb (Conformation Specific) (Cell Signaling Technology, Inc., # L27A9), which recognizes the native rabbit IgG and does not recognize the denatured and reduced rabbit IgG heavy (about 50 kDa) or light (about 25 kDa) chains on Western immunoblotting. Data are representative of three separate experiments.

7. Isolation of Adult Mouse and Rat Cardiomyocytes

Adult ventricular myocytes were isolated as described previously⁶⁵. Adult cardiac ventricular myocytes were isolated from 20-30 g male or female FVB, WT mice and adult male 2-3 month old Sprague-Dawley rats as described previously⁶⁵. Rod-shaped, Ca²⁺-tolerant myocytes were counted and assayed for

viability by trypan blue exclusion assay. Myocytes were plated in M199 (Mediatech) and supplemented with 5 mmol/L creatine, 2 mmol/L L-carnitine, 5 mmol/L taurine (Sigma), 50 units of penicillin, and 50 units of streptomycin (Mediatech) at a density of 1.4×10^5 cells per 35-mm dish (Falcon) or a one-chambered slide (Nalge) coated with 15 μ g of mouse laminin (Invitrogen). After 2h of plating at 37 °C, 5% CO₂ unattached cells were removed by two washes with media, leaving only attached rod-shaped viable myocytes.

8. Immunofluorescence

Endogenous Pak-1 protein was visualized by immunofluorescence in isolated mouse cardiomyocytes using goat polyclonal antibodies to Pak-1 (Abcam Inc., anti-Pak-1 Antibody # ab77096) and chicken anti-goat IgG (H+L) secondary antibodies (Invitrogen™, Alexa Fluor® 488, # A-21467). Endogenous Erk1/2 protein (in red) was identified in isolated mouse cardiomyocytes using rabbit polyclonal Erk1 + Erk2 antibody and goat anti-rabbit IgG (H+L) secondary antibody (Invitrogen™, Alexa Fluor® 568, # A-11011).

9. AdPak-1 Construction, Viral Amplification, and Plaque Assays

AdPak-1 construction, viral amplification, plaque assays as well as adenoviral infection of adult rat cardiac myocytes have been described previously⁵¹. To prepare recombinant adenovirus that expresses CA Pak-1 (AdPak-1), we used the polymerase chain reaction method to tag the human Pak-1 cDNA with an HA epitope (YPYDVPDYA) at the N-terminal region and next to the translational initiation codon. Threonine 423 was mutated to glutamic acid in Pak-

1 cDNA to convert the Pak-1 protein into a CA form²⁸. The cDNA was cloned into a shuttle vector pAdCMV to obtain pAdCMVPak-1. The AdPak-1 was made by homologous recombination between pAdCMVPak-1 and the viral backbone DNA DL7001⁶⁶. The lysate of virus amplified from a clone was used to infect cells cultured in 100-mm dishes, and 5 to 10 large dishes (150 mm) were infected with the lysate from 100-mm dishes. The cells were harvested 28 to 35 h after infection. The lysates from the large dishes were applied at the top of a discontinuous CsCl gradient and centrifuged at 20 000 g for 2 h. The band containing virus was harvested and dialyzed against HBS buffer. Virus in the dialysis unit was used directly or mixed with storage buffer and stored at -80°C. The viral titer (plaque-forming units per ml) and multiplicity of infection (moi) were determined by standard techniques⁶⁷.

10. Adenoviral Infection of Adult Rat Cardiac Myocytes

Freshly isolated rat cardiac myocytes were plated in 100-mm tissue culture plates at room temperature in Tyrode's solution (pH 7.4) consisting of (in mmol/L) 0.5 CaCl₂, 132 NaCl, 4.8 KCl, 1.2 NaH₂PO₄, 1 MgSO₄, 10 HEPES, and 11 glucose. The cells were infected with AdPak-1 and incubated for 8 hours. In control experiments, we found that the expression of Pak-1 protein in cardiac myocytes began as early as 2 hours after infection with AdPak-1. The expression reached a maximum at 6 to 8 hours and remained at this level for >15 hours. Cell proteins were extracted with lysis buffer (50 mmol/L Tris, pH7.5; 150 mmol/L

NaCl; 0.1% Nonidet P40; Complete Protease Inhibitor Cocktail Tablets [Roche Molecular Biochemicals])^{31, 50}.

11. Preparation of Skinned Fibers

Left ventricular papillary muscles were dissected and detergent-extracted fiber bundles were prepared as previously described⁶⁸. Male and female mice weighing approximately 25 g and 3-4 month old were anesthetized using intraperitoneal injection of pentobarbital sodium (5-9 mg/100g). The chest was opened by bilateral thoracotomy, the heart was rapidly excised and rinsed in ice-cold relaxing solution (pH 7.0) composed of (in mmol/L) 10 EGTA, 41.89 K-Prop, 6.57 MgCl₂, 100 BES, 6.22 ATP, 5 Na Azide, and 10 creatine phosphate. The solution also contained 1 µg/ml leupeptin, 2.5 µg/ml pepstatin A, and 50 µmol/L phenylmethylsulfonyl fluoride. Left ventricular papillary muscles were dissected and fiber bundles were prepared as previously described⁶⁸. The fiber bundles were extracted overnight in relaxing solution plus 1% (vol/vol) Triton X-100 at 4°C. The fiber bundles were mounted between a force transducer and a stationary rod using glue. The sarcomere length was set to 2.2 µm using He-Ne laser diffraction⁶⁹. The width and diameter were each measured at three points along the fiber bundle. Force per cross-sectional area was used to determine tension. The fiber was initially contracted at a saturating calcium concentration (50 µmol/L) and sarcomere length was again adjusted to 2.2 µm. Sarcomere length remained constant throughout the rest of the experiment.

12. 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 previously⁷⁰. Myofibrils were purified from liquid nitrogen frozen mouse tissue and homogenized twice in standard relax buffer (10 mmol/L Imidazole pH 7.2, 75 mmol/L KCL, 16 mmol/L MgCl₂, 2 mmol/L EDTA, and 1 mmol/L NaN₃) with 1% (v/v) Triton X-100. Myofibrils were centrifuged and the supernatant was removed. The pellet was resuspended in UTC buffer (8 mol/L urea, 2 mol/L thiourea, 4% chaps) and protein concentration was determined with an RC DC assay kit (BioRad, Hercules, CA). Samples in UTC buffer (100 µg) were cleaned up with GE Healthcare's 2D clean-up kit (Piscataway, NJ) and then resuspended in UTC buffer. Since it is possible to separate up to three different samples within the same 2-D gel, two samples and an internal standard were included in every gel. Tissue samples were randomly labeled to receive either Cy3 or Cy5 to control for any dye differences, while the internal standard was labeled with Cy2. The CyDye™ DIGE Fluors Cy2, Cy3, and Cy5 were from GE Healthcare (Piscataway, NJ). Protein samples were labeled by adding 100 pmol of CyDye to 50 µg of protein and then quenched with 10 mmol/L L-lysine. The gel's first dimension was focused using the Protean IEF cell (BioRad, Hercules, CA) with pH 4-7 or 7-11 IPG strips. The samples were actively rehydrated at 50 V in 180 mm IPG strips pH 4-7, or 7-11NL 10-16 hrs with 90 µg (30 µg per channel) of total protein. The focusing in the first dimension was achieved using

a preset linear program: 250 V rapid ramping for 15 min, 10,000 V linear ramping for 3 hours, 10,000 V rapid ramping until 60,000 V•hours. The strips were equilibrated and laid onto 12% SDS-PAGE gels. Gels were imaged with a Typhoon 9410 scanned at 100 μ m and analyzed with PDQuest software (v7.1, BioRad, Hercules, CA). PDQuest software analyzes the gel images by determining the optical densities of each spot. The spot density was computed by dividing the density of a particular protein spot by the total of all the spot densities for that protein.

13. 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.

C. Results

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 (Figure 2A) and determined the levels of protein expression of Pak-1, Pak-2 and Pak-3 (two less abundant protein isoforms of Pak) in the heart (Figures 2B through 2E). 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.

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 3) and are reported in 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 β 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. 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 4A through 4C).

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 5A) 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 5B and 5C). 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 5B and 5D). 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 5E through 5G). 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.

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 (Figures 6A and 6B). In addition, Erk1/2 and PP2A also associate in the absence of Pak-1 (Figure 6C). Pak-1 and Erk1/2 co-localize in the adult mouse cardiac myocyte as demonstrated by immunofluorescence (Figure 6D).

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 (Figures 6E and 6F). 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.

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 PP2A⁵³. 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 5H and 5I). 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.

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 3) and are reported in 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 4A through 4C). 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 (Figure 7). These findings support the initial observation that Erk1/2 is a major kinase driving ISO-induced myocardial hypertrophy in the absence of Pak-1.

9. **Myofilament Response to Ca²⁺ 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 8A and 8B, 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_o ($p\text{Ca}50$) 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 ; $p = 0.99$).

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 existing 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 9A and 9B). 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 ⁷⁰. Results (Figures 9A and 9C) 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 8B and 8D) 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 9B and 9E) 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 8 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.

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 (Figures 10A through 10C). 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).

D. 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^{71, 72}. 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 PP2A³¹, which in turn dephosphorylates cTnI³¹, myosin-binding protein C³¹, phospholamban⁷³, inwardly rectifier potassium channels⁷⁴, and L-type Ca²⁺ channels⁷⁵. In the current study, we demonstrated association of Pak-1, PP2A, and Erk1/2 *in vivo*. Previous studies have found β -adrenergic stimulation in cardiomyocytes⁷⁶, HEK293 cells⁷⁷, COS-7 cells⁷⁸, and Chinese hamster ovary cells⁷⁹ 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 hypertrophy⁸⁰. 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 PP2A⁵⁹, and a role for PP2A has been shown in regulating Mek and Erk in a receptor-independent manner⁸¹. 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 11 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^{82, 83}. 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 phosphorylation⁸². However, others report Akt regulation of Pak²⁹, and yet others report that these two proteins may possibly regulate each other⁸³. 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. Our experiments demonstrated that phosphorylation of MAPK JNK1/2/3 were similar in all the models 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 p38⁸⁴. Yet our data contrast with studies that reported activation of JNK in COS-7 cells⁸⁵. Pak-1 and Pak-2 in HEK 293 cells induced activity of p38 MAPK but also JNK/SAPK⁸⁶. CA Pak-3 was also reported to activate JNK1 in COS1 cells⁸⁷. In that study, activated Cdc42 stimulated the activity of p38 MAPK, but was a less effective activator of Erk2⁸⁷. 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 pathway⁸⁵. 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. 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-3⁴⁴ and phosphorylation of cTnI by Pak-1³¹. However, we also reported that activation of PP2A by Pak-1 induces dephosphorylation of cTnI and myosin binding protein C³¹. 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^{44, 88}, 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 depress⁸⁹, whereas phosphorylation at S150 of cTnI is known to enhance myofilament Ca-sensitivity⁴⁴. 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 6).

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.

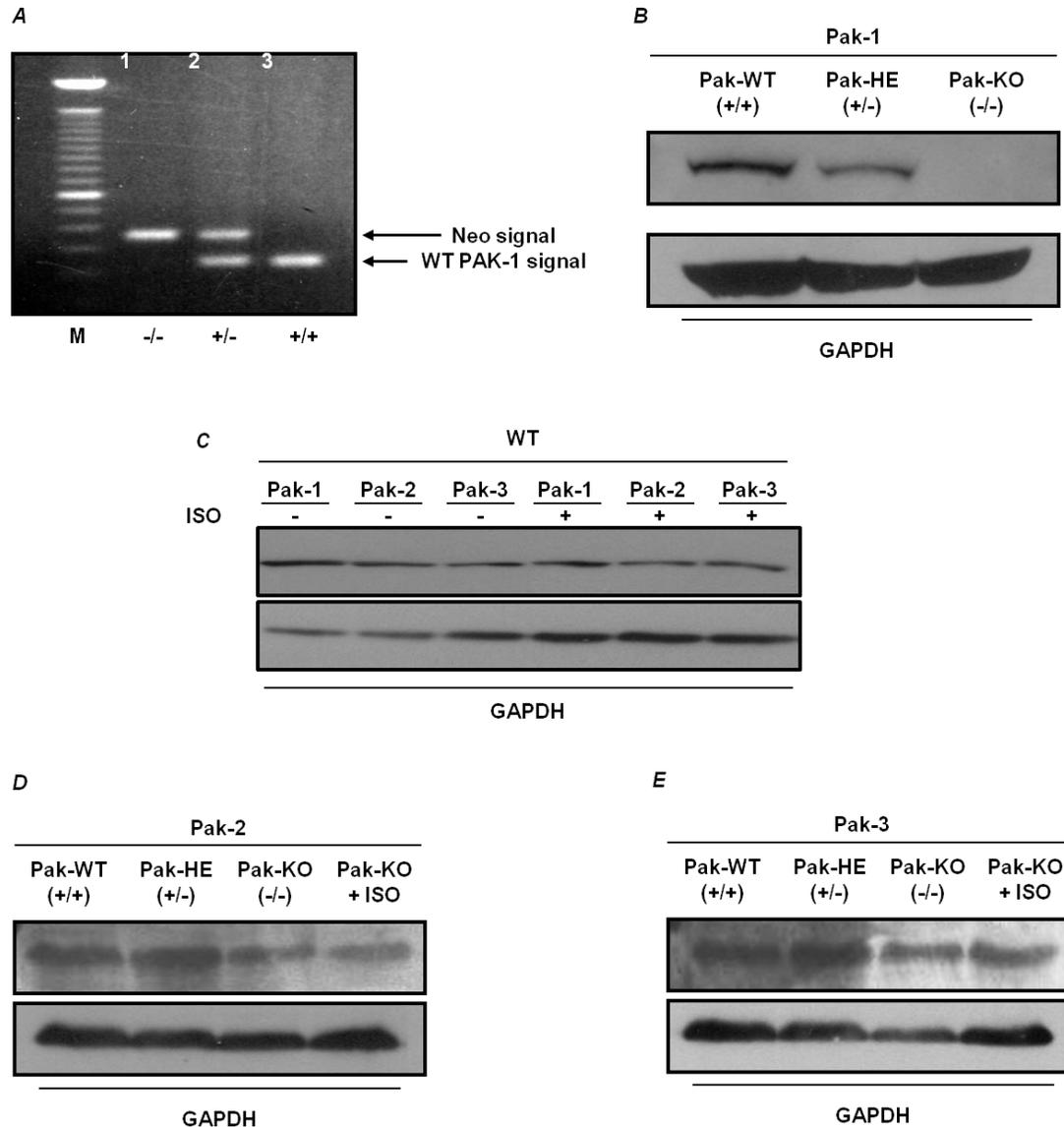


Figure 2. Genotyping of Pak-1-KO mice and expression levels of Pak-1, Pak-2, and Pak-3 protein isoforms in myocardial tissue lysate. A, PCR analysis indicates successful deletion of the Pak-1 gene in Pak-1-KO mice (-/-, lane 1). Genotypes for HE (+/-, lane 2) and WT (+/+, lane 3) mice are also illustrated. B, Western immunoblotting of Pak-1 protein obtained from WT, HE, and Pak-1-KO mouse LV cardiac tissue; Pak-1 expression is reduced by 50% in HE mice and is undetectable in Pak-1-KO mice. C, Levels of expression of Pak-1, Pak-2, Pak-3 in ISO-treated WT mice. D-E, Levels of expression of Pak-2 and Pak-3 (two less abundant Pak isoforms) are unaffected in WT, HE, and Pak-1-KO mice, in the presence or in the absence of ISO.

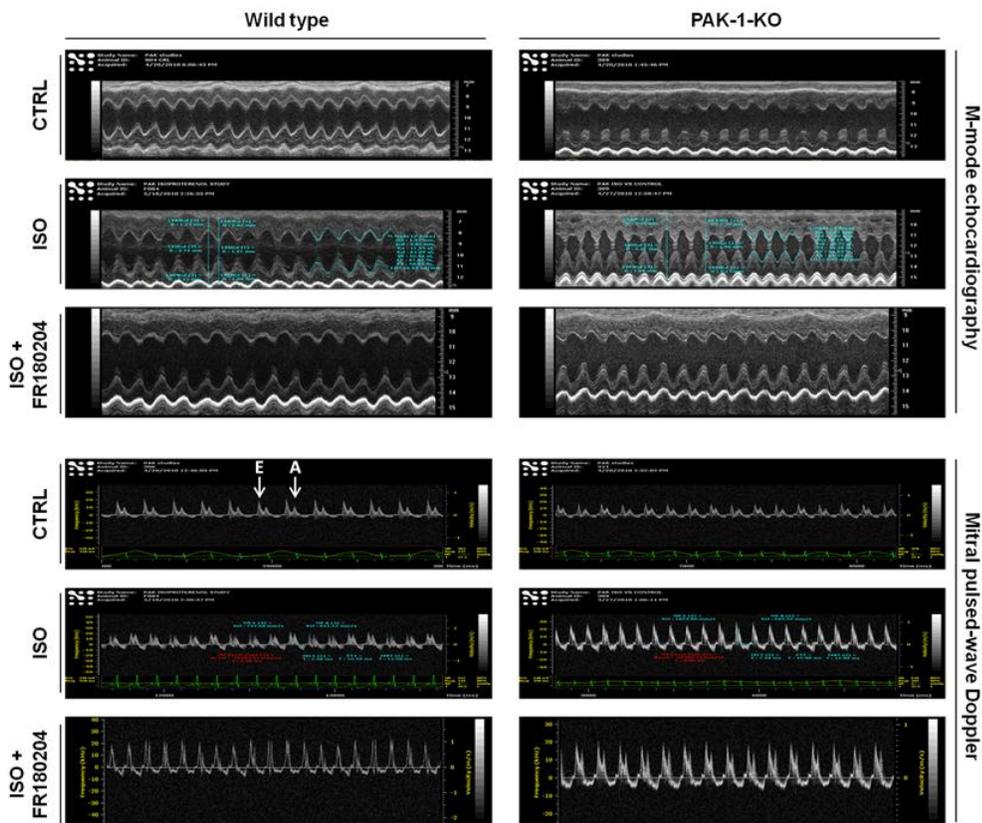


Figure 3. Cardiac hemodynamics. M-mode echocardiography obtained from the parasternal short axis view at the mid-papillary level (top), and mitral pulsed-wave Doppler recordings showing early (E) and late (atrial-A) ventricular filling velocity (bottom), in WT and Pak-1-KO mice, in the absence (CTRL) 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.

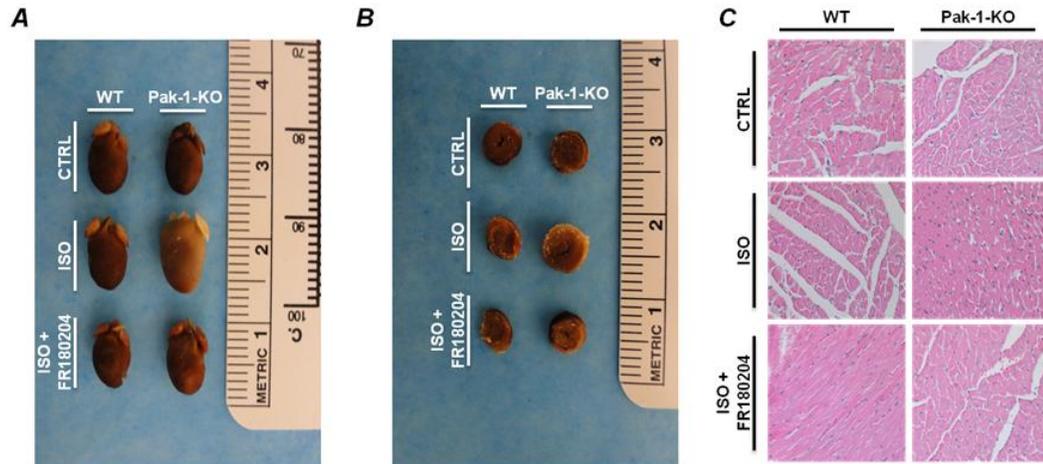


Figure 4. Gross anatomy and histology of WT and Pak-1-KO hearts. A, Gross anatomy of WT and Pak-1-KO hearts illustrating the increased cardiac mass in ISO-treated Pak-1-KO hearts (middle, right) compared to WT and Pak-1-KO hearts that received ISO or CTRL. ISO-treated Pak-1-KO hearts that received FR180204, 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.

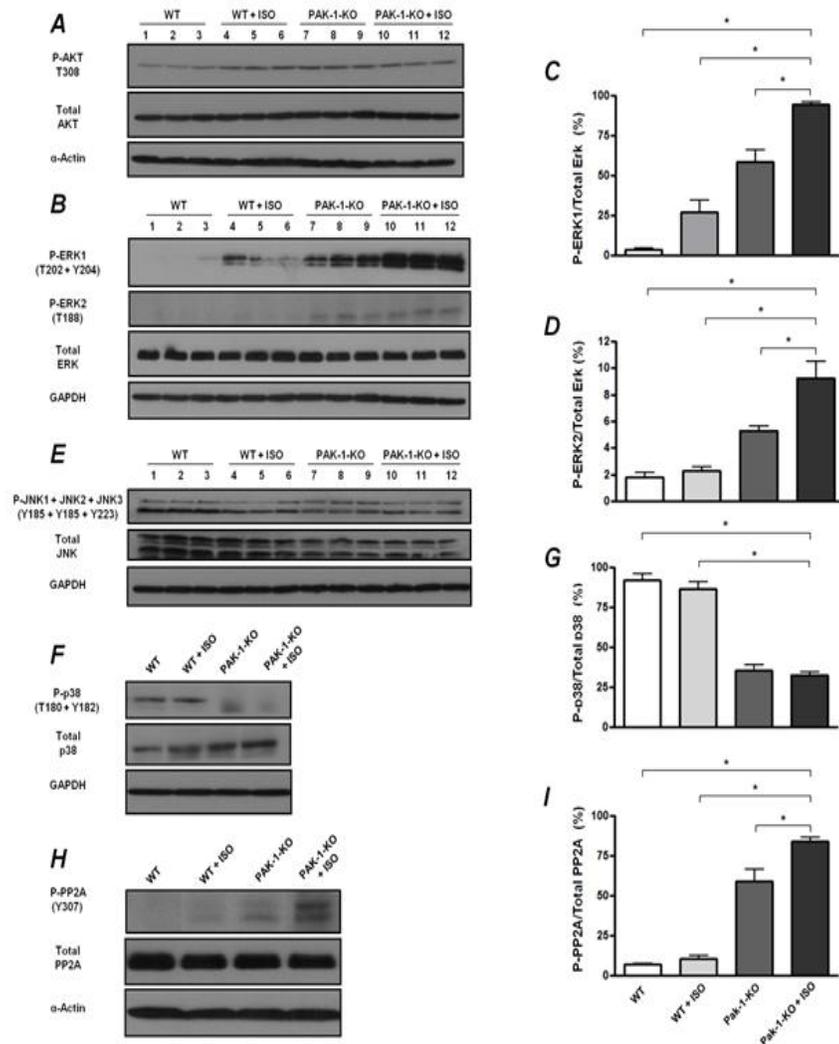


Figure 5. Western immunoblotting analysis of AKT, MAPKs, and PP2A indicate that cardiac hypertrophy is due to enhanced Erk1/2 activation in Pak-1-KO mice. A, AKT phosphorylation at T308 was not significantly different among any of the experimental groups. B-D, Western immunoblotting of Erk1/2 phosphorylation 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 $p < 0.05$.

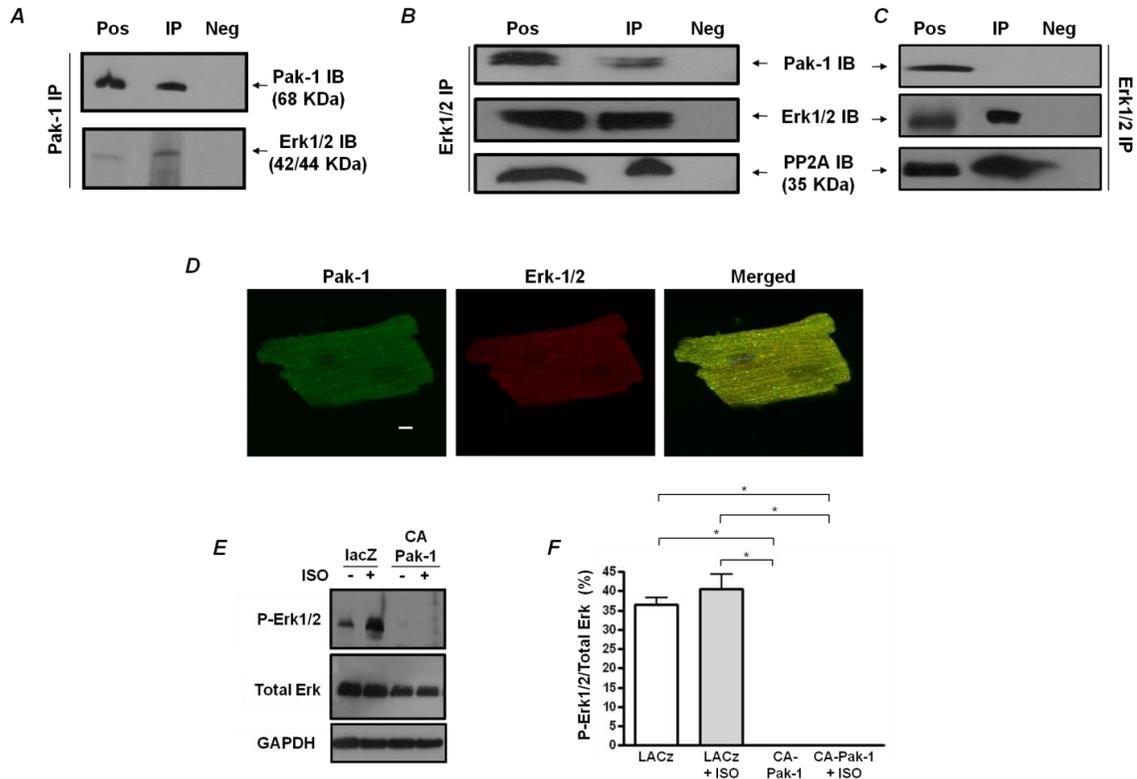


Figure 6. Protein association between Pak-1, PP2A, and Erk1/2. A, Pak-1 co-immunoprecipitates (IP) with Erk1/2 in WT myocardial tissue. B, Erk1/2 co-immunoprecipitates with Pak-1 and PP2A in WT myocardial tissue. C, Erk1/2 co-immunoprecipitates with PP2A in Pak-1-KO myocardial tissue. A-B-C, Pak-1 and Erk1/2 were immunoprecipitated (IP) from ISO-stimulated myocardial tissue lysate by overnight incubation with total anti-Pak-1 (A), or total anti-Erk1/2 antibodies (B-C). Complexes were then precipitated and analyzed by SDS-PAGE. In (A), Western immunoblotting (IB) was performed using an antibody recognizing total Pak-1 or Erk1/2 and; in (B) and (C), antibodies anti-total Pak-1, anti-total Erk1/2 and anti-total PP2A. In (A), (B), and (C), positive control (Pos) represents a brain lysate loading control for total Pak-1, Erk1/2, and PP2A respectively; negative control (Neg) represents a LV lysate loading control incubated overnight in the absence of immunoprecipitating primary antibody. Data are representative of three separate experiments. D, Pak-1 co-localizes with Erk1/2 in isolated adult mouse cardiomyocytes demonstrating that Pak-1 and Erk1/2 have similar intracellular localization. The shown images are representative of at least three independent experiments. Bar = 5 μ m. E-F, Adenovirally-infected adult rat cardiac myocytes overexpressing CA-Pak-1 exhibit the loss of Erk1/2 phosphorylation. Erk1/2 phosphorylation is consistently reduced in lysates obtained from adenovirally-infected adult rat cardiomyocytes that express CA-Pak-1, in the presence and in the absence of ISO, compared to cardiomyocytes expressing only the empty vector lacZ. * indicates $p < 0.05$.

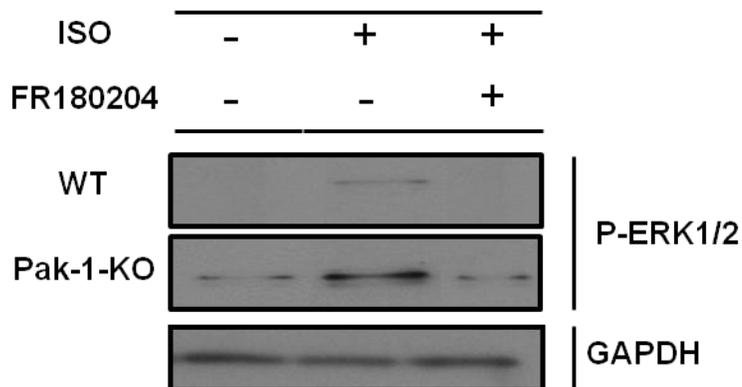


Figure 7. Western immunoblotting analysis of P-Erk1/2 in WT and Pak-1-KO mice receiving ISO alone, ISO and FR180204, or control saline. Results indicate that phosphorylation of Erk1/2 is increased in WT and Pak-1-KO hearts that received ISO alone vs. untreated WT and Pak-1-KO hearts. Administration of FR180204 to ISO-treated WT and Pak-1-KO mice resulted in reduction of Erk1/2 phospho-levels.

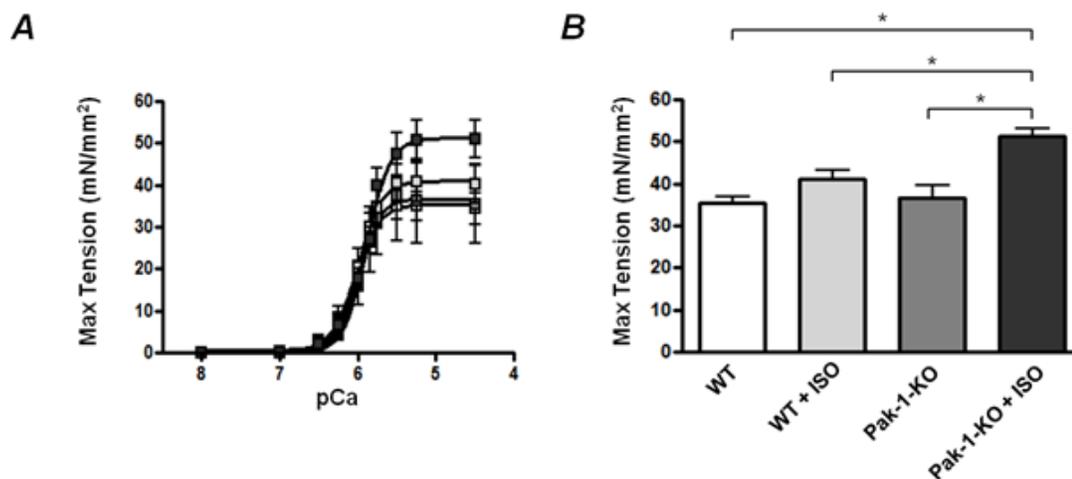


Figure 8. Ca²⁺-dependent isometric tension development of detergent-extracted cardiac fibers. A-B, ISO-treated Pak-1-KO hearts develop a statistically significantly higher maximal Ca²⁺ activated tension compared to all other experimental groups. * indicates $p < 0.05$.

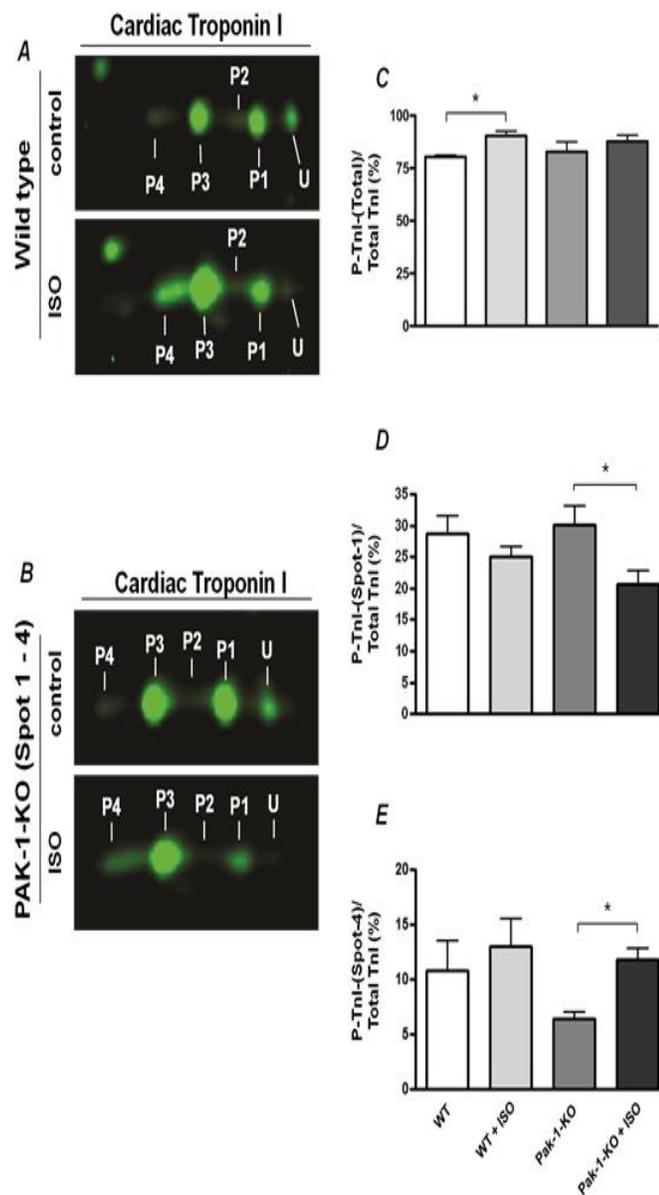


Figure 9. Analysis of cTnI by 2-D DIGE. A-B, Two-dimensional map of the cTnI in WT and Pak-1-KO mice. C, Total phosphorylation of cTnI increased in WT mice that received ISO vs. untreated WT control. No change in total cTnI phosphorylation was observed in Pak-1-KO mice after ISO treatment. D, Individual 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 $p < 0.05$.

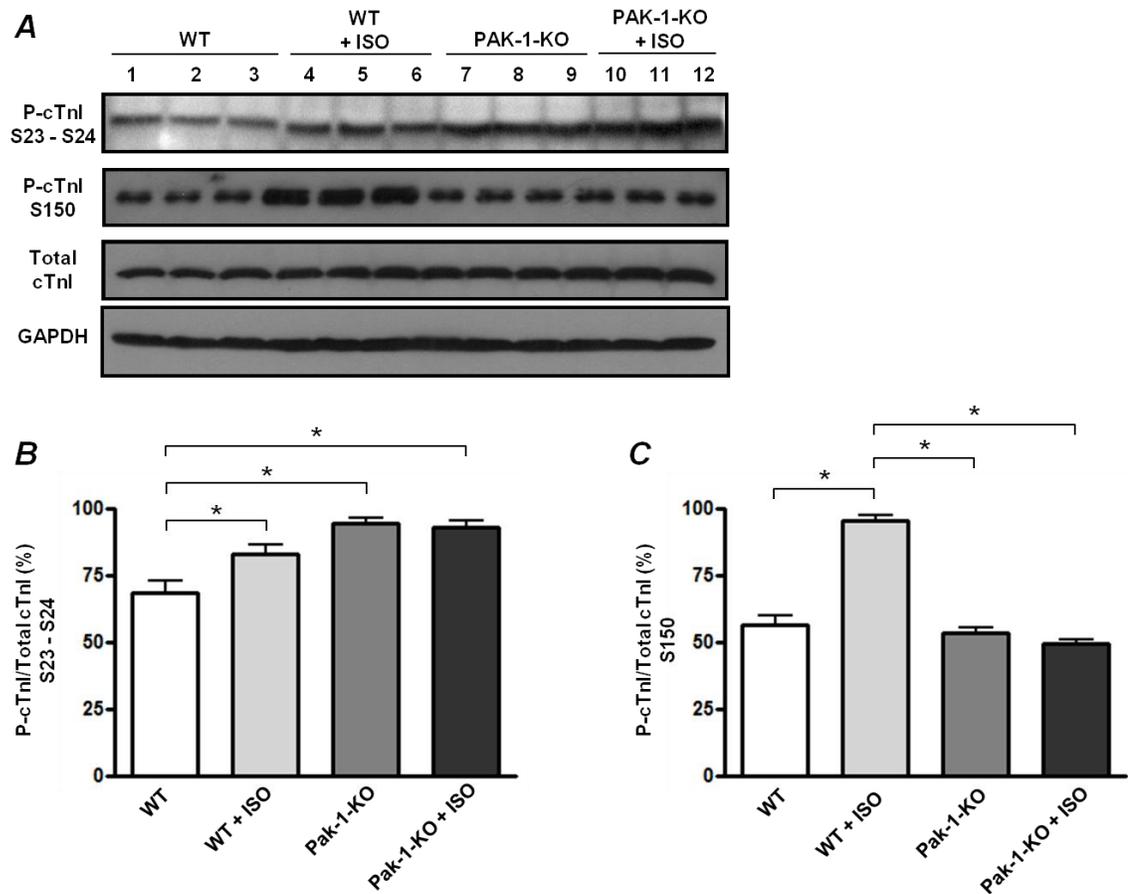


Figure 10. Western immunoblotting analysis of cTnl at serine residues 23, 24 and 150. Results indicate that phosphorylation of serine 23 and 24 is increased during ISO stimulation in WT mice, and in Pak-1-KO mice compared to untreated WT. Phosphorylation of serine 150 is increased in ISO-treated WT mice, however no response to ISO is observed in Pak-1-KO mice. * indicates $p < 0.05$.

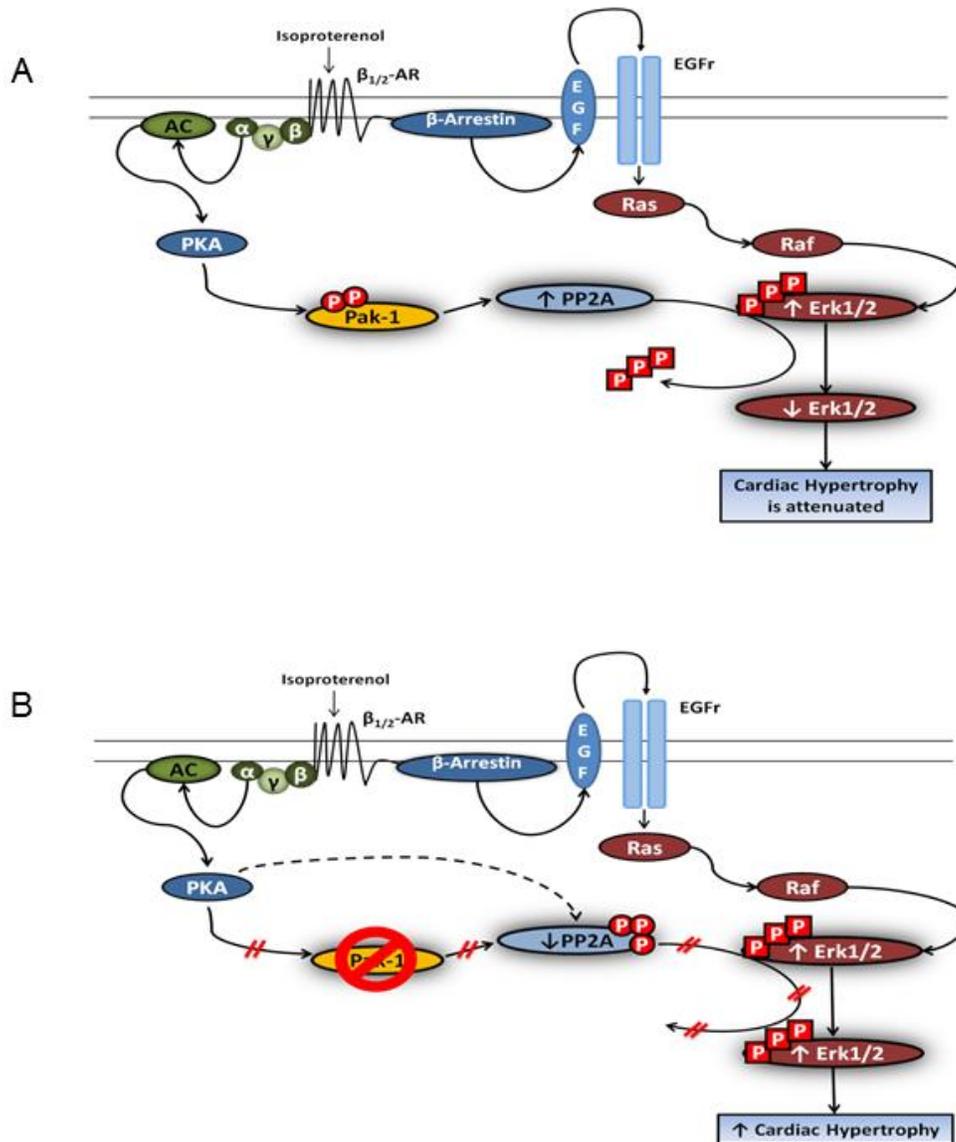


Figure 11. Proposed mechanism of LV myocardial hypertrophy in a Pak-1-KO mouse model. A, In the presence of Pak-1, ISO stimulation of the β -adrenergic receptor activates protein kinase A (PKA) signaling via activation of heterotrimeric G protein (α , β , and γ) and adenylate cyclase (AC). Also, ISO 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.

Table 1. Echocardiographic characteristics of WT and Pak-1-KO mice at baseline and after subcutaneous administration of ISO for seven days.

	WT (N = 6)	Pak-1-KO (N = 6)	WT + ISO (N = 6)	Pak-1-KO + ISO (N = 6)
Heart rate, bpm	482 ± 10	493 ± 15	685 ± 10	690 ± 12
LV FS, %	35.19 ± 2.70	28.02 ± 4.01	46.12 ± 3.29	59.25 ± 3.71 (*)
LV ESV, µL	22.30 ± 7.30	25.21 ± 7.29	16.31 ± 2.15	4.31 ± 0.56 (*)
LV EDV, µL	52.31 ± 4.58	61.31 ± 4.56	73.33 ± 2.5	37.31 ± 3.15 (*)
LV mass, mg	124.14 ± 5.76	125.41 ± 5.56	151.31 ± 4.72	198.11 ± 20.15 (*)
LVAWD (mm)	0.86 ± 0.07	0.84 ± 0.09	1.06 ± 0.05	1.17 ± 0.05 (*)
LVPWD (mm)	0.80 ± 0.08	0.80 ± 1.0	0.95 ± 0.05	1.10 ± 0.07 (*)
EDT, mm/s ²	-28653 ± 3862	-21550 ± 3560	-30412 ± 3914	-80460 ± 13620 (*)

* indicates $p < 0.05$ vs. WT + ISO.

LV: Left ventricular; FS: Fractional shortening; ESV: End-systolic volume; EDV: End-diastolic volume; LVAWD: Left ventricular diastolic anterior wall thickness; LVPWD: Left ventricular diastolic posterior wall thickness, EDT: Transmitral early filling deceleration time.

Table 2. Echocardiographic characteristics of WT and Pak-1-KO mice, at baseline, after receiving subcutaneously ISO only, or subcutaneous administration of ISO and the selective Erk1/2 inhibitor FR180204 intraperitoneally, for seven days.

	WT (before treatment) (N = 3)	Pak-1-KO (before treatment) (N = 3)	WT + ISO (N = 2)	Pak-1-KO + ISO (N = 3)	WT + ISO + FR180204 (N = 3)	Pak-1-KO + ISO + FR180204 (N = 3)
Heart rate, bpm	488 ± 40	490 ± 12	673	650 ± 22	600 ± 12	650 ± 18
LV FS, %	30.30 ± 5.20	28.12 ± 4.30	42.02	62.33 ± 6.00	30.76 ± 5.55	32.45 ± 6.12 (*)
LV ESV, µL	22.12 ± 4.12	25.30 ± 8.10	18.76	7.31 ± 1.10	19.40 ± 6.18	20.12 ± 5.44 (*)
LV EDV, µL	47.30 ± 6.30	50.22 ± 4.30	61.26	30.15 ± 7.51	60.78 ± 7.50	58.40 ± 6.33 (*)
LV mass, mg	99.30 ± 10.50	110.30 ± 12.80	155.80	215.11 ± 25.12	130.77 ± 6.80	125.78 ± 7.30 (*)
LVAWD (mm)	0.80 ± 0.05	0.75 ± 0.06	1.00	1.20 ± 0.08	0.85 ± 0.10	0.83 ± 0.14 (*)
LVPWD (mm)	0.76 ± 0.07	0.74 ± 0.08	0.80	1.15 ± 0.13	0.75 ± 0.13	0.78 ± 0.08 (*)
EDT, mm/s ²	-25720 ± 3031	-21749 ± 3695	-40355	-85540 10370	± -25338 5720	± -22350 ± 4451 (*)

* indicates $p < 0.05$ vs. Pak-1-KO + ISO. For the WT + ISO group, the mean value only is reported.

LV: Left ventricular; FS: Fractional shortening; ESV: End-systolic volume; EDV: End-diastolic volume; LVAWD: Left ventricular diastolic anterior wall thickness; LVPWD: Left ventricular diastolic posterior wall thickness, EDT: Transmitral early filling deceleration time.

III. P21-ACTIVATED KINASE IMPROVES CARDIAC CONTRACTILITY DURING ISCHEMIA-REPERFUSION INJURY CONCOMITANT WITH CHANGES IN MYOFILAMENT PROTEIN PHOSPHORYLATION

A. Introduction

When patients experience myocardial infarction, reperfusion injury occurs when coronary flow is re-established, resulting in reduced myocardial mechanical function. However, despite a large number of studies, the signaling mechanisms identified so far have contributed little to the improvement of the clinical outcome of these patients. Studies in our laboratory have discovered a potentially significant signaling cascade involving p21-activated kinase (Pak-1)⁵³. Pak-1 is a serine/threonine kinase which has been previously demonstrated by our laboratory⁵³ and others^{8, 90} to be activated by Cdc42/Rac1. Pak-1 activates protein phosphatase 2a (PP2A)^{31, 91} and leads to the dephosphorylation of cardiac proteins, including cTnI and myosin-binding protein C (MyBP-C)³¹, which in turn increases myofilament Ca²⁺ sensitivity in adult rat cardiac myocytes^{31, 50} and in cardiac intact trabeculae⁹². Activation of Pak-1 is also associated with reduced L-type Ca²⁺ current, but no change in phospholamban levels of phosphorylation⁵¹.

Emerging evidence indicates a role for Pak-1 in ischemia/reperfusion (I/R), but it is not clear whether Pak-1 activation during ischemia reduces or increases injury. On the one hand, Pak-1 and PP2A activation have been suggested to be downstream signaling molecules for sphingosine-1-phosphate (S1P) and bradykinin signaling in cardiac tissue, and be cardioprotective during I/R injury⁹³. It has been demonstrated that the phosphoinositide 3-kinase (PI3K) dependent

pathway can activate Pak-1⁹⁴, and that activation of the PI3K/Akt signaling pathway protects against I/R injury⁹⁵. Additionally, Pak-1 may reduce the rise in Ca²⁺ or enhance myofilament response to Ca²⁺ with I/R, which may improve the response to I/R⁹⁶. On the other hand, Pak-1 has been implicated to be a crucial upstream regulator of NADPH oxidase activation in neutrophils^{97, 98}, and an upstream mediator of JNK^{99, 100}, which is activated during myocardial I/R and leads to cardiomyocyte death¹⁰¹. One particular prior study found Pak-1 to prevent I/R-associated arrhythmias⁵. However, the authors in this latter study perfused the heart with a hypoxic solution, rather than performing true ischemia. Thus, the role of Pak-1 had yet to be studied under true ischemic conditions in cardiac tissue. In the present study, we determined the effect of Pak-1 on cardiac function during true ischemia using a stop-flow method. Additionally, the generation of mouse models without Pak-1 expression provides a powerful tool to investigate this question.

We hypothesize that activation of the Pak-1 signaling pathway induces post-translational modifications in myofilament proteins, which promote cardioprotective mechanisms that prevent or reverse the detrimental effects of ischemic injury in mouse cardiac tissue. In the present study, the contractility of hearts after I/R injury from Pak-1-KO mice was significantly further reduced compared to wild type (WT). ProQ analysis revealed a significant decrease in MLC2 phosphorylation in Pak-1-KO hearts compared to WT after I/R injury. Understanding the role of Pak-1 activation and its downstream effects is critical for the development of pharmaceutical interventions for use in patients with

ischemic events. The current study has advanced the field by providing direct evidence that Pak-1 signaling acts upon myofilament proteins, and is beneficial during I/R injury, in that in the absence of Pak-1, a rise in end-diastolic pressure is exacerbated and left ventricular developed pressure is further decreased after I/R injury. Thus, the current study provides a novel target for future studies to explore as a therapeutic strategy for patients with ischemic events.

B. Materials and Methods

All protocols were in accordance with the guidelines of the Animal Care and Use Committee of the University of Illinois and comply with the laws of The United States of America.

1. Pak-1 Knockout Mouse Model

Mice with Pak-1 gene disruption at both alleles were created in SV129 background and have been described previously^{61, 62}. Pak-1-KO mice were previously rederived in FVB background and genotyped to confirm the deletion of the Pak-1 gene, and the levels of protein expression of Pak-1, Pak-2, and Pak-3 (two less abundant protein isoforms of Pak in the heart) were measured in the LV cardiac tissue lysate by Western immunoblotting¹⁰². Additionally, hemodynamic parameters were previously assessed by echocardiography, which demonstrated no morphometric and hemodynamic differences between Pak-1-KO mice and WT controls¹⁰². In the current study, young adult (16.2 ± 0.3 weeks) female mice were used for acquisition of

functional and Western immunoblotting analysis. In the current study, age- and gender-matched FVB non-transgenic mice purchased from Charles River Laboratories were used as wild-type controls.

2. **Ex Vivo Left Ventricular Pressure Measurements During Global Cardiac Ischemia/Reperfusion**

Female wild-type (n = 9) and Pak-1-KO (n = 16) mice, were intraperitoneally injected with 50 IU/g heparin 20 min before being anesthetized with 0.5 mg/g pentobarbital sodium by intraperitoneal injection. The hearts were rapidly excised, the aorta cannulated, and the heart perfused using ice-cold modified Krebs-Henseleit (KH) solution containing (in mM) 118 NaCl, 4.7 KCl, 1.2 MgSO₄, 1.2 KH₂PO₄, 0.5 Na EDTA, 25 NaHCO₃, 10 glucose, and 2.5 CaCl₂. Once perfused, the heart was rapidly transferred to the Langendorff setup and perfused with KH solution, which was gassed with 95% O₂ / 5% CO₂ and at 37°C.

The left atrium was removed, and a balloon connected to a pressure transducer was inserted into the left ventricle for the measurement of left ventricular pressure as described previously⁹⁶. Electrodes were attached to the right atrium to field-stimulate and pace the myocardium. The heart was paced for 20 min at the lowest rate necessary to capture the rhythm (usually 7-9 Hz) until stabilization of pressure measurements was observed. Pacing was stopped 1 min before a stop-flow method was used to induce global ischemia. After 20 min of ischemia, flow was re-established, and field-stimulation for pacing was resumed 5 min after re-establishment of flow. Reperfusion continued for a total of

30 min, with continuous recording of left ventricular pressure and heart rate. The left ventricular pressure and heart rate were multiplied so as to report the rate-pressure constant, to account for varying heart rates in the different hearts. After 30 min of reperfusion, hearts were flash frozen in liquid nitrogen and saved for later analysis of protein phosphorylation and redox states. The tail clip was saved for PCR genotype confirmation.

3. Analysis of Phosphorylation and Redox State of Myofilament Proteins

Pro-Q Diamond (Invitrogen) stain was used to detect changes in phosphorylation states of myofilament proteins. Myofibrils were prepared from WT and Pak-1-KO mouse hearts, and pellets were solubilized in a non-reducing 2X Laemmli buffer (4% SDS, 20% glycerol, 0.004% bromophenol blue, and 0.125 M Tris HCl pH 6.8)¹⁰³. 25 mM N-ethylmaleimide (NEM) was added to the standard rigor buffer with Triton X-100, the standard rigor wash buffer and the 2X Laemmli buffer. An RC-DC assay (Bio-Rad) was used to determine protein concentrations. Samples were diluted at a 1:1 ratio in reducing sample buffer (8 M urea, 2 M thiourea, 0.05 M tris pH 6.8, 75 mM DTT, 3% SDS, and 0.05% bromophenol blue)¹⁰⁴, and approximately 15 µg of protein was loaded on to a 12% resolving 1D SDS-PAGE gel^{105, 106}. The gels were stained with Pro-Q Diamond and destained according to the manufacturer's recommendations prior to imaging with a Typhoon 9410 scanner (GE Healthcare). Coomassie R-250 staining was used to normalize protein load to actin. Optical density of the

proteins was determined using ImageQuant TL (GE Healthcare) software and results were exported to Excel for further analysis with statistical software JMP.

Western blot analysis was used to detect glutathionylated proteins. Myofibrils were prepared from WT and Pak-1-KO mouse hearts and pellets were solubilized in a non-reducing 2X Laemmli buffer¹⁰³. 25 mM N-ethylmaleimide (NEM) was added to the standard rigor buffer with Triton X-100, the standard rigor wash buffer and the 2X Laemmli buffer. Using the protein concentration determined from an RC-DC (Bio-Rad) assay, approximately 40 µg of total protein was applied to 1D 12% resolving SDS-PAGE gel and transferred onto a 0.2 µM PVDF membrane. The blot was blocked in 5% nonfat dry milk with 2.5 mM NEM for 1 hour. Anti-glutathione mouse monoclonal primary antibody (Virogen) was used at 1:1000 dilution along with anti-mouse HRP-conjugated secondary antibody (Sigma) at 1:100,000 dilution to detect for S-glutathionylation¹⁰⁷.

Western blot analysis was used to detect the phosphorylation level of MLC2. Myofibrils were prepared from WT and Pak-1-KO mouse hearts, and pellets were solubilized in a non-reducing 2X Laemmli buffer (4% SDS, 20% glycerol, 0.004% bromophenol blue, and 0.125 M Tris HCl pH 6.8)¹⁰³. 25 mM N-ethylmaleimide (NEM) was added to the standard rigor buffer with Triton X-100, the standard rigor wash buffer and the 2X Laemmli buffer. An RC-DC assay (Bio-Rad) was used to determine protein concentrations. Samples were diluted at a 1:1 ratio in reducing sample buffer (8 M urea, 2 M thiourea, 0.05 M tris pH 6.8, 75 mM DTT, 3% SDS, and 0.05% bromophenol blue)¹⁰⁴, and approximately 20 µg of protein was loaded on to a 12% resolving 1D SDS-PAGE gel^{105, 106} and

transferred onto a 0.2 μ M PVDF membrane. The blot was blocked in 5% nonfat dry milk for 1 hour. Anti-phospho-MLC S20 (Abcam) was used at 1:5000 dilution to detect phosphorylated levels of MLC2. Cardiac-specific Anti-MLC2 (Abcam) was used at 1:10,000 to detect total levels of MLC2 protein. Ponceau Image was used to normalize MLC2 level to actin.

Total proteins (200 μ g) obtained from WT left-ventricular cardiac tissue were incubated overnight with rabbit monoclonal total Pak-1 antibody (Cell Signaling) followed by incubation with protein A sepharose beads (70 μ l of 50% bead slurry) with gentle rocking for 4 hours at 4° C. Complexes were then precipitated and analyzed by SDS-PAGE and Western blot. Cardiac-specific anti-MLC2 (Abcam) was used at 1:10,000 to detect co-immunoprecipitation of cardiac MLC2 with Pak-1. Anti-troponin-T (Santa Cruz Biotechnology, Inc.) was used at 1:200 to detect co-immunoprecipitation of TnT with Pak-1.

4. **Statistics**

Data were statistically analyzed by two-way ANOVA followed by Student's t-test using JMP statistical software where all groups are considered (Figures 1B-1C and 3A-3F). Data were statistically analyzed by t-test using JMP statistical software where only two groups were considered (Figure 1D). Data were statistically analyzed by an independent samples t-test (ANOVA with two groups) using JMP statistical software to determine if there is a difference for the two groups WT and Pak-1-KO over the entire reperfusion phase (Figure 1E). A value of $P < 0.05$ was considered significantly different (P value required for

significance appropriately adjusted by JMP statistical software for ANOVA then followed by student's t-test). Data are represented as mean \pm SEM.

B. Results

1. Pak-1 Is Involved In Regulation of Developed Pressure during Ischemia/Reperfusion In Mouse Heart

A representative tracing of left ventricular pressure in Langendorff-perfused hearts during global ischemia is shown in Figure 1, Panel A. Left ventricular developed pressure (systolic pressure minus diastolic pressure) in WT and Pak-1-KO ex vivo hearts was significantly reduced after 20 min ischemia and 30 min reperfusion relative to baseline values before ischemia in each group (Figure 1, Panel B). The rate-pressure product in WT and Pak-1-KO ex vivo hearts was also significantly reduced after 20 min ischemia and 30 min reperfusion relative to baseline values in each group (Figure 1, Panel C), in agreement with the values for left ventricular developed pressure. Left ventricular developed pressure measurements after I/R were divided by the left ventricular developed pressure at baseline before ischemia to determine the percent recovery of myocardial function. The percent recovery of left ventricular developed pressure after I/R injury was significantly reduced in Pak-1-KO hearts compared to WT controls (Figure 1, Panel D). End systolic pressure in WT and Pak-1-KO ex vivo hearts was significantly reduced after I/R relative to baseline values in each group. (WT decreased from 85.6 ± 1.7 mmHg at baseline to 69.8 ± 1.8 mmHg after I/R injury. Pak-1-KO decreased from 90.8 ± 3.7 mmHg at

baseline to 68.8 ± 3.2 mmHg after I/R injury.) One-way ANOVA results indicate that the change in end diastolic pressure across all points over the course of reperfusion was significantly higher in Pak-1-KO hearts compared to WT hearts (Figure 1, Panel E). However, this increase was not significantly different between WT and Pak-1-KO hearts at any of the particular points measured during reperfusion as shown in Figure 1, Panel E ($P = 0.07$ at 5 min reperfusion, $P = 0.11$ at 10 min reperfusion, $P = 0.08$ at 15 min reperfusion, $P = 0.09$ at 20 min reperfusion, $P = 0.11$ at 25 min reperfusion, and $P = 0.18$ at 30 min reperfusion). Positive dP/dt significantly decreased (in WT from 2290.4 ± 94.8 mmHg/sec at baseline to 1127.3 ± 37.2 mmHg/sec after I/R and in Pak-1-KO from 2585.8 ± 236.9 mmHg/sec at baseline to 841.0 ± 103.4 mmHg/sec after I/R) while negative dP/dt significantly increased after 20 min ischemia and 30 min reperfusion (in WT from -1733.8 ± 72.0 mmHg/sec at baseline to -946.2 ± 47.0 mmHg/sec after I/R injury, and in Pak-1-KO from -2174.1 ± 164.7 mmHg/sec at baseline to -867.2 ± 105.1 mmHg/sec after I/R injury) relative to baseline values before ischemia in each group.

2. Change of Phosphorylation of Myofilament Proteins in Pak-1-KO Heart

All hearts used in the analysis of the phosphorylation and redox states of the myofilament proteins were frozen at 8 Hz. A representative gel with ProQ and Coomassie staining can be seen in Figure 13. Compared to WT hearts not subjected to ischemia, WT hearts after I/R demonstrated a significant

increase in phosphorylation of TnT (Figure 14, Panels B-C), and a significant decrease in phosphorylation of Tm, Tnl, and MLC2 (Figure 14, Panels D, E, and F, respectively). Pak-1-KO hearts after I/R injury exhibited a significant decrease in phosphorylation of Tnl and MLC2 compared to Pak-1-KO hearts not subjected to ischemia (Figure 14, Panels E and F, respectively). However, TnT and Tm phosphorylation was not significantly different in Pak-1-KO hearts at baseline and after I/R injury (Figure 14, Panels B-D). TnT phosphorylation was increased at baseline in Pak-1-KO hearts compared to WT (Figure 14, Panels B-C). MLC2 phosphorylation was significantly decreased in Pak-1-KO hearts compared to WT after I/R injury (Figure 14, Panel F). MyBP-C phosphorylation was not significantly different between any of the groups studied (Figure 14, Panel A). No significant changes in S-glutathionylation of the myofilament proteins were detected in any of the groups studied (results not shown).

Western blot analysis of phosphorylation levels of MLC2 at Serine 20 was performed to confirm the results obtained from the ProQ analysis (Figure 15). Consistent with our ProQ findings, phosphorylation levels of MLC2 decreased after I/R injury in hearts from both WT and Pak-1-KO mice compared to baseline levels (Figure 16). MLC2 phosphorylation levels were even further decreased in hearts from Pak-1-KO mice after I/R injury compared to WT after I/R injury. Again consistent with our ProQ results, MLC2 phosphorylation was not significantly different between hearts from WT and Pak-1-KO mice at baseline.

3. Co-Immunoprecipitation

To test whether a direct association exists between Pak-1 and cardiac MLC2, we performed a co-immunoprecipitation and demonstrate that these two proteins establish a direct protein-protein interaction in left ventricular WT cardiac tissue (Figure 17A and 17B). Pak-1 and TnT also establish a direct protein-protein interaction in left ventricular WT cardiac tissue (Figure 17A and 17C).

D. Discussion

An important finding of our current study is the demonstrations of a beneficial effect of the Pak-1 signaling pathway, which improves myocardial performance concomitant with a modulation of troponin-T and myosin light chain 2 during global myocardial ischemia and reperfusion injury. In the absence of Pak-1, there was an exacerbation of the increased end diastolic pressure and reduced left ventricular developed pressure occurring after I/R injury. Thus, results of our study provide a basis for targeting a novel pathway including Pak-1 in the therapies for patients with ischemic events.

The mechanism by which the absence of Pak-1 induces a depressed recovery of function after I/R may involve a depressed level of MLC2 phosphorylation. This was the only difference we found in sarcomeric protein phosphorylation between WT and Pak-KO hearts following I/R (Fig. 3F). Our lab has shown that ablation of MLC2 phosphorylation decreases ventricular power, lengthens the duration of ventricular ejection, and may also modify other

sarcomeric proteins (e.g. cardiac troponins) as substrates for kinases and phosphatases³²⁻³⁴. The other differences that may be of significance are that Tm phosphorylation fell significantly with I/R in WT but not in Pak-KO hearts and TnT phosphorylation increased with I/R in WT but not in Pak-KO. In previous studies, we have reported that a loss in MLC2 phosphorylation in fibers from a transgenic mouse model expressing a cardiac specific nonphosphorylatable regulatory light chain results in a decrease in maximum Ca^{2+} -activated tension and tension cost (ATP hydrolysis/unit tension) with no change in Ca^{2+} sensitivity³². Moreover there is evidence that phosphorylation of cTnT may depress cardiac function¹⁰⁸.

An interesting finding of our study was the demonstration that under baseline conditions the major difference between WT and Pak-KO hearts was the phosphorylation levels of TnT3 and TnT4, both of which were higher in the Pak-1 KO myofilaments compared to WT (Figs. 3B and 3C). TnT3 and TnT4 phosphorylation levels both increased in WT following I/R injury, but this increase in phosphorylation levels was not seen in Pak-KO hearts after I/R injury, suggesting a role for Pak-1 in the regulation of TnT3 and TnT4 phosphorylation. Additionally, we also demonstrate that Pak-1 and TnT associate in a protein-protein interaction, as assessed by co-immunoprecipitation. The results of this study suggest Pak-1 plays a regulatory role in the phosphorylation level of TnT.

We demonstrate that the ablation of Pak-1 causes a reduction in the percent recovery of left ventricular developed pressure after I/R injury, probably due largely to an exacerbated increase in end diastolic pressure. There is a significant baseline increase in TnT phosphorylation in the Pak-1-KO hearts

compared to WT, and this difference is not observed after I/R injury. Overall protein phosphorylation was detected by ProQ, and it is important to note that phosphorylation at one site of any of the proteins studied may have increased while phosphorylation at another site in the same protein may have decreased.

Phosphorylation of Tm, Tnl, and MLC2 is significantly reduced in WT hearts after I/R injury compared to phosphorylation levels in WT before ischemic insult. I/R injury has been previously reported to be associated with a decrease in myofilament calcium sensitivity¹⁰⁹, and the concurrent de-phosphorylation of Tm, Tnl, and MLC2 has previously been reported to be associated with a decrease in myofilament calcium sensitivity¹¹⁰. This could provide a possible mechanism for the decrease in left ventricular systolic and developed pressures observed in WT hearts after I/R injury. Specific increases in myofilament calcium sensitivity in transgenic mouse hearts that express slow skeletal Tnl treated with EMD-57033 have shown to diminish the effects of I/R on cardiac function⁹⁶. Additionally, phosphorylation of MLC2 is further decreased in hearts from Pak-1-KO mice compared to WT controls after I/R injury, and a decrease in phosphorylation of MLC2 has been previously associated with a decrease in myofilament calcium sensitivity^{33, 111}. A previous study demonstrated the phosphorylation of intact non-muscle myosin II regulatory light chain by γ -Pak¹¹². This decrease in MLC2 phosphorylation and the co-immunoprecipitation demonstrating an association between MLC2 and Pak-1 suggests that Pak-1 plays a role in the phosphorylation of MLC2 in cardiac tissue during I/R injury and could provide a

possible mechanism for the reduced recovery of pressure observed in hearts from Pak-KO mice.

The main emphasis of the current study is to demonstrate that Pak-1 plays a role in I/R injury. Future studies will investigate the other mechanisms by which protein phosphorylation can be altered during ischemia/reperfusion. Pak-1 is involved in a number of cell types and signaling pathways, and has been shown to form a complex with an important cytoskeletal protein ¹¹³. Future studies will distinguish the individual effects of these various signaling proteins and the crosstalk which occurs between pathways.

In conclusion, the present study demonstrates that Pak-1 signaling acts upon myofilament proteins and is beneficial during I/R injury, in that Pak-1 reduces end diastolic pressure and increases left ventricular developed pressure after I/R injury. Thus, the current study provides a novel pathway including the potential target Pak-1 for development of pharmaceutical interventions for use in the clinic for patients with ischemic events.

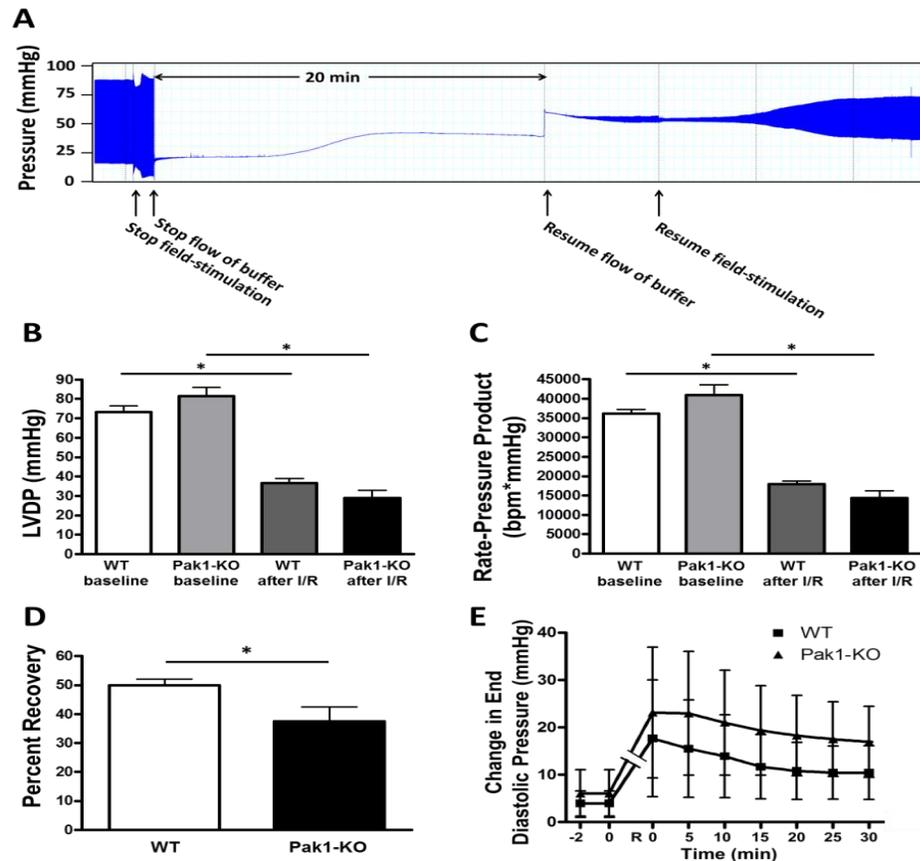
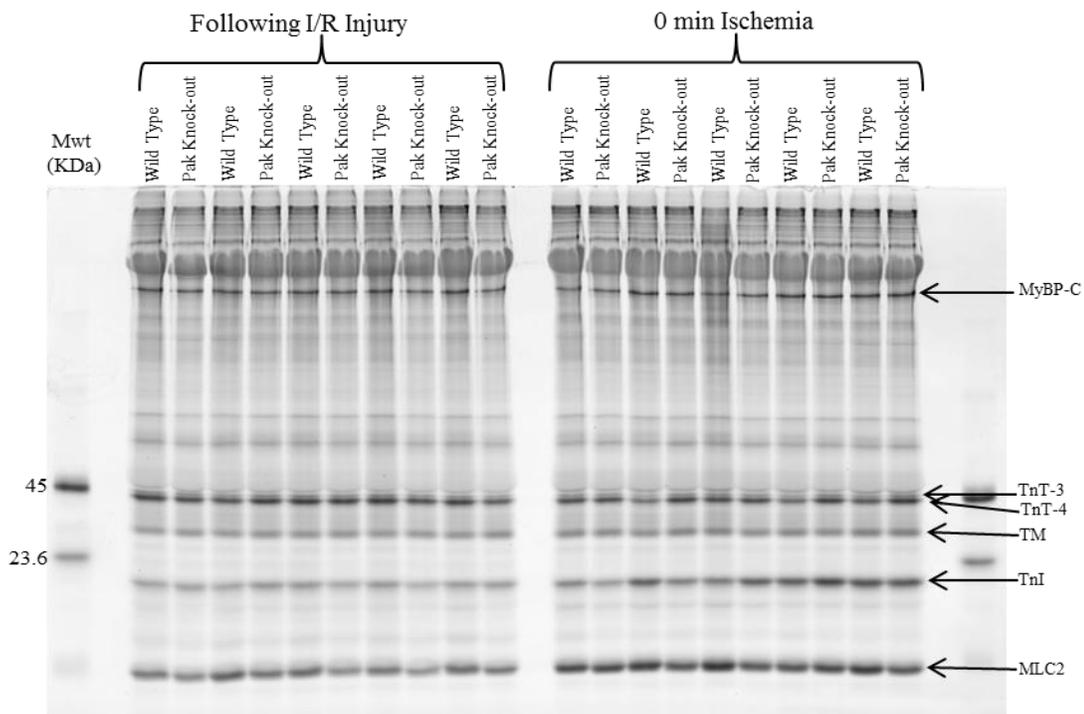


Figure 12. Left ventricular pressure measurements. A) Representative tracing of pressure during ischemia, WT female mouse, 8 Hz, 37°C, experiment number 110311e. B) Left ventricular developed pressure in WT and Pak-1-KO ex vivo hearts is significantly reduced after 20 min ischemia and 30 min reperfusion relative to baseline values in each group. Statistics was performed by two-way ANOVA followed by Student's t-test using JMP statistical software. C) The rate-pressure product, calculated as heart rate multiplied by left ventricular developed pressure, in WT and Pak-1-KO ex vivo hearts is significantly reduced after 20 min ischemia and 30 min reperfusion relative to baseline values in each group. Statistics was performed by two-way ANOVA followed by Student's t-test using JMP statistical software. D) Percent recovery of left ventricular developed pressure after I/R injury, as calculated by LVDP after I/R divided by LVDP at baseline, is significantly less in Pak-1-KO hearts compared to WT controls. Statistics was performed by t-test using JMP statistical software. E) Change in left ventricular end diastolic pressure during reperfusion is increased in Pak-1-KO hearts compared to WT. Data were statistically analyzed by an independent samples t-test (ANOVA with two groups) using JMP statistical software to determine if there is a difference for the two groups WT and Pak-1-KO over the entire reperfusion phase (but not at any particular timepoint). Data collected at 2.5 mM $[Ca^{2+}]_o$, 37°C, n = 9 for WT, n = 16 for Pak-1-KO. Data reported as mean \pm SEM, P < 0.05 considered significant.

Pro-Q Gel



Coomassie R-250 Gel

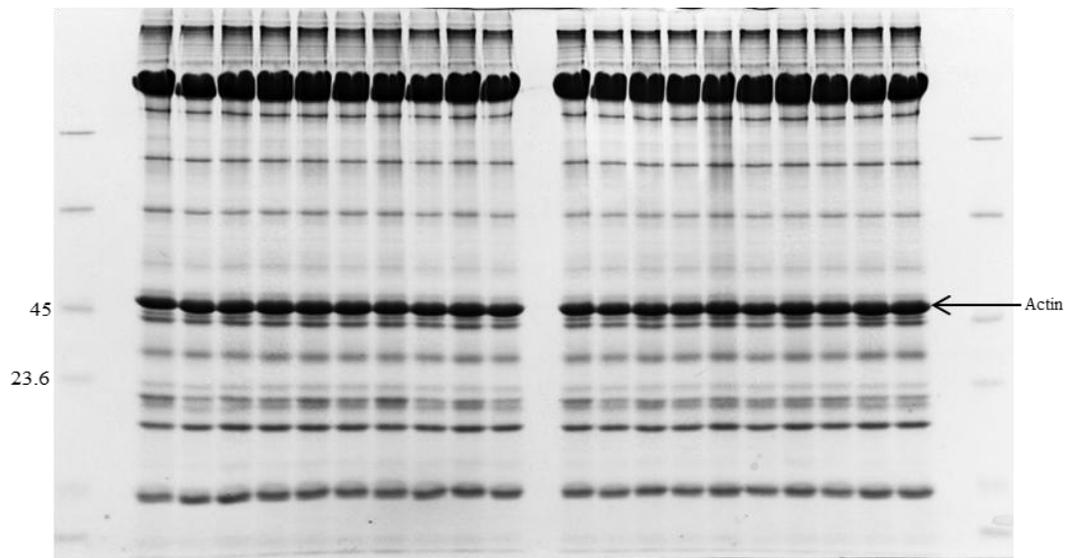


Figure 13. Representative Pro-Q and Coomassie Gels. Gels demonstrate changes in phosphorylation of various myofilament proteins in WT and Pak-1-KO hearts after 20 min ischemia and 30 min reperfusion compared to hearts not subjected to ischemia.

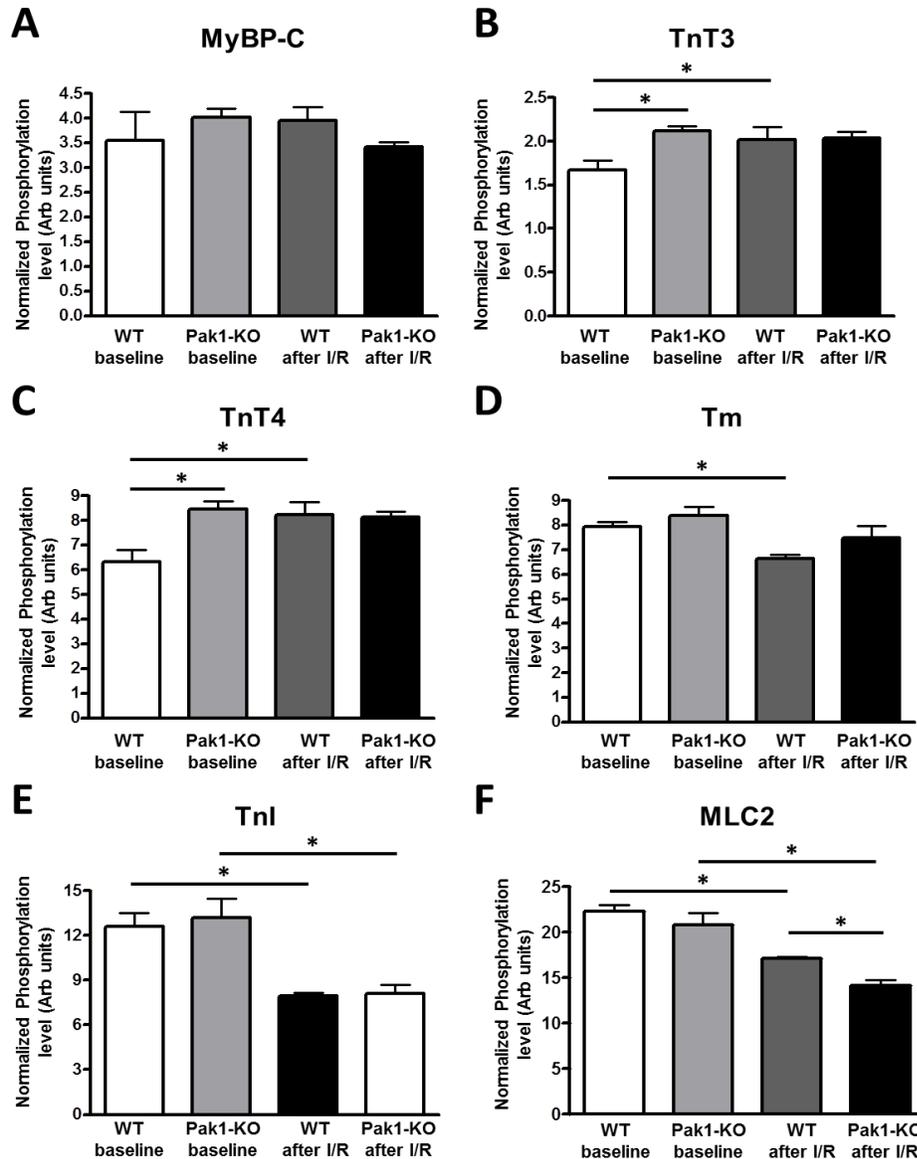


Figure 14. Phosphorylation of myofilament proteins as detected by ProQ.

A) MyBP-C phosphorylation was not significantly different after I/R in either WT or Pak-1-KO hearts. B-C) TnT3 and TnT4 phosphorylation was increased at baseline in Pak-1-KO hearts compared to WT hearts, and phosphorylation of WT hearts was increased after I/R compared to WT hearts at baseline. D) Tm phosphorylation decreased in WT hearts after I/R, but was not significantly different in Pak-1-KO hearts after I/R. E) TnI phosphorylation decreased after I/R in both WT and Pak-1-KO hearts, but was not significantly different between WT and Pak-1-KO. F) MLC2 phosphorylation decreased after I/R in both WT and Pak-1-KO hearts, and was significantly decreased in Pak-1-KO compared to WT after I/R. Data normalized to actin. Statistics was performed by two-way ANOVA followed by Student's t-test using JMP statistical software. Data reported as mean \pm SEM.

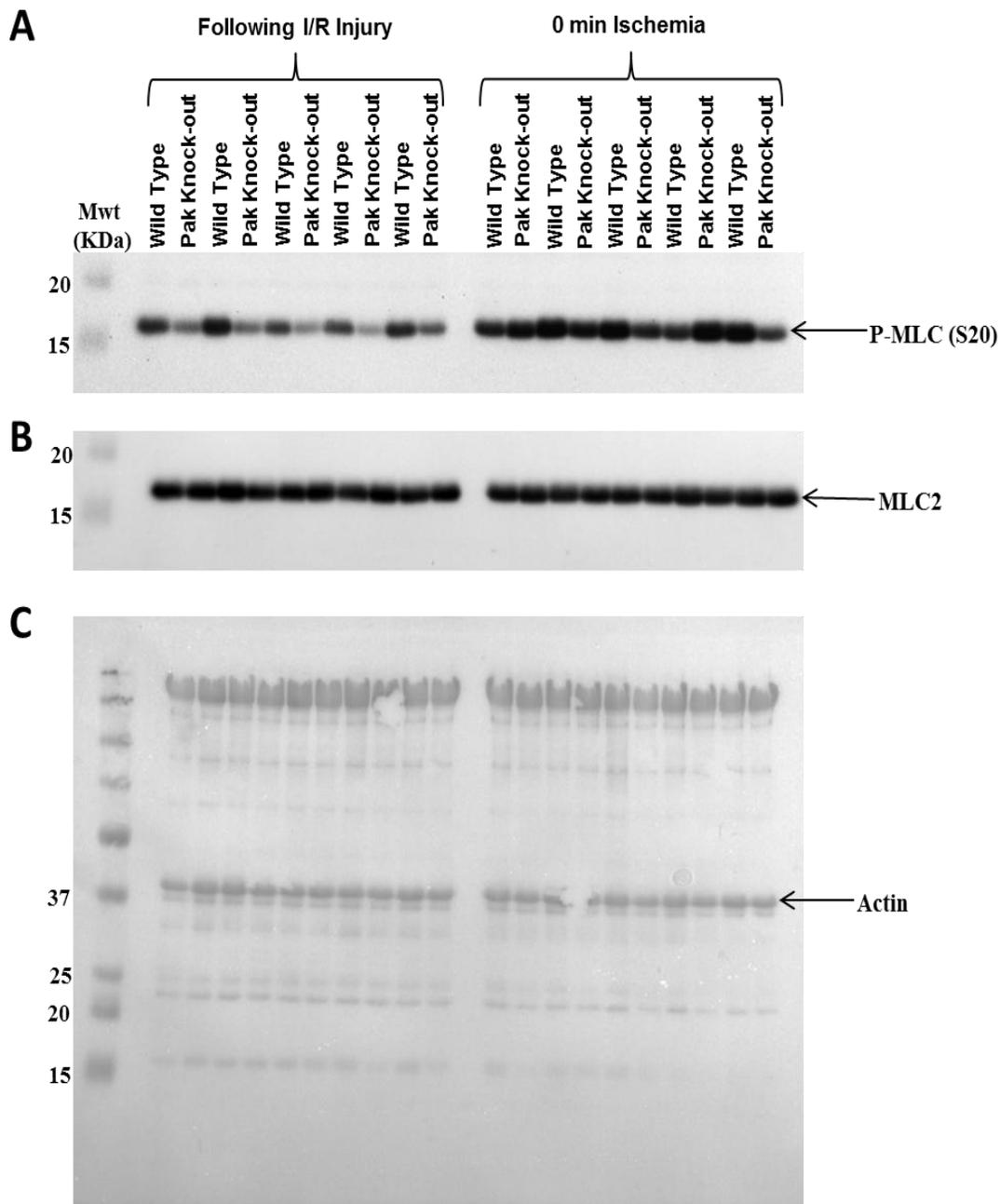


Figure 15. Representative Western Blot demonstrating changes in MLC2 phosphorylation. A) Western Blot probed for phospho-MLC2 at Serine 20. B) Western Blot probed for total MLC2. C) Ponceau image used for normalization of MLC2 to actin.

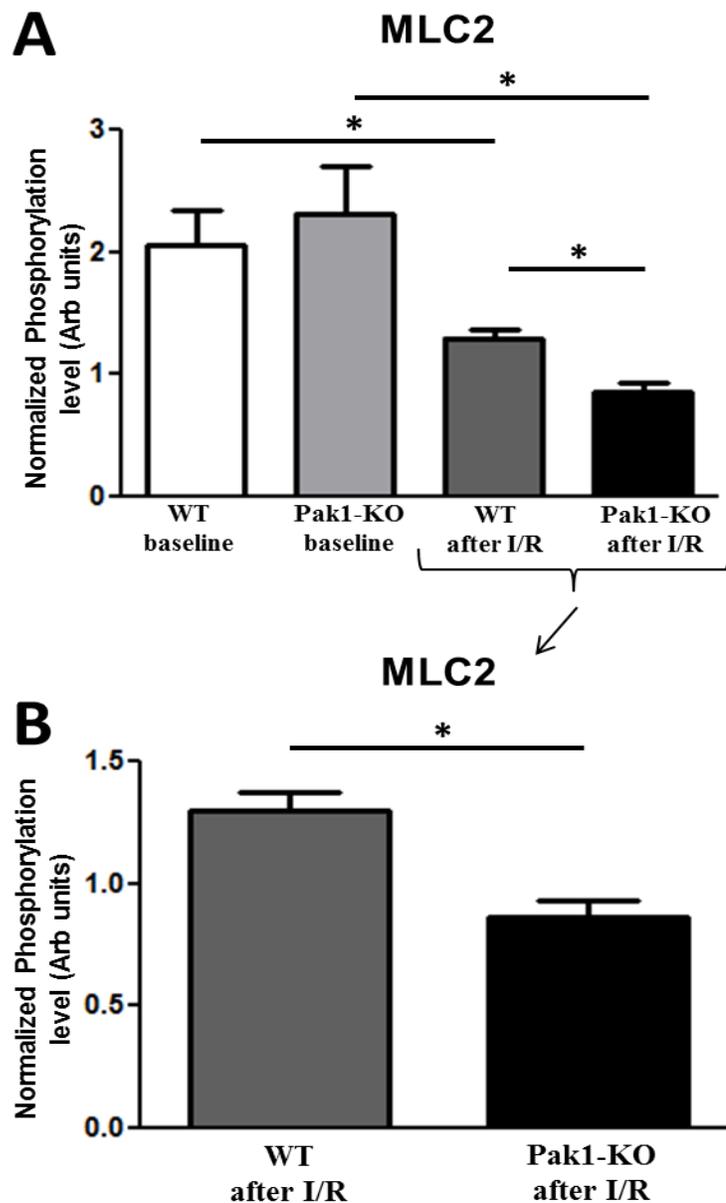


Figure 16. Phosphorylation of MLC2 as detected by Western Blot. A) MLC2 phosphorylation decreased after I/R injury in hearts from both WT and Pak-1-KO mice. MLC2 phosphorylation was not significantly different between hearts from WT and Pak-1-KO mice at baseline. B) MLC2 phosphorylation was further reduced in hearts from Pak-1-KO mice after I/R injury compared to hearts from WT mice after I/R injury. Statistics were performed by two-way ANOVA followed by Student's t-test using JMP statistical software. Data reported as mean \pm SEM.

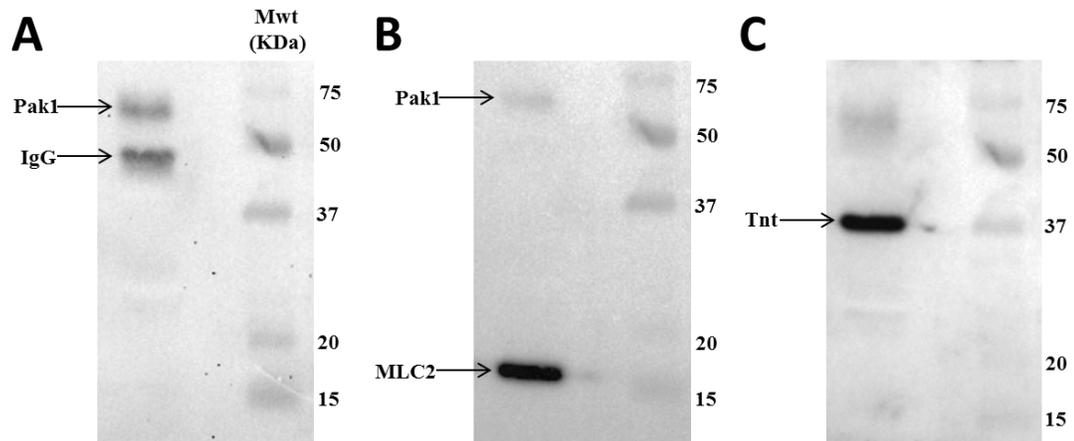


Figure 17. Co-immunoprecipitation demonstrating an association between Pak-1 and MLC2, and Pak-1 and TnT. A) Western blot probed with total Pak-1 antibody (Cell Signaling). B) Western blot probed with total cardiac-specific MLC2 antibody (Abcam). C) Western blot probed with total TnT antibody (Santa Cruz Biotechnology, Inc.).

IV. INHIBITION OF ANGIOTENSIN II-INDUCED VENTRICULAR HYPERTROPHY AND FIBROSIS THROUGH ACTIVATION OF PAK-1 AND MODULATION OF ERK1/2 AND P38 SINGALING

A. Introduction

Activation of the renin-angiotensin-aldosterone system (RAAS) induces hypertrophic remodeling in the heart^{114, 115}. Activity of the RAAS is increased in patients with heart failure, and its maladaptive mechanisms may lead to adverse effects such as cardiac remodeling and sympathetic activation. Elevated renin activity has been demonstrated in patients with dilated cardiomyopathy. In addition, angiotensin II is believed to influence a number of molecular and structural changes in the heart, mostly mediated through the AT₁-receptor and the importance of the RAAS in heart failure is shown by the survival benefit conferred by treatment with ACE inhibitors and Angiotensin II Receptor Blockers (ARBs). Bradykinin (BK) is thought to contribute to the cardioprotective effect of ACE inhibition through modification of nitric oxide release, calcium handling and collagen accumulation. Our recent evidence supports the idea that BK signals alters protein phosphorylation in adult rat cardiac myocytes through the activation and translocation of Pak-1 in as much as treatment of myocytes with BK resulted in the activation of Pak-1 and a diminished striated localization, which is present in the basal state in isolated cardiac myocytes⁵². In the current studies, we provide evidence that a link between angiotensin II and Pak-1 activities exists and that Pak-1 activation counteracts angiotensin II-induced myocardial hypertrophy and fibrosis.

B. Methods

1. WT and Pak-1-KO Mouse Model

A description of the WT and Pak-1-KO mouse models can be found in the methods section of chapter II.

2. Infusion System and Pharmacological Treatments

Alzet micro-osmotic pumps Model 1002 (0.25 ul per hour, 14 days) were used in to infuse angiotensin II and the peptides described below. Peptides were dissolved in solution containing 50% ethanol and 0.45% NaCl. The peptide dose of infusion was set at 1 ug/g/day unless otherwise specified. The surgical procedure for miniosmotic pump implantation is reported in chapter II. We used the following peptides:

1. Angiotensin II. Asn-Arg-Val-Tyr-Val-His-Pro-Phe (NRVYVHPF) was purchased from Sigma.
2. The Pak-1 proline-rich region PRR-1-1 (Tat-PR-1 region): YGRKKRRQRRRGKPPAPPMRNTSTM (25 amino acids) ²³.
3. Control peptide: YGRKKRRQRRRGXXXXXXXXXXXXXA (X=random amino acid).

All the peptides except Angiotensin II were synthesized in the proteomics core lab in Research Resource Center (RRC) at UIC. The synthesized peptides were examined by MS (mass spectrometry).

3. **Transthoracic Two-Dimensional And M-Mode And Pulsed Doppler Echocardiography**

Echocardiographic analysis has been described previously in the methods section of chapter II.

4. **Western Immunoblotting**

Generation of protein samples from tissue, as well as Western immunoblotting and chemifluorescent detection have been described previously in the methods section of chapter II.

5. **Hydroxyproline Assay**

Hydroxyproline (HOP) content was determined as previously described with some minor modifications¹¹⁶. Liquid nitrogen frozen mouse cardiac tissue was minced into a pre-weighed Pyrex 9826 screw cap vial to determine the wet weight of the tissue (5–10 mg). The tissue was hydrolyzed overnight in 200 μ L of 6 M HCL in an oven set to 110 °C. After the hydrolysis 80 μ L of isopropanol was added to 5 μ L of hydrolysate, and then 40 μ L of chloramine-T solution (7% chloramine-T in water) mixed with acetate citrate buffer (0.695 M anhydrous C₂H₃O₂Na, 0.174 M C₆H₈O₇, 0.435 M NaOH, and 38.5% [v/v] 2-propanol) in a 1:4 ratio was added, vortexed, and allowed to incubate and oxidize for 5 min at room temperature. Next 0.5 ml of Ehrlich's reagent (3 g of 4-(dimethylamino) benzaldehyde, 10 ml ethanol, 675 μ l sulfuric acid) mixed with 2-propanol in a 3:13 ratio was added, vortexed, and incubated

for 30 min at 55 °C then quenched in an ice bath. The samples were then centrifuged at 5000g for 1 min and the optical density of the supernatant was determined at 558 nm. A standard curve of trans-4-hydroxy-L-proline (0–500 nM) was included in each assay to determine the nM HOP/mg of tissue.

C. Results

1. Transthoracic Two-Dimensional, M-Mode, And Doppler Echocardiography

Transthoracic M-mode and Doppler echocardiography were performed in WT mice that received AT-II, AT-II + PRR-1 peptide, or control treatment. M-mode measurements and transmitral flow velocities were obtained from parasternal short-axis view and are reported in Table 1. Our data demonstrate that, compared to mice that received control treatment, chronic treatment with AT-II results in increased LV myocardial mass, increased anterior and posterior wall thickness, and reduced LV volume in diastole and systole. Also, AT-II-treated mice, compared to those that received control treatment, developed a significantly faster transmitral early filling deceleration time (EDT), suggesting an increased LV operating stiffness and a restrictive myocardial pattern. On the contrary, hearts of WT mice treated with both AT-II and PRR-1 peptide showed an attenuated AT-II myoproliferative response for all hemodynamic variables and no statistically significant changes were observed compared to control hearts.

Table 3. Echocardiographic characteristics of WT mice that received control treatment and after administration of AT-II, AT-II + PIP peptide for seven days. (*) indicates $P < 0.05$ vs. ATII group.

	CTRL (N = 6)	AT-II (N = 6)	AT-II + PRR-1 peptide (N = 6)
Heart rate, bpm	606 ± 10	595 ± 22	599 ± 13
LV FS, %	35.28 ± 5.16	32.77 ± 1.61	32.82 ± 4.49
LV ESV, μ L	23.43 ± 4.34	14.52 ± 2.33	30.18 ± 8.12 (*)
LV EDV, μ L	65.93 ± 4.50	38.47 ± 4.86	64.91 ± 6.3 (*)
LV mass, mg	97.27 ± 5.80	168.66 ± 13.93	106.30 ± 9.43 (*)
LVAWD (mm)	0.87 ± 0.06	1.62 ± 0.11	1.05 ± 0.06 (*)
LVPWD (mm)	0.82 ± 0.08	1.40 ± 0.07	0.78 ± 0.07 (*)
EDT, mm/s^2	-46615 ± 13469	-95616 ± 31486	-32875 ± 9319 (*)

2. Analysis of MAPK Phosphorylation by Western Immunoblotting

Analysis of MAPK phosphorylation evidenced that phosphorylation of Erk1/2, P38 and JNK was increased in WT mice that received continuous infusion of angiotensin II. However, concomitant administration of PRR-1 peptide along with angiotensin II was associated with a reduced activation of the MAPKs, in particular Erk1/2.

3. Hydroxyproline Assay

We assessed the level of collagen production in WT untreated hearts and hearts treated with AT-II, in the absence and the presence of PRR-1 peptide. Results showed that the administration of AT-II (n=6) was associated with increased fibrosis compared to untreated mice (n=6) ($P < 0.05$). Mice that

received AT-II and PRR-1 (n=7) were characterized by reduced collagen expression in the extracellular matrix ($P < 0.07$).

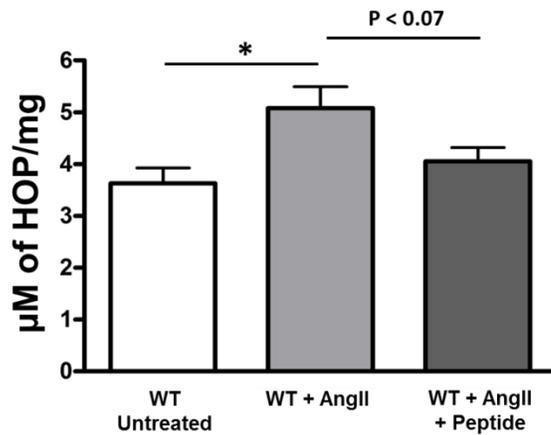


Figure 18. Hydroxyproline Assay. Analysis of collagen content in untreated hearts and hearts treated with ATII, in the absence and the presence of PRR-1 peptide.

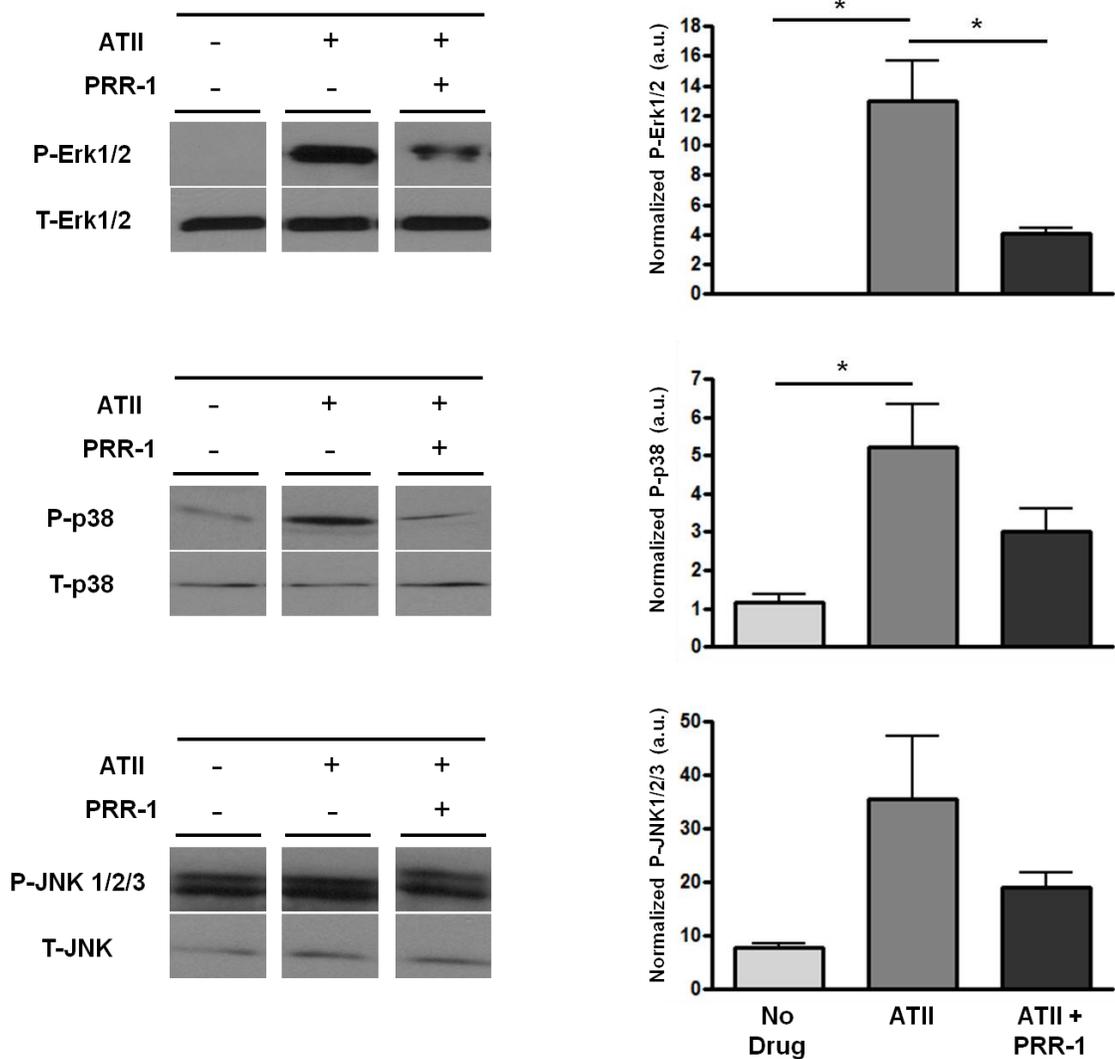


Figure 19. Analysis of MAPK phosphorylation and activation. Phosphorylation of Erk1/2, P38 and JNK was increased in WT mice that received continuous infusion of angiotensin II. However, concomitant administration of PRR-1 peptide along with angiotensin II was associated with reduced activation of the three MAPKs, especially Erk1/2 phosphorylation.

D. DISCUSSION

Our findings indicate that modulation of Pak-1 activities by PRR1 significantly attenuates angiotensin II induced ventricular hypertrophy and fibrosis in mouse. It is known that angiotensin II stimulation induces an increase of protein phosphorylation on different targets including MAPKs and myofilament proteins in the heart¹¹⁷⁻¹²⁰. In addition, protein phosphatase 1 (PP1) and protein phosphatase 2A (PP2A), two major types of phosphatases in the heart¹²¹, dephosphorylate signaling molecules including Erk1/2¹²². Our laboratory has previously demonstrated that Pak-1 induces activation of PP2A in cardiomyocytes with consequent modulation of myofilament phosphorylation and sensitivity to Ca²⁺^{31, 50} and that Pak-1 has an important role in cardiac hypertrophy. On the contrary, PP1 is regulated in the heart by RhoA/Rock signaling; phosphorylation of PP1 by Rho/Rock reduces its phosphatase activity¹²³. The results presented indicate that by achieving modulation of Pak-1 signaling by bioactive peptides it is possible to achieve significant inhibitory effect on angiotensin II induced ventricular hypertrophy and fibrosis. Our current hypothesis is that the PRR-1 peptide works through activation of Pak-1 since the peptide had no inhibitory effect on either fibrosis or hypertrophy in Pak-1-KO mice²³. The evidence provided here may indicate alternative pathways to AT₁-receptor antagonism to modulate the renin-angiotensin-aldosterone system. Earlier observations reported that antibody targeting the first proline rich region of Pak-1 activates the Pak-1 kinase activity¹²⁴. Previously, published evidence evidenced that the PRR-1 peptide had a role on formation of growth of new blood

vessels ²³, and that a peptide derived from the Cdc42 switch I region also showed similar inhibitory effects on the development of cardiac hypertrophy. It is possible that the PRR-1 as well as switch I and KI peptides used in this study may release the autoinhibition and activate Pak-1 by interacting with Pak-1 dimer. The PRR-1 peptide did not completely inhibit the AT-II effect in mouse ventricle possibly because the PRR-1 peptide is three times larger than AT-II and it must pass cell membrane to directly interact with Pak-1 ³¹.

V. EXPRESSION OF P21-ACTIVATED KINASE IS ASSOCIATED WITH EFFICIENT CARDIAC CONTRACTILITY AND LONGER SURVIVAL OUTCOME AFTER POST-ISCHEMIC CARDIAC REMODELING

A. Introduction

An important aspect in my studies was to determine the yet unexplored functional significance of Pak-1 in relation to post-ischemic myocardial remodeling. MAPKs and Wnt/ β -catenin signaling have been implicated in signaling during maladaptive remodeling following and ischemic insult^{35, 125, 126}. For example, Pak-1 has been shown to be an upstream mediator of JNK, which is activated during myocardial I/R and leads to cardiomyocyte death¹²⁷. Activation of p38 α MAPK and JNK1/2 promote pathological cardiac remodeling that includes cardiomyocyte apoptosis in the infarct border zone, infarct expansion, and fibrosis at the site of the infarction and in the unaffected myocardium³⁵. Erk1/2 is believed to be intimately related to the PKC family, which is ultimately responsible for phosphorylating myofilament proteins and for contributing to the development of myofilament dysfunction observed in cardiomyocytes with progression to heart failure¹²⁸. Inhibition of the Wnt/ β -catenin canonical pathway results in attenuated cardiac hypertrophy, and a direct binding between Pak-1 and β -catenin has been demonstrated in non-cardiac systems¹²⁹. Our lab previously reported that Pak-1/PP2A activation increases myofilament Ca²⁺-sensitivity in rat myocytes. Also, enhanced myofilament Ca²⁺-sensitivity is cardioprotective in post-ischemia myocardial dysfunction⁹⁶. We reasoned that the actions of Pak-1 on the MAPKs and Wnt/ β -catenin may be important in

understanding dysfunction of myocardial contractility after ischemic injury, as well as in identifying novel therapeutic strategies.

Therefore, our objective is to determine the functional significance of the Pak-1/MAPKs and Wnt/ β -catenin signaling cascades during post-ischemic myocardial remodeling to promote amelioration of the depressed myocardial mechanics. Our approach involves the use of a Pak-1-KO mouse model of post-ischemic myocardial remodeling obtained through irreversible left anterior descending (LAD) coronary artery ligation for 10 weeks. This knowledge will advance the field by providing a significant contribution in the understanding of Pak-1's role in regulating maladaptive control of cardiac contractility.

B. Materials and Methods

Experiments were conducted in accordance with the Institutional Animal Care and Use Committee and National Institutes of Health guidelines.

1. Animals

Fifty eight 3–4 mo old, sexually mature female and male FVB WT or Pak-1-KO mice weighing 20–25 g were used: 26 females and 32 males. Six females were designated as control without coronary artery ligation and 20 females underwent coronary artery ligation. There were 2 acute deaths, but 18 (90%) of female mice survived to their respective end points. Seven males were designated as control and 25 males underwent coronary artery ligation. In the males, there were 3 acute deaths, 1 mouse died 1 wk post-MI, but 21 (~88%)

mice survived to their respective end points. On the basis of post-procedural inspection, 2 (~8%) males did not develop MI, and an additional two mice (one control and one 4 wk post-MI) did not yield PV loops with an end-systolic pressure higher than 60 mmHg in the open chest configuration, so they were excluded from final analysis. There were eight experimental groups of female and male mice ($n = 6$): control, 2 wk, 4 wk, and 10 wk post-MI. Mice were kept in a temperature- and humidity-controlled environment with standard chow and water given ad libitum and on a 12:12-h light-dark cycle.

2. Surgical Preparation

Anesthesia was induced with 3% isoflurane inhaled in a closed chamber. After intubation, mice were connected to a rodent ventilator with a stroke volume 50 ml/min and respiration rate 135 breaths/min. A plane of anesthesia for surgery was maintained by delivery of 1.5% isoflurane through a vaporizer with 100% oxygen. Both coronary artery ligations and pressure volume loop measurements were performed under the same anesthesia, and a Zeiss dissecting microscope maintaining aseptic conditions on a heated surgical pad at 40 C.

3. Myocardial Infarction

A left thoracotomy was performed and the left main coronary artery ligated with 8-0 prolene suture 1–2 mm below the ostium as previously described. Myocardial blanching indicated a lack of perfusion. The chest cavity

was closed in three layers (intercostal muscles, pectoral muscles, and skin) with 7-0 silk sutures. Mice were gradually weaned off the respirator, and once spontaneous respiration resumed, the endotracheal tube was removed and were placed in a cage on a heating pad until fully conscious.

4. Transthoracic Two-Dimensional And M-Mode And Pulsed Doppler Echocardiography

Induction and maintenance of anesthesia were performed as indicated above. Mice were placed in the dorsal decubitus position on a warming pad to maintain normothermia. Transthoracic two-dimensional, M-mode and pulsed Doppler images were acquired by using a high-resolution echocardiographic system (VeVo 770, Visual Sonics, Toronto, ON, Canada) equipped with a 30-MHz mechanical transducer. Echocardiographic measurements were performed in all groups, before and eight days after miniosmotic pump implantation. All measurements were taken in compliance with the American Society of Echocardiography guidelines. Results were based on the average of at least three cardiac cycles.

5. Statistics

Data were statistically analyzed using either Student's t-test or one-way ANOVA followed by Holm-Sidak test where appropriate. The survival time in different experimental groups was calculated by Kaplan-Meier analysis and compared for statistical significance by using the Log-Rank test. $P < 0.05$ is the criterion for statistical significance. Data are reported as means \pm SEM.

C. Results

1. Transthoracic Two-Dimensional, M-Mode, and Doppler Echocardiography and Survival Analysis

Transthoracic two-dimensional, M-mode, and Doppler echocardiography were performed in WT and Pak-1-KO mice. M-mode measurements and transmitral flow velocities were obtained from parasternal short-axis view and reported in Supplemental Table 1. Our data demonstrate that 10 weeks following irreversible ligation of the LAD coronary artery, Pak-1-KO hearts showed reduced global systolic and diastolic function (\downarrow EF, \downarrow FS, \uparrow ESV and EDV, \downarrow DT) and reduced LV mass compared to WT, indicative of impaired cardiac remodeling. The reduced amount of viable myocardium results in a restrictive mitral inflow pattern, which, in turn, results in poorer survival outcome in Pak-1-KO mice than in WT (Fig.14).

2. Co-Immunoprecipitation

We demonstrated that Pak-1 and β -catenin establish a protein-protein interaction in LV cardiac tissue, as assessed by co-immunoprecipitation in whole lysates obtained from WT (Figures 15).

3. Western immunoblotting analysis of MAPKs

Preliminary western blotting analysis illustrates that Erk5 is increased in Pak-1-KO hearts after 10 weeks from LAD coronary artery ligation. P-p38 is also increased in Pak-1-KO mice.

D. DISCUSSION

A role for Pak-1 in relation to β -catenin/Wnt has been suggested in the literature; however, this relation in the heart has never been explored so far. It has been suggested that Pak-1 functions as a novel signaling mediator for insulin and Wnt crosstalk in PI3Kdependent but Akt/PKB-independent manner. Pak-1 knockdown using dominant negative Pak-1 (K299R) or Pak-1 shRNA resulted in significantly reduced effects of insulin on proto-oncogene expression, nuclear β -catenin content and binding of β -catenin to the c-Myc gene promoter¹³⁰. Pak-1 was initially recognized as a downstream target of the Rho family GTPases, Rac1 and Cdc42, in regulating actin remodeling^{16, 25, 131}. It is also known that Pak-1 is involved in the development and metastasis of tumors from various tissues including the intestine. Elevated Pak-1 expression is observed in a number of cancers and has been coined as a potential malignancy marker. Recently, Pak-1 over-expression was shown to be correlated with intestinal tumor metastasis¹³². The role of Pak-1 in insulin-mediated β -catenin signaling may provide a possible explanation for observed correlation between Pak-1 expression levels in neoplastic tissues.

However, further studies are needed to clarify the potential effects of Pak-1 and possible involvements of Wnt/ β -catenin pathway components in this process.

	Baseline		10 weeks after LAD coronary artery ligation	
	WT (N = 13)	Pak1-KO (N = 18)	WT (N = 12)	Pak1-KO (N = 11)
Heart rate, bpm	494 ± 12	459 ± 15	467 ± 20	477 ± 25
Fractional shortening, %	37.84 ± 2.02	42.16 ± 3.44	25.76 ± 3.47	13.52 ± 2.79*
Ejection fraction, %	68.35 ± 2.67	70.97 ± 4.27	49.34 ± 5.56	28.18 ± 5.42*
ESV, microL	16.32 ± 1.84	20.56 ± 4.17	32.54 ± 4.31	65.85 ± 11.37 *
EDV, microL	50.47 ± 2.83	62.08 ± 4.65 †	73.15 ± 7.33	85.02 ± 11.30
LV mass, mg	118.6 ± 2.70	129.8 ± 5.48	246.6 ± 29.75	183.5 ± 11.45 *
LVIDs, mm	2.16 ± 0.1	2.23 ± 0.19	3.0 ± 0.21	3.75 ± 0.28 *
LVIDd, mm	3.47 ± 0.08	3.76 ± 0.12	4.02 ± 0.17	4.24 ± 0.24 *
MV deceleration time, ms	13.63 ± 2.33	12.27 ± 2.27	21.32 ± 3.36	9.0 ± 2.58 *

Table 4. Echocardiographic measurements of cardiac hemodynamics of WT and Pak-1-KO mice at baseline and 10 weeks after surgical ligation of the LAD coronary artery. ESV = End-Systolic Volume; EDV = End-Diastolic Volume; LV = Left Ventricular; LVIDd = LV internal diameter in diastole, LVIDs internal diameter in systole, MV = Mitral Valve. * indicates $p < 0.05$ vs. WT after 10-week LAD coronary artery ligation. † indicates $p < 0.05$ vs. baseline WT.

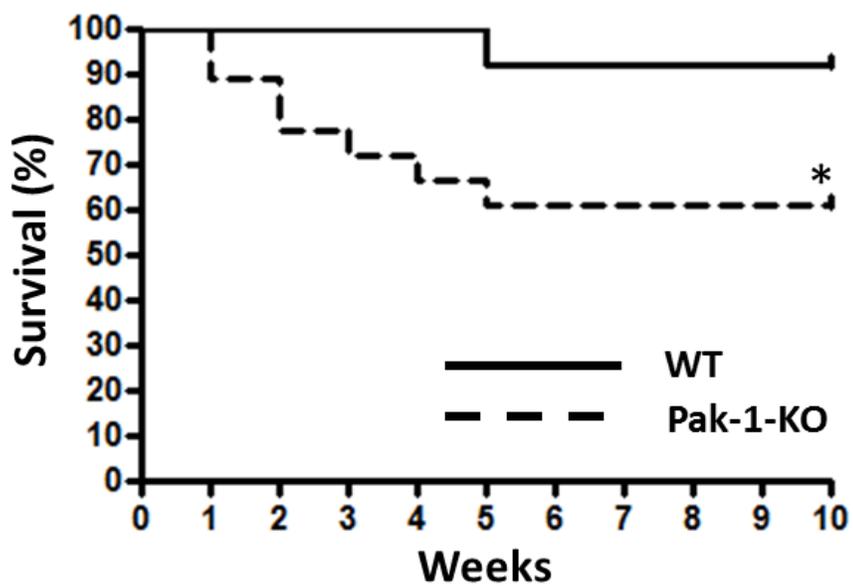


Figure 20. Kaplan–Meier Survival Analysis. Pak-1-KO mice subjected to LAD coronary artery ligation shows increased mortality vs. WT mice.

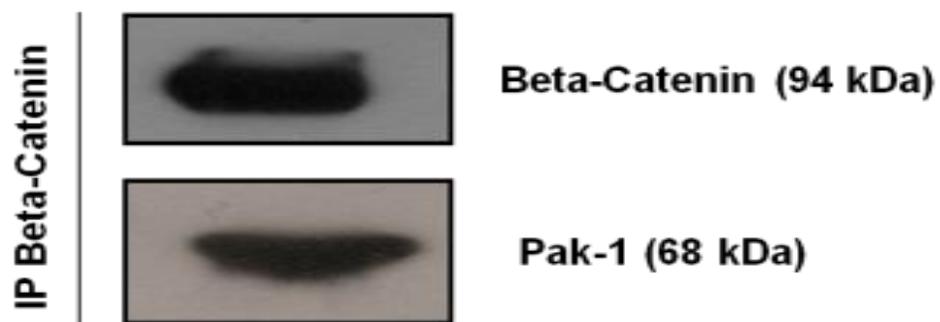


Figure 21. Protein association between Pak-1 and β -catenin. Pak-1 co-immunoprecipitates with β -catenin in WT LV tissue.

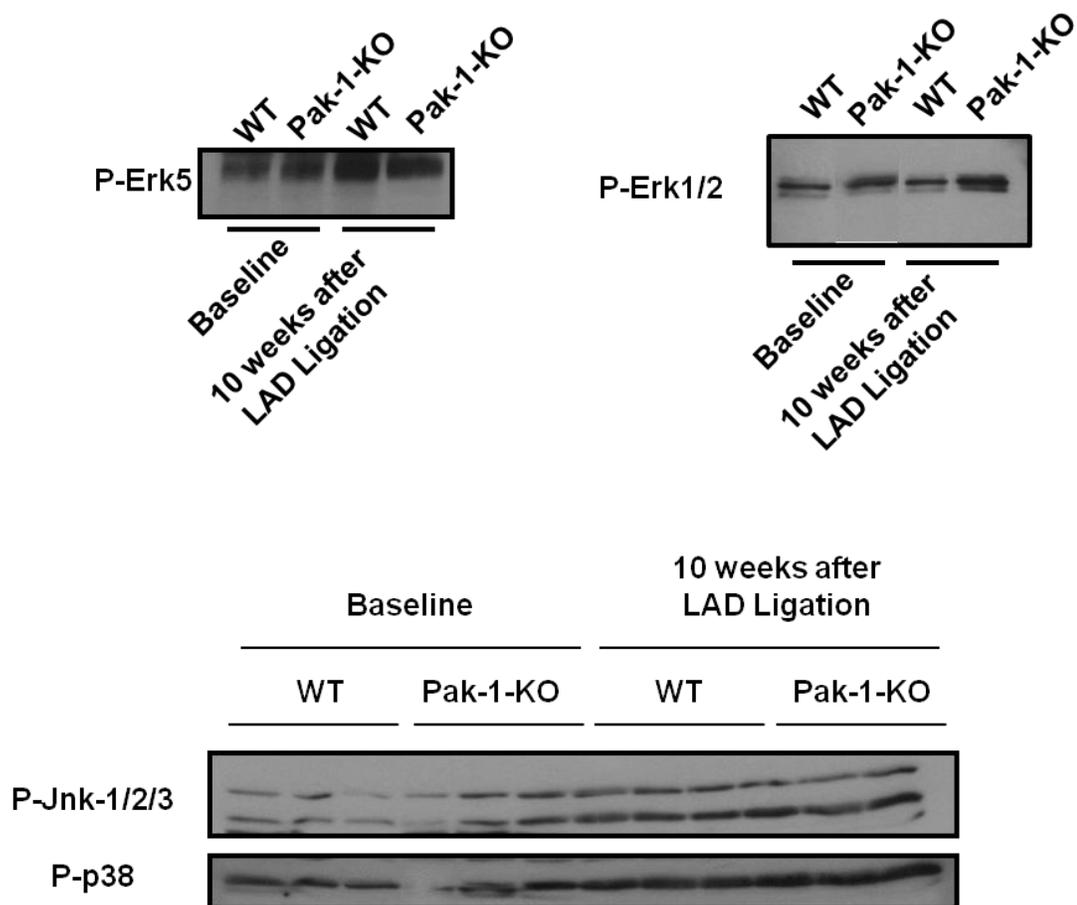


Figure 22. Western immunoblotting analysis of MAPKs. Initial findings support the idea that Erk5 may have a role in the regulation of the severe heart failure in Pak-1-KO hearts at 10 weeks after LAD ligation. Phospho-p38 may also have a role in the pathogenesis of heart failure of heart failure in Pak-1-KO mice.

VI. OVERALL DISCUSSION AND FUTURE DIRECTIONS

A. Principle Findings

In summary, the most noteworthy findings in this thesis are:

1. Pak-1 plays a significant role as a determinant of growth signaling and sarcomeric function in the myocardium.
2. Pak-1 plays a significant contribution in the mechanism of adaptive control of cardiac contractility.
3. 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.
4. Chronically ISO-treated Pak-1-KO hearts present an increased LV mass, increased systolic contractile performance, and increased LV operational stiffness.
5. Erk1/2 activation is increased by ISO stimulation in WT hearts, and further increased in ISO-stimulated hearts from Pak-1-KO mice.
6. Erk1/2 and Pak-1/PP2A bind to carry out their biological function
7. Phosphatase activity of PP2A is reduced in ISO-treated WT hearts, and further reduced in ISO-treated Pak-1-KO hearts.
8. Pak-1 has an inhibitory role on Erk1/2 activation, either directly or indirectly via PP2A.
9. Maximum Ca^{2+} -activated tension is higher in ISO-treated Pak-1-KO mice than in all other groups.

10. ISO stimulation increases phosphorylation of cTnI-S150 in hearts from WT, but not in Pak-1-KO mice, suggesting a role for Pak-1 in regulating phosphorylation at this site.
11. Pak-1 signaling acts upon myofilament proteins and is beneficial during I/R injury, in that Pak-1 reduces end diastolic pressure and increases left ventricular developed pressure after I/R injury.
12. Pak-1 improves myocardial function following I/R injury concomitant with changes in TnT and MLC2 phosphorylation.
13. PRR-1 peptide attenuates AT-II induced cardiac remodeling and fibrosis through activation of Pak-1 and dephosphorylation of Erk1/2 and possibly p38 MAPK.
14. Pak-1 is beneficial during cardiac remodeling when the left anterior descending coronary artery is ligated for 10 weeks.
15. Pak-1-KO mice had increased mortality compared to WT after the left anterior descending coronary artery was ligated for 10 weeks.
16. Pak-1 signaling is protective in multiple disease models (isoproterenol-induced hypertrophy, acute global I/R injury, and left anterior descending coronary artery ligation).
17. Our studies provide a novel target for future investigations of the molecular signaling during stress.

B. Pak-1 as a Determinant of Growth Signaling and Anti-Hypertrophic Signaling Enzyme

Chapter II describes data, which for the first time, demonstrate direct association of Pak-1 to Erk1/2 in WT hearts. 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.

It is known that in addition to growth factor-mediated signaling through RTKs, signaling via G protein-coupled receptors (GPCRs) has also been shown to promote cardiac hypertrophy, and in a number of settings, this has been shown to be mediated via Erk signaling. β -Adrenergic agonists promote cardiomyocyte hypertrophy via direct interaction between Erk and β -arrestin. Interestingly, signaling from β -adrenergic receptors, which can lead to detrimental effects in the failing heart, utilize β -arrestin to transactivate RTK signaling via Erk. This β -

arrestin-dependent, G protein-independent signaling by those receptors is thought to be cardioprotective. This is exemplified by the recent discovery that carvedilol, a nonsubtype-selective β -adrenergic receptor antagonist that has been shown to be particularly effective in treatment of heart failure, promotes signaling via β -arrestin-dependent Erk1/2 activation in the absence of G protein activation. Likewise, other GPCRs, including α -adrenergic receptors, angiotensin receptors and endothelin receptors have been shown to signal through Erk to promote cardiomyocyte hypertrophy. In addition to arrestin-mediated Erk activation, Wright et al. provided evidence that nuclear targeted α -adrenergic receptor might activate Erk located in caveolae, although the underlying molecular basis remains unclear¹³³. More recently, Lorenz et al. have identified heterotrimeric G protein-mediated autophosphorylation of Erk as yet another hypertrophic signaling mechanism leading to Erk activation. In these studies, activation of G_q -coupled receptors was sufficient to mediate a protein-protein interaction between $G\beta\gamma$ and Erk, leading to autophosphorylation and translocation to the nucleus and activation of prohypertrophic substrates. This novel autophosphorylation-mediated Erk activation was sufficient to induce hypertrophy both in vitro and in vivo and was also shown to be present in failing human hearts. Finally, a recent report by Cervante et al. suggests that cross-talk of GPCRs can be orchestrated by arrestin to achieve spatiotemporal activation of Erks in nucleus versus cytoplasm, leading to different functional outcome. In addition to ligand-mediated mechanisms, Ras activation can be facilitated by direct oxidative modification of its thiol groups, thus providing another possible

molecular link between oxidative stress and the onset of cardiac hypertrophy. In short, Ras-Raf-Mek1-Erk1/2 pathway is generally regarded as a prohypertrophic and prosurvival pathway that can be a significant but not a necessary signaling component in cardiomyocyte hypertrophy.

C. Pak-1–Mediated Phosphorylation and Regulation of Myofilament Contractility and Sarcomeric Function

1. Regulation of Cardiac TnI And TnT Phosphorylation

It is known that kinases can modulate calcium (de)sensitization of myofilament proteins¹³⁴. It is known that protein kinase A (PKA) phosphorylation of cTnI at Ser22 and 23 decreases myofilament calcium sensitivity, whereas protein kinase C (PKC) phosphorylation of cTnI at Ser41 and 43 and Thr142 lowers the maximum actin-activated ATPase activity of the myofilament in vitro¹³⁵. PKC also phosphorylates cTnT at Thr190, 199, and 280 (phosphorylation of Ser22 and 23 by PKC occurs with high concentrations of the kinase) causing inhibition of maximum actin-activated ATPase activity of the contractile apparatus, in vitro¹³⁶. These findings have suggested that novel mechanisms of modulation of calcium sensitivity during cardiac muscle contraction may involve other cellular kinases, besides PKA and PKC, in modulation of myofilament calcium responsiveness of cardiac muscle via modification of Tn. Buscemi et al. have shown that constitutively active GST-mPAK3 increases the calcium sensitivity of Triton-skinned cardiac muscle fiber bundles via a mechanism involving phosphorylation of four myofilament proteins, namely, desmin, TnI,

TnT, and an unidentified 70-kDa protein⁴⁴. These authors suggests that PAK3-induced calcium sensitization of cardiac muscle contraction may involve phosphorylation of Ser149 of cTnI which is located within the second TnC binding domain of TnI (cTnI 148–163). This region of TnI forms a calcium-sensitive interaction with the N-terminal regulatory domain of TnC. Specifically, in the presence of elevated intracellular calcium, the second TnC binding domain of TnI binds the N-domain of TnC resulting in preferential binding of the inhibitory region of TnI (cTnI 128–147) with the C-domain of TnC instead of actin-TM. The release of TnI inhibitory region (cTnI 136–147) binding to actin-TM facilitates crossbridge attachment and, ultimately, contraction. Introduction of a negatively charged phosphate group into the second TnC binding domain of cTnI may result in greater calcium affinity of cTnC via (1) increased affinity of the second cTnC binding domain of cTnI (cTnI 147–163) with cTnC and/or (2) a reduction in the calcium off-rate of cTnC. In addition desmin was found to be a substrate for PAK3 in the permeabilized skinned fiber system which has also been shown in other systems. In vitro characterization of desmin phosphorylation by PAK-1 reveals that PAK-1 phosphorylates mainly serine residues of the head region of desmin (residues 1–88). It is known that sarcomere length and interfilament lattice spacing are intimately linked to calcium sensitivity and ATPase activity of muscle fiber strips. It is also known that TnT can be phosphorylated by PAK3 in the permeabilized cardiac fibers, in isolation, and within the context of a biochemically reconstituted system and may, therefore, be involved in a mechanism of PAK-induced calcium sensitization of force. Identification of amino

acid residues phosphorylated by PAK3, in vitro, as determined by MALDI-TOF mass spectrometry, revealed that hcTnT is phosphorylated in both the N- and C-terminal domains.

2. Regulation of Myosin Light Chain 2 Phosphorylation

Another prominent Pak downstream target includes myosin light chain 2 (MLC2). Phosphoryl group transfer to MLC2 is controlled by a distinct cardiac myosin light chain kinase (cMLCK) whereas dephosphorylation is regulated by PP2A or a specific myosin phosphatase¹³⁷. Our lab has shown that ablation of MLC2 phosphorylation decreases ventricular power, lengthens the duration of ventricular ejection, and may also modify other sarcomeric proteins (e.g. cardiac troponins) as substrates for kinases and phosphatases³². In addition, myofilament calcium sensitivity increases when MLC2 is phosphorylated, a mechanisms that may contribute to the improvement of myocardial contractility during I/R injury. However, even though our knowledge on MLC2 regulation by upstream kinases and phosphatases in normal hearts is progressing, the significance of MLC2 phosphorylation in hearts subjected to I/R injury has not yet been fully understood. Studies now included in the current series of investigations indicate a significant reduction in myosin light chain-2 (MLC2) phosphorylation after ischemia and reperfusion injury in Pak-1-KO mice, compared to wild-type mice subjected to the same ischemic insult. Also, recovery of left ventricular developed pressure is significantly less in Pak-1-KO mice compared to WT. Our lab has shown that reduction in MLC2 phosphorylation

promotes severe myocardial dysfunction, providing deep insight into the understanding of MLC2 regulation of myocardial contractility. Previously, we provided evidence that increased MLC2 phosphorylation results in increased myofilament calcium sensitivity, a mechanism that could be exploited to improve myocardial function³⁴. Since it is not possible to investigate phosphorylation of every myofilament protein in a single study, we have conducted preliminary studies to shed light on which proteins should be studied in more detail first. Therefore, in the current proposal we have specifically aimed at understanding the relationship between Pak-1 and MLC2, given our strong expertise in MLC2 regulation and Pak-1/PP2A signaling. We propose to use a drug which targets Pak-1 to increase MLC2 phosphorylation and ameliorate functional outcome after ischemic injury. We believe this gain-of-function approach complements well our loss-of-function studies with the intent to elucidate the relationship between the Pak-1 signaling and the force-regulating protein MLC2. In conclusion, under normal conditions of the heart, Pak may be involved in balancing the effects of the calcium-desensitizing kinases, such as PKA and PKC, so as to fine-tune the myofilament response to calcium. In support of such an interaction, it has been demonstrated that PKA negatively regulates PAK in nonadherent cells, inhibiting MAPK activation, in vivo. Under pathological conditions of the heart, such as heart failure, it is possible that PAK acts to increase force production via two mechanisms, namely, increased calcium sensitivity of the myofibril apparatus and morphological alterations of the cytoskeleton so as to promote protective remodeling of the heart.

D. The Role of Pak-1 In Acute Myocardial Ischemia and Post-Ischemic Remodeling

1. Pak-1 in Acute Global Ischemia and Reperfusion Injury

Chapter III describes data demonstrating the beneficial effects of the Pak-1 signaling pathway, which improves myocardial performance concomitant with phosphorylation changes of troponin-T and myosin light chain 2 during global myocardial ischemia and reperfusion injury. In the absence of Pak-1, there was an exacerbation of the increased end diastolic pressure and reduced left ventricular developed pressure occurring after I/R injury. Thus, results of our study provide a basis for targeting a novel pathway including Pak-1 in the therapies for patients with ischemic events.

The main emphasis of the data in chapter III is to demonstrate that Pak-1 plays a role in I/R injury. Future studies will investigate the other mechanisms by which protein phosphorylation can be altered during ischemia/reperfusion. Pak-1 is involved in a number of cell types and signaling pathways, and has been shown to form a complex with an important cytoskeletal protein¹¹³. Future studies will distinguish the individual effects of these various signaling proteins and the crosstalk which occurs between pathways.

2. Pak-1 in ischemia and long-term myocardial remodeling

One subject of current and future studies is the important aspect of Pak-1 signaling in its potential role in relation to Mitogen-Activated Protein Kinases (MAPKs, namely Erk1/2/5, p38 MAPK, and JNK1/2/3) and Wnt/ β -catenin signaling during post-ischemic myocardial remodeling¹³⁸. We have preliminarily begun to study the direct role of Pak-1/PP2A signaling in I/R injury and/or post-ischemic cardiac remodeling and the relation between Pak-1/PP2A and the MAPKs and Wnt/ β -catenin signaling cascades in this process. Pak-1 has been shown to be an upstream mediator of JNK^{99, 100}, which is activated during myocardial I/R and leads to cardiomyocyte death¹⁰¹. Activation of p38 α MAPK and JNK1/2 promote pathological cardiac remodeling that includes cardiomyocyte apoptosis in the infarct border zone, infarct expansion, and fibrosis at the site of the infarction and in the unaffected myocardium. Erk1/2 is believed to be intimately related to the PKC family¹³⁹, which is ultimately responsible for phosphorylating myofilament proteins and for contributing to the development of myofilament dysfunction observed in cardiomyocytes with progression to heart failure^{140, 141}. Inhibition of the Wnt/ β -catenin canonical pathway results in attenuated cardiac hypertrophy^{142, 143}, and a direct binding between Pak-1 and β -catenin has been demonstrated in non-cardiac systems^{129, 130}. Our lab previously reported that Pak-1/PP2A activation increases myofilament Ca²⁺-sensitivity in rat myocytes³¹. Also, enhanced myofilament Ca²⁺ sensitivity is cardioprotective in post-ischemia myocardial dysfunction⁹⁶.

E. Future Directions

1. Identification of Pak-1 Regulation of the MAPK Signaling

We reasoned that the actions of Pak-1 on the MAPKs and Wnt/ β -catenin may be important in understanding dysfunction of myocardial contractility after ischemic injury, as well as in identifying novel therapeutic strategies. Our first future objective is to determine the functional significance of the Pak-1/MAPKs and Wnt/ β -catenin signaling cascades during post-ischemic myocardial remodeling to promote amelioration of the depressed myocardial mechanics. Our approach involves the use of the Pak-1-KO mouse model of post-ischemic myocardial remodeling obtained through irreversible left anterior descending (LAD) coronary artery ligation for 10 weeks. This knowledge will advance the field by providing a significant contribution in the understanding of Pak-1's role in regulating maladaptive control of cardiac contractility.

To generate a mouse model of post-ischemic cardiac remodeling, 3- to 4-month-old WT and Pak-1-KO mice were randomized into one of the following groups to receive irreversible LAD coronary artery ligation for 10 weeks:

- ❖ “GROUP 1” and “GROUP 2”: represent WT mice whose hearts have been excised at baseline, immediately after LAD coronary artery ligation or sham operation, respectively.
- ❖ “GROUP 3” and “GROUP 4”: represent Pak-1-KO mice whose hearts have been excised at baseline, immediately after LAD coronary artery ligation or sham operation, respectively.

- ❖ “GROUP 5” and “GROUP 6”: represent WT mice whose hearts have been excised after 10 weeks since LAD coronary artery ligation or sham operation, respectively.
- ❖ “GROUP 7” and “GROUP 8”: represent Pak-1-KO mice whose hearts have been excised after 10 weeks since LAD coronary artery ligation or sham operation, respectively.

So far we have assessed by Doppler echocardiography, 1 week before and 10 weeks after coronary ligation, and long-term survival was also assessed. Table 3 illustrates hemodynamic data which demonstrate that Pak-1-KO hearts showed reduced global systolic and diastolic function (reduced ejection fraction and fractional shortening, increased end-systolic and end-diastolic left ventricular volumes and diameters, and reduced transmitral early filling deceleration time) and reduced LV mass compared to WT, indicative of impaired cardiac remodeling and severe dysfunction. The reduced amount of viable myocardium was associated with a restrictive mitral inflow pattern, which, in turn, resulted in poorer survival outcome in Pak-1-KO mice than in WT, as demonstrated by Kaplan–Meier survival analysis (Figure 14). To test whether Pak-1 has a role in MAPK and Wnt signaling pathways, we analyzed the association of Pak-1 with Erk1/2, PP2A, and β -catenin in myocardial tissue crude lysate by co-immunoprecipitation³¹. Co-immunoprecipitation analysis showed an association between Pak-1, Erk1/2 and PP2A (Figure 15A and 15B), as well as between

Erk1/2 and PP2A (Figure 15C). Finally, Pak-1 associates with β -catenin (Figure 15D).

Therefore we have hypothesized that Pak-1 is at the center of signaling cascades controlling stress-induced cardiac growth. Future investigations will aim to determine the role of Pak-1 during post-ischemic myocardial remodeling in regulating cardioprotective signal transduction pathways that promote amelioration of the depressed myocardial mechanics.

We propose to test whether Pak-1 is a regulator of the Erks. The Mek5-Erk5 axis is currently considered a major mediator of eccentric hypertrophy leading heart failure¹⁴⁴. A dual approach will allow the identification of the effects of Pak-1 on Mek5-Erk5 by using a gain-of-function approach (constitutively active Pak-1)³¹ and a loss-of-function approach (Pak-1-KO mouse model) to identify a possible role for Pak-1 as a regulator of the Mek5-Erk5 axis. We propose to infect isolated mouse cardiac myocytes with an adenoviral vector expressing constitutively active Pak-1 and assess the level of phosphorylation and activation of Mek5-Erk5 by WB. Similarly, we will explore Mek5-Erk5 axis by WB in the perilesional tissue of the hearts obtained from the groups described above. The viable myocardium will be identified by Evans blue staining¹⁴⁵. Our preliminary studies support the idea that the phosphatase PP2A may have a role in regulating the phosphorylation levels of the Erks, as an association exists between the two proteins (Figure 6C). Therefore, I plan to assess the level of PP2A activation by measuring phosphorylation at Y307, since phosphorylation at this amino acid is a reporter of PP2A relative activity³¹. A similar approach will be

used to identify the functional relation of p38 to Pak-1, which plays an important role in cardiac remodeling after injury. We will perform histological and myofilament phosphorylation analysis, since p38 can regulate extracellular matrix remodeling and myocyte contractility ⁴⁶.

2. Identification of Pak-1 Regulation of the Wnt Signaling

We propose to assess the levels of phosphorylation of β -catenin in the perilesional tissue of the hearts obtained from the groups described above. Since PP2A is part of the destruction complex that regulates β -catenin phosphorylation and since Pak-1 associates with PP2A (Figure 6B)³¹, we will investigate PP2A activation by WB. We will analyze the subcellular distribution of β -catenin and Pak-1 as literature would suggest a possible role for these two proteins as regulators of gene expression ¹²⁹. To do so, we will adapt a protocol of subcellular separation based on differential centrifugation currently in use in our lab ¹⁴⁵. To assess the relative abundance of Pak-1 and β -catenin in the cytosol and in the nucleus, we will compare the levels of phosphorylated and total expression of these proteins in the nuclear, cytoplasmic, and sarcomeric enriched fractions of ischemic and non-ischemic tissues. If Pak-1 and β -catenin relative abundance in the nuclear-enriched fraction will be significantly changed, DNA microarray analysis of gene expression will be performed. The role of Pak-1 in relation to non-canonical Wnt pathways will also primarily focus on regulators of cytoskeleton remodeling. Measurements of RNA expression of genes that regulate cytoskeleton remodeling will be obtained for all experimental groups by

RT-PCR. Phosphorylation and activation of cofilin will be assessed at Ser3¹⁴⁶, JNK1/2 at Thr 183 + Tyr 185, and CaMKII at Thr286 by WB, all of which are indicators of modulation of cytoskeleton dynamics. A gain-of-function approach (by using constitutively active Pak-1) will be employed in cultured adult mouse cardiac myocytes to discern whether Pak-1 has a role in modulating these kinases. Cultured adult mouse cardiac myocytes will be used to assess the role of profilin in relation to Pak-1, employing immunofluorescence analysis of cardiomyocytes isolated from WT and Pak-1-KO mice to analyze changes in actin polymerization. We anticipate that Pak-1 plays a role as an inhibitor of the Erks. In this view, we predict that in the absence of Pak-1, the levels of expression of Erk5 will be increased. A future direction is a pharmacological approach aimed at employing a specific inhibitor of Erk5 such as BIX02189 in WT and Pak-1-KO mice¹⁴⁷ to rescue the progression towards heart failure. In our chronic model of angiotensin-induced cardiac remodeling (Y Ke and RJ Solaro, in preparation), we demonstrate that activation of Pak-1 inhibits MAPK activation and alleviates myocardial dysfunction. We predict that in our Pak-1-KO mouse model of post-ischemic cardiac remodeling MAPKs are increased. Previously we have demonstrated that in the absence of Pak-1, PP2A is inactive¹⁰². Therefore one could anticipate β -catenin phosphorylation levels are increased in Pak-1-KO mice, a mechanism that leads to β -catenin ubiquitination, proteasome degradation, and repression of gene expression. Evidence exists that the Rac1/Pak-1/LIMK1 signaling pathway controls cofilin activity¹⁴⁶. Therefore, we

anticipate that in the absence of Pak-1, the severing activity of cofilin is increased or deregulated since LIMK phosphorylation at Ser3 of cofilin is reduced.

F. Concluding Remarks

Coordination of Pak-1 and PP2A activities is not only potentially involved in regulation of normal cardiac function, but is likely to be important in pathophysiological conditions. Further studies will provide further insight into the mechanism on the cascades signaling through PP2A by Pak-1, which also add a new dimension to the search for potential therapeutic agents for cardiac disorders.

VII. List of References

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VIII. Appendix

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Office of Animal Care and Institutional
Biosafety Committee (M/C 672)
Office of the Vice Chancellor for Research
206 Administrative Office Building
1737 West Polk Street
Chicago, Illinois 60612

October 6, 2011

R. John Solaro
Physiology & Biophysics
M/C 901

Dear Dr. Solaro:

The modifications requested in modification indicated below pertaining to your approved protocol indicated below have been reviewed and approved in accordance with the Animal Care Policies of the University of Illinois at Chicago on **10/05/2011**.

Title of Application: Modulation of Calcium Control of Cardiac Myofibrils

ACC Number: 09-003

Modification Number: 09-003-16

Nature of Modification: *Personnel addition: Niambe Scruggs, Lindsey Duncan*
PI is requesting 315 rats (number of rats requested were 660, but was prorated to 315, since the protocol will expires in less than 6 months), to isolate cardiomyocytes from neonatal pups. 5/litters per month (5 dams/50 pups will be needed to isolate enough cells for cultures). Cells will be used to study the down regulation of ssTnl after both. Dams will be euthanized via anesthesia with 100 mg/kg pentobarbital and cardectomy. Adult tissue will be used a controls. Pups will be euthanized via decapitation and hearts removed immediately to avoid cardiac

Protocol Approved: 2/20/09

Current Approval Period: 2/20/11 2/20/12.

Current Funding: *Portions of this protocol are supported by the funding sources indicated in the table below.*

Number of funding sources: 7

Funding Agency	Grant Title			Portion of Grant Matched
NIH	Modulation of Calcium Control in Cardiac Myofibrils			Matched
Grant Number	Current Status	UIC PAF NO.	Performance Site	Grant PI
RO1 HL022231 (years 29-33)	Funded	200604017	UIC	R. John Solaro
Funding Agency	Grant Title			Portion of Grant Matched

Phone (312) 996-1972 • Fax (312) 996-9088

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Appendix (continued)

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<i>NIH</i>	<i>Troponin Modulation In Heart Failure</i>			<i>Matched</i>
Grant Number	Current Status	UIC PAF NO.	Performance Site	Grant PI
<i>ROI HL64035</i>	<i>Funded</i>	<i>200503710</i>	<i>UIC</i>	<i>R. John Solaro</i>
Funding Agency	Grant Title			Portion of Grant Matched
<i>AHA- American Heart Association</i>	<i>Reactive Oxygen Species in Cardiac Pathophysiology: Modification of Sarcomeric Proteins</i>			<i>Matched</i>
Grant Number	Current Status	UIC PAF NO.	Performance Site	Grant PI
<i>A0810131Z</i>	<i>Funded</i>	<i>200706682</i>	<i>UIC</i>	<i>Benjamin Avner</i>
Funding Agency	Grant Title			Portion of Grant Matched
<i>AHA- American Heart Association</i>	<i>Cardiac Myofibril Regulation By Kappa-Tropomyosin: A Newly Discovered Isoform In The Human Heart</i>			<i>Matched</i>
Grant Number	Current Status	UIC PAF NO.	Performance Site	Grant PI
<i>AHA 0810134Z</i>	<i>Funded</i>	<i>200800137</i>	<i>UIC</i>	<i>Chehade Nehman Karam</i>
Funding Agency	Grant Title			Portion of Grant Matched
<i>NIH</i>	<i>Troponin Modulation In Heart Failure (Tied to Form G 09-049)</i>			<i>Matched</i>
Grant Number	Current Status	UIC PAF NO.	Performance Site	Grant PI
<i>ROI HL064035 (years 11-15)</i>	<i>Funded</i>	<i>200901189</i>	<i>UIC</i>	<i>R. John Solaro</i>
Funding Agency	Grant Title			Portion of Grant Matched
<i>NIH</i>	<i>Integrated Mechanisms Of Cardiac Maladaptation (Tied to ACC Form G 10-098)</i>			<i>Matched</i>
Grant Number	Current Status	UIC PAF NO.	Performance Site	Grant PI
<i>PO1 HL062426 (years 11-15)</i>	<i>Funded</i>	<i>200906478</i>	<i>UIC</i>	<i>R. John Solaro</i>
Funding Agency	Grant Title			Portion of Grant Matched
<i>NIH</i>	<i>Modulation of Calcium Control in Cardiac Myofibrils</i>			<i>Matched</i>
Grant Number	Current Status	UIC PAF NO.	Performance Site	Grant PI
<i>ROI HL022231 (years 33-37) Original</i>	<i>Pending</i>	<i>201101862</i>	<i>UIC</i>	<i>R. John Solaro</i>

R. John Solaro
ACC 2009003

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10/6/2011

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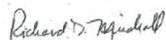
Appendix (continued)

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This institution has Animal Welfare Assurance Number A3460.01 on file with the Office of Laboratory Animal Welfare, NIH. **This letter may only be provided as proof of IACUC approval for those specific funding sources listed above in which all portions of the grant are matched to this ACC protocol.**

Thank you for complying with the Animal Care Policies and Procedures of UIC.

Sincerely yours,



Richard D. Minshall, PhD
Chair, Animal Care Committee

RDM/sj
cc: BRL, ACC File, Lindsey Duncan, Amin Rmeileh

R. John Solaro
ACC 2009003

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10/6/2011

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Appendix (continued)

UIC UNIVERSITY OF ILLINOIS
AT CHICAGO

February 25, 2009

R. John Solaro
Physiology & Biophysics
M/C 901

Office of Animal Care and Institutional
Biosafety Committees (MC 672)
Office of the Vice Chancellor for Research
206 Administrative Office Building
1737 West Polk Street
Chicago, Illinois 60612-7227

Dear Dr. Solaro:

The protocol indicated below was reviewed at a convened ACC meeting in accordance with the Animal Care Policies of the University of Illinois at Chicago on 2/17/2009. The protocol was not initiated until final clarifications were reviewed and approved on 2/18/2009. The protocol is approved for a period of 3 years with annual continuation.

Title of Application: Molecular Signaling in Cardiac Sarcomeres

ACC Number: 09-004

Initial Approval Period: 2/18/2009 to 2/17/2010

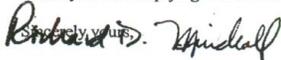
Current Funding: Portions of this protocol are supported by the funding sources indicated in the table below.

Number of funding sources: 1

Funding Agency	Grant Title			Portion of Grant Matched
NIH	Integrated Mechanisms Of Cardiac Maladaptation			Matched
Grant Number	Current Status	UIC PAF NO.	Performance Site	Grant PI
PO1HL62426	Funded	2005-05464	UIC	R. John Solaro

This institution has Animal Welfare Assurance Number A3460.01 on file with the Office of Laboratory Animal Welfare, NIH. This letter may only be provided as proof of IACUC approval for those specific funding sources listed above in which all portions of the grant are matched to this ACC protocol.

Thank you for complying with the Animal Care Policies and Procedures of UIC.

Sincerely yours,


Richard D. Minshall, PhD
Chair, Animal Care Committee
RDM/SS

cc: BRL, ACC File, Amin Rmeilch, Angela Reese, Mariam Farjah, **PAF# 2005-05464**

IX. VITA

NAME: Domenico M. Taglieri

EDUCATION: M.D. Università degli Studi Messina, 2000

Residency in Anesthesiology and Intensive Care Medicine.
Università degli Studi Trieste, 2004

Ph.D, Physiology and Biophysics, University of Illinois at Chicago,
pending

AWARDS: University of Illinois at Chicago, Center for Cardiovascular
Research Day Poster Award, 2011

International Society for Heart Research Young Investigator
Award Finalist, *Travel award to present at ISHR conference, 2011*

Mark R. Lambrecht Award for Research
*Research award to support lab expenses in basic medical
sciences, 2011*

Graduate Student Council - Travel Award

Student Activities Funding Committee (SAFC)
*Awarded while serving as Treasurer of the PBGSA to sponsor
weekly PBGSA Science Seminars at UIC, 2010*

Mark R. Lambrecht Award for Scholarship and Commitment
*Highest award bestowed on a graduate student from the
Department of Physiology and Biophysics at the University of
Illinois at Chicago, 2010*

Chancellor's Student Service and Leadership Award
*In recognition of outstanding volunteer service to the University of
Illinois at Chicago and the community, 2010*

Student Activities Funding Committee (SAFC)
*Awarded while serving as Treasurer of the PBGSA to sponsor
weekly PBGSA Science Seminars at UIC, 2010*

University of Illinois at Chicago Fellowship – University of Illinois at
Chicago, Chicago, IL, 2010

Student Activities Funding Committee (SAFC)
*Awarded while serving as Treasurer of the PBGSA to sponsor
weekly PBGSA Science Seminars at UIC, 2009*

University of Illinois at Chicago Fellowship – University of Illinois at Chicago, Chicago, IL, 2007

Young Investigator Award – American Heart Association, Chicago, IL *Top-scoring abstract, 2006*

PROFESSIONAL MEMBERSHIPS:

American Association for the Advancement of Science
2007-present, Member

American Heart Association
2005-present, Member

Biophysical Society
2007-present, Member

International Society for Heart Research (North American Section)
2011, Member

PEER-REVIEWED FULL PAPERS:

MM Monasky, **DM Taglieri**, BG Patel, J Chernoff, BM Wolska, Y Ke, RJ Solaro. P21-Activated Kinase Improves Cardiac Contractility during Ischemia-Reperfusion concomitant with changes in Troponin-T and Myosin Light Chain 2 Phosphorylation. *Am J Physiol Heart Circ Physiol*. In Press.

W Liu, M Zi, R Naumann, S Ulm, J Jin, **DM Taglieri**, S Prehar, J Gui, H Tsui, R-P Xiao, L Neyses, RJ Solaro, Y Ke, EJ Cartwright, M Lei, X Wang. Pak1 as a Novel Therapeutic Target for Anti-Hypertrophic Treatment in the Heart. *Circulation*. In Press.

DM Taglieri, MM Monasky, I Knezevic, KA Sheehan, M Lei, X Wang, J Chernoff, BM Wolska, Y Ke, RJ Solaro. 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. *J Mol Cell Cardiol* (2011), 2011 Sep 24 doi: 10.1016/j.yjmcc.2011.09.016 PMID: 21971074

S Wang, J Radhakrishnan, IM Ayoub, JD Kolarova, **DM Taglieri**, RJ Gazmuri. Limiting sarcolemmal Na⁺ Entry during Resuscitation from Ventricular Fibrillation Prevents Mitochondrial Ca²⁺ Overload and Attenuates Myocardial Dysfunction. *J Appl Physiol* 2007; 103(1):55-65. PMID: 17431086

INVITED REVIEWS: **D Taglieri**, RA Lachmann, B Lachmann (2004) Research tools for the study of therapeutic intervention in acute respiratory failure. In: Gullo A (ed) Anaesthesia, Pain, Intensive Care and Emergency Medicine - APICE 18. Springer-Verlag Italia, Milan pp. 37-61.

JJ Haitsma, **D Taglieri**, B Lachmann (2004) Present status of surfactant replacement therapy. In: Gullo A (ed) Anaesthesia, Pain, Intensive Care and Emergency Medicine - APICE 18. Springer-Verlag Italia, Milan pp. 325-340.

ABSTRACTS: MM Monasky, **DM Taglieri**, BG Patel, J Chernoff, BM Wolska, Y Ke, RJ Solaro. P21-Activated Kinase Improves Myocardial Performance through Modulation of Troponin-T and Myosin Light Chain 2 during Global Myocardial Ischemia and Reperfusion Injury. *Circulation* In Press.
This abstract will be presented at the American Heart Association Scientific Sessions.

A Labate, E Mazzon, M Mesiti, **DM Taglieri**, G Certo. Angiosarcoma Secondary to Breast Cancer with Contralateral Dorsal Recurrence. *Pathologica* In Press.
This abstract will be presented at National Annual Congress SIAPEC IAP, Palermo, Italy, October 2011.

A Labate, E Mazzon, T Leardo, S Federico, **DM Taglieri**, G Certo. Incidental Finding of Uterine Cardinosarcoma in a Young Woman: A Case Report. *Pathologica* In Press.
This abstract will be presented at National Annual Congress SIAPEC IAP, Palermo, Italy, October 2011.

A Labate, E Mazzon, G Certo, RM Mamo, **DM Taglieri**, Mazzitelli. Neuroendocrine Carcinoma of the Colon. *Pathologica* In Press.
This abstract will be presented at National Annual Congress SIAPEC IAP, Palermo, Italy, October 2011.

MM Monasky, **DM Taglieri**, BG Patel, J Chernoff, B Wolska, Y Ke, RJ Solaro. Ablation of p21-activated kinase reduces left ventricular remodeling, contractile function, and survival rates following myocardial ischemia and reperfusion injury. *J Mol Cell Cardiol* 2011: 51(3) pp. S12-13.
This abstract was presented at the International Society of Heart Research Meeting, Philadelphia, PA, May 2011.

DM Taglieri, MM Monasky, I Knezevic, M Lei, X Wang, J Chernoff, Y Ke, RJ Solaro. 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. *J Mol Cell Cardiol* 2011; 51(3) pp. S46-47. This abstract was presented at the International Society of Heart Research Meeting, Philadelphia, PA, May 2011.

Y Ke, **DM Taglieri**, RJ Solaro. Inhibition of angiotensin II-induced ventricular hypertrophy and fibrosis by modulation of Pak1 activity. *J Mol Cell Cardiol* 2011; 51(3) pp. S8. This abstract was presented at the International Society of Heart Research Meeting, Philadelphia, PA, May 2011.

DM Taglieri, II Knezevic, J Chernoff, RJ Solaro, Y Ke. Erk1-Mediated Development of Left Ventricular Cardiac Hypertrophy in a P21 Activated Kinase-1 (Pak1) Knockout Mouse Model. *Biophysical Journal* 2011;100(3) pp. 289a. This abstract was presented at the Biophysical Society Annual Meeting.

DM Taglieri, IM Ayoub, JD Kolarova, S Wang, J Radhakrishnan, RJ Gazmuri. Early Administration of Epinephrine During Closed-Chest Resuscitation Fails to Improve Return of Spontaneous Circulation or Short-Term Survival. *Circulation* 2006; 114:18:II-1207. This abstract was presented at the American Heart Association Scientific Sessions.

DM Taglieri, IM Ayoub, JD Kolarova, J Radhakrishnan, S Wang, RJ Gazmuri. Failure of Propranolol and Epinephrine Combination to Improve Resuscitability and Short-Term Survival in a Rat Model of Ventricular Fibrillation. *Circulation* 2006; 114:18:II-1206. This abstract was presented at the American Heart Association Scientific Sessions.

J Radhakrishnan, IM Ayoub, S Wang, JD Kolarova, **DM Taglieri**, RJ Gazmuri. Myocardial Protection by Glycogen Synthase Kinase-3 β Inhibition during Resuscitation from Cardiac Arrest. *Circulation* 2006; 114:18:II-606. This abstract was presented at the American Heart Association Scientific Sessions.

J Radhakrishnan, IM Ayoub, S Wang, JD Kolarova, **DM Taglieri**, RJ Gazmuri. Activation of the Mitochondrial Apoptotic Pathway Without Evidence of Apoptotic Cell Death after Resuscitation from Ventricular Fibrillation. *Circulation* 2006; 114:18:II-1194. This abstract was presented at the American Heart Association Scientific Sessions.

S Wang, J Radhakrishnan, IM Ayoub, JD Kolarova, **DM Taglieri**, RJ Gazmuri. Post-Resuscitation Mitochondrial Ca²⁺ Overload is Prevented by Limiting Sarcolemmal Na⁺ Entry During Resuscitation from Ventricular Fibrillation. *Circulation* 2006; 114:18:II-1202.

This abstract was presented at the American Heart Association Scientific Sessions.

JD Kolarova, IM Ayoub, S Wang, J Radhakrishnan, **DM Taglieri**, RJ Gazmuri. AVE4454 (a New NHE-1 Inhibitor) Administered During Resuscitation from VF Ameliorates Post-Resuscitation Myocardial Dysfunction. *Circulation* 2006; 114:18:II-1197.

This abstract was presented at the American Heart Association Scientific Sessions.

IM Ayoub, S Wang, JD Kolarova, J Radhakrishnan, D Singh, **DM Taglieri**, RJ Gazmuri. Decreased ventilatory requirements during cardiac resuscitation. *Circulation* 2005; 112:17:II-433.

This abstract was presented at the American Heart Association Scientific Sessions.