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RhoA as a mediator of cardiomyocyte survival and apoptosis

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in

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by

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Dedication

This work is dedicated to my parents. Without your steadfast support, encouragement and love, this would not be possible. And to Grace. You challenge me, humble me, lift me and inspire me daily. Thank you.

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List of Abbreviations

ABTS	2,2’-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid)
ANF	atrial natriuretic factor
Ang II	angiotensin II
APAF-1	apoptotic protease-activating factor-1
AP-1	activator protein 1
βAR	β adrenergic receptor
Bak	Bcl-2 homologous antagonist killer
Bax	Bcl-2 associated X protein
Bcl-2	B-cell lymphoma 2
BNP	B-type natriuretic protein
BSA	bovine serum albumin
CHO	Chinese hamster ovary
DISC	death-inducing signaling complex
DMEM	Dulbecco’s Modified Eagle’s Medium
dn	dominant negative
DNA	deoxyribonucleic acid
dUTP	2'-deoxyuridine 5'-triphosphate
EDTA	ethylenediaminetetraacetic acid
EGTA	ethylene glycol tetraacetic acid
ELISA	enzyme-linked immunosorbent assay

ERM	ezrin/radixin/moesin
eNOS	endothelial nitric oxide synthase
ERK	extracellular signal-regulated kinase
ET-1	endothelin
FACS	fluorescence-activated cell sorting
FAK	focal adhesion kinase
FBS	fetal bovine serum
FITC	fluorescein isothiocyanate
FoxO1	Forkhead box O1
FRNK	FAK-related nonkinase
GAP	GTPase activating protein
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GDP	guanosine diphosphate
GEF	guanine nucleotide exchange factor
Glut-1	glucose transporter 1
GPCR	G-protein coupled receptor
GSK3α/β	glycogen synthase kinase α/β
GST	glutathione S-transferase
GTP	guanosine 5'-triphosphate
H_2O_2	hydrogen peroxide
HA	hemagglutinin
HDAC	histone deacetylase
HEK	human embryonic kidney

HUVEC	human umbilical vein endothelial cell
I/R	ischemia/reperfusion
IP	immunoprecipitation
IPC	ischemic preconditioning
kb	kilobase
kD	kilodalton
LARG	leukemia-associated Rho guanine nucleotide exchange factor
LPA	lysophosphatidic acid
M	molar
MAP kinase	mitogen-activated protein kinase
mDia	mammalian diaphanous
Mdm2	murine double minute 2
MEF2	myocyte enhancer factor-2
MI	myocardial infarction
MOI	multiplicity of infection
mPTP	mitochondrial permeability transition pore
mTOR	mammalian target of rapamycin
mTORC2	mammalian target of rapamycin complex 2
MYPT-1	myosin phosphatase target subunit 1
NF-κB	nuclear factor kappa B
NO	nitric oxide
NRVM	neonatal rat ventricular myocyte
PBS	phosphate buffered saline

PCR	polymerase chain reaction
PDK1	phosphoinositide dependent kinase 1
PE	phenylephrine
PHLPP	PH domain and leucine rich repeat protein phosphatase
PI3K	phosphoinositide 3-kinase
PIP_2	phosphatidylinositol 4,5-bisphosphate
PIP_3	phosphatidylinositol 3,4,5-trisphosphate
PKN	protein kinase N
PtdIns	phosphatidylinositol
PTEN	phosphatase and tensin homolog
RBD	Rho binding domain
RhoGDI	Rho guanine nucleotide dissociation inhibitor
RNA	ribonucleic acid
ROCK	Rho-associated coiled coil containing protein kinase
ROS	reactive oxygen species
RTK	receptor tyrosine kinase
S1P	sphingosine 1-phosphate
SDS PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SHIP-1	SH2-containing inositol phosphatase 1
SHIP-2	SH2-containing inositol phosphatase 2
SRF	serum response factor
TAC	transverse aortic constriction
TG	transgenic

TMRE	tetramethylrhodamine ethyl ester
tTA	tetracycline-responsive transactivator
TUNEL	terminal deoxynucleotidyl transferase dUTP nick end labeling
UV	ultraviolet
VEGF	vascular endothelial growth factor
WT	wild-type

List of Figures

Acknowledgments

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Abstract of the Dissertation

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Heart failure is a major cause of death and a serious and growing public health problem in the United States. The development of heart failure is often preceded by cardiac hypertrophy, and the presence of hypertrophy strongly correlates with increased risk for failure. It is of great importance to continue gaining insight into the underlying mechanisms responsible for the development of both hypertrophy and heart failure, and even more so, to define signaling pathways that contribute to the transition or progression of hypertrophy to failure. RhoA has been strongly implicated in the development of hypertrophy in cultured primary cardiomyocytes. However, the role of RhoA signaling in the *in vivo* heart is not as clear. Data from our lab and others suggests that the primary role of RhoA in the heart may be the regulation of cell survival. To directly test whether RhoA can modulate cardiomyocyte survival, an adenoviral construct was used to express a constitutively active RhoA in primary

neonatal rat ventricular myocytes. Expression of RhoA resulted in robust activation of FAK, which engaged a protective signaling pathway by binding and activating PI3K and subsequently, Akt. Inhibition of the RhoA effector Rho kinase, FAK, or PI3K abolished the protection afforded by RhoA. Interestingly, however, with prolonged expression/ activation of RhoA, protective responses were lost and frank apoptosis developed. Progression from protection to apoptosis correlated with down regulation of FAK and PI3K/Akt signaling and induction of pro-apoptotic events. RhoA, specifically acting via Rho kinase, increased the expression of the Bcl-2 family protein Bax. Increases in activated Bax at mitochondria initiate apoptosis through a mitochondrial death pathway characterized by increases in cytochrome *c* release and caspase-9 and -3 activation. Thus, based on these findings we conclude that RhoA is a pleiotropic molecule that can initiate both protective and apoptotic signaling cascades in cultured cardiomyocytes. To explore the role of increased RhoA signaling in the *in vivo* myocardium, a tetracycline-inducible line of cardiac specific RhoA transgenic mice have been generated. These mice will be used to examine temporal and dose dependent protective versus deleterious effects of myocardial RhoA expression in a physiological setting.

I. Introduction

I.A. General Introduction to the Dissertation

This dissertation focuses on studying the involvement of RhoA signaling pathways in regulating cardiomyocyte survival and apoptosis. My findings demonstrate that increased RhoA expression and activation is sufficient to activate a protective signaling program that promotes cell survival. I have elucidated the mechanism that underlies this effect and demonstrate that RhoA induces cytoskeletal changes leading to focal adhesion kinase activation, culminating in activation of Akt and cardiomyocyte protection from apoptotic insult. Interestingly, however, this protective phenotype is not sustained, as chronic activation of RhoA ultimately induces programmed cell death and the initial protection afforded by RhoA is lost. My studies also elucidate the signaling mechanism responsible for the apoptotic outcome, which involves upregulation of the pro-apoptotic protein Bax and mitochondrial dysfunction. Finally, we have successfully generated an inducible transgenic mouse model that will be used to examine which of these pathways are operative in the heart *in vivo*. This introduction provides background information regarding RhoA signaling in the cardiovascular context and offers insight into the rationale guiding this dissertation research.

I.B. Introduction to cardiac hypertrophy and heart failure

Cardiac hypertrophy, or increased heart muscle mass that is distinct from normal myocardial growth, is thought to be an adaptive response to the increased demands of load or pressure which serves to preserve heart function. Currently, cardiac hypertrophy is classified as either physiologic or pathologic, and these processes are manifest in distinct ways. Physiologic hypertrophy is characterized by enlargement of the heart, with maintained or elevated cardiac function, that occurs during normal heart development and in response to physiologic stimuli such as exercise (39; 115). Pathologic hypertrophy is both structurally and functionally distinct from physiologic hypertrophy. Pathologic hypertrophy occurs in response to various stressors including hypertension, excess hormonal stimulation, or defects in contractile or calcium handling proteins, and is maladaptive. Increased fibrosis, dilation of ventricular chambers, thinning of ventricular walls, activation of the fetal gene program, and impaired contractile function are all hallmarks of pathologic hypertrophy (38). Pathologic hypertrophy may be initially compensatory, but when prolonged or chronic the processes set into motion cause decompensation resulting in heart failure (98).

Cardiac hypertrophy develops as a result of the enlargement of cardiomyocytes within the heart. Because these cells are thought to be terminally differentiated, and therefore unable to proliferate, they respond to growth stimuli through non-mitotic cell growth. Hallmarks of hypertrophy include increased cell size, increased protein synthesis, organization of myofilaments, and increased expression of embryonic genes including atrial natriuretic factor (ANF) and B-type natriuretic protein (BNP), both of which are used as markers for cardiac hypertrophy.

Heart failure is a complex disease that results from an impaired ability of the heart to fill with, or pump, blood. Failure can be caused by any of a number of structural or functional defects. Heart failure is a major health problem in the United States with approximately 5 million patients currently diagnosed with this disease (81). Main causes of heart failure include coronary artery disease, hypertension, valvular defects and dilated cardiomyopathy. Because pathologic hypertrophy leads to cardiac remodeling and dilation of ventricle chambers, it is generally seen, not only as a precursor, but a causative agent in the development of heart failure.

I.C. Introduction to RhoA

RhoA is a small G-protein with an approximate molecular weight of 21 kD. It is a member of the Ras superfamily of small G-proteins, of which there are five subfamilies (Ras, Rho, Rab, Arf and Ran). The Rho subfamily consists of Rho, Rac and cdc42. RhoA, like its other small G-protein counterparts, has intrinsic GTPase activity and is roughly half the size of the Gα subunit GTPase of the heterotrimeric G-proteins. RhoA and other small G-proteins act as molecular switches that transduce signals from the extracellular environment to inside the cell and elicit a variety of cellular responses (44).

Whether or not RhoA is active and can initiate downstream signaling events is dependent on its guanine nucleotide binding status. Rho proteins cycle between an active GTP-bound state and an inactive GDP-bound state. The activation state of RhoA is controlled by three main types of regulators; guanine nucleotide exchange factors (GEFs), GTPase activating proteins (GAPs), and GDP dissociation inhibitors

(GDIs) (Figure 1). GEFs serve to positively regulate, that is to turn on, RhoA activity by catalyzing the exchange of GDP to GTP bound to RhoA (155). There are several known Rho GEFs including p190RhoGEF, p115RhoGEF, p63RhoGEF, LARG, PDZ-RhoGEF and AKAP-Lbc (37; 53; 54; 58; 67; 91; 105; 147). Conversely, GAPs and GDIs negatively regulate, or turn off, RhoA signaling. GAPs catalyze the intrinsic GTPase activity of RhoA causing increased hydrolysis of GTP to GDP leading to inactivation of the protein. GDIs are thought to inhibit Rho signaling by preventing translocation to the plasma membrane and subsequent RhoGEF-mediated GTP loading, thereby inhibiting Rho activation.

RhoA is ubiquitously expressed and is present in all cell types in the heart, including cardiomyocytes. The same has been demonstrated for certain RhoGEFs (10; 163). There are a number of agonists that have been shown to induce RhoA activation in neonatal rat ventricular myocytes, including sphingosine 1-phosphate (S1P) (35), lysophosphatidic acid (LPA) (Del Re DP and Brown JH unpublished data), phenylephrine (PE) (29), and endothelin (ET-1) (73) (Figure 2). Whereas S1P and LPA signal through receptors that can couple strongly to Gα12/13, PE and ET-1 are ligands for GPCRs that couple primarily to Gαq. A number of RhoGEFs have been shown to interact with Gα12/13 subunits and this can explain why S1P and LPA are robust activators of RhoA. However, more recent work has demonstrated that p63RhoGEF can also couple to Gαq and is, perhaps, the link between Gαq agonists and RhoA activation (17; 27; 105; 106; 174). Additionally, mechanical stretch has been demonstrated by our group and others to activate RhoA and downstream RhoA

signaling pathways in neonatal rat cardiomyocytes (2; 88), although the GEFs involved have not been well defined.

Once activated, RhoA stimulates downstream signaling pathways. The best studied effector of RhoA is Rho kinase, or ROCK. There are two Rho kinase isoforms, ROCK1 and ROCK2, which share high amino acid sequence and structural homology, and their substrate specificity is still largely unknown (102). Activated GTP-bound RhoA binds to and activates Rho kinase (82; 97; 111), which phosphorylates multiple downstream substrates including the myosin binding subunit of myosin light chain phosphatase, MYPT-1, ezrin/radixin/moesin (ERM), LIM kinase, and rhotekin influencing cell adhesion, motility, proliferation and differentiation (89; 104; 141). Active RhoA can also bind and activate another downstream effector mammalian diaphanous (mDia) which promotes actin polymerization and rearrangement of the cytoskeleton (132).

In addition to its ability to alter structural components of the cell, a sizable literature describes the ability of RhoA to increase gene transcription. RhoA has been shown to alter both c-Jun and c-fos expression, thereby affecting AP-1 mediated gene transcription in non-muscle cells (22; 108; 109). RhoA may also regulate NF-κB induced transcription (8; 14). In cardiomyocytes, there are reports suggesting that RhoA can mediate GATA-4 activity to potentiate hypertrophic gene expression, although the mechanism of this regulation is not clear (24; 183). More recent work has suggested that RhoA may mediate MEF2 dependent gene expression by altering the localization of HDAC5 (23; 66). The ability of RhoA to modulate the activity of serum response factor (SRF) has also been demonstrated (3; 63; 74; 103; 107; 177).

Work from our laboratory suggests that this regulation may be in part through the activation of another RhoA effector, PKN (127).

I.D. RhoA-mediated hypertrophic signaling

Activation of RhoA and RhoA signaling pathways elicit a variety of cellular responses including cell migration and proliferation in dividing cells. However, in terminally differentiated cardiomyocytes, RhoA mediates cellular hypertrophy. Using neonatal rat ventricular myocytes as a system to study cardiomyocyte hypertrophy, a role for RhoA in mediating the hypertrophic effect downstream of both Gαq and Gα12/13 has been clearly demonstrated. Indeed, inhibition of RhoA, using C3 toxin or expression of a dn RhoA construct, or Rho kinase (treatment with Y-27632) is sufficient to prevent PE-induced increases in cell size, gene and protein expression, and myofilament organization (110; 149; 169; 183), with similar results obtained for ET-1 (24; 94) and angiotensin (Ang) II (9). PE, ET-1 and Ang II act primarily as Gαq agonists to induce hypertrophy. Interestingly, these agonists that couple to Gαq elicit a robust hypertrophic effect but only modestly activate RhoA. In contrast, S1P and LPA, primarily Gα12/13 agonists, are relatively weak hypertrophic agents but are very effective activators of RhoA in cardiomyocytes. Work from our lab has shown that blocking RhoA signaling attenuates the weak myocyte hypertrophy induced by LPA (73). The hypertrophic effect of mechanical stretch also appears to be RhoA dependent (2), and may be mediated in part through the regulation of ERK nuclear translocation via RhoA (88).

Several animal models have been generated to study the role of RhoA in cardiac hypertrophy *in vivo*. Using a cardiac specific RhoA transgenic mouse model, our laboratory demonstrated that expression of either wild-type or constitutively activated (L63)RhoA led to a development of dilated cardiomyopathy, accompanied by arrhythmias, severe edema and premature death (150). Strikingly, no ventricular hypertrophy was observed in these mice, either because it was very early, transient, or never induced. Another cardiac specific transgenic model utilized ectopic expression of RhoGDI to do the converse experiment, i.e. inhibit Rho activation. The RhoGDI transgenic animals did have decreased RhoA, Rac1 and cdc42 activation, although the total expression of these proteins was increased roughly 10-fold perhaps to compensate for transgene expression (176). The predominant phenotype observed was an AV conduction defect and only in aged mice did a mild ventricular hypertrophy develop. More recent work using ROCK1 hemizygous null (146) and ROCK1 knockout mice (157) demonstrated a role for this RhoA effector (and by implication, for RhoA) in the development of cardiac fibrosis in response to pressure overload or chronic agonist treatment. Again its role in mediating hypertrophy of the heart remains uncertain in part because ROCK1 was deleted from all tissues, not just the cardiomyocytes.

Another mouse model in which a GDP dissociation stimulator (Smg GDS) was globally ablated offers further insight regarding Rho signaling in an *in vivo* context (165). Smg GDS is thought to function like a GEF, so its deletion should act to limit the activation of RhoA. Few Smg GDS$^{-/-}$ mice survived postnatally and the hearts of these mice showed thinning of the atrial, ventricle and septal walls, as well as

increased apoptosis. These data indicate that decreased RhoA activation is maladaptive, suggesting that RhoA signaling could play a protective role in the heart. However, again because this was a global knockout, all cell types were affected and there could be developmental changes that occurred influencing the overall phenotype.

I.E. RhoA signaling in cell survival and apoptosis

Involvement of RhoA in mediating cell proliferation, migration, and gene expression has been well established, but there is a limited literature addressing the question of whether RhoA can influence cell survival or apoptosis. In fact, the published findings are conflicting, suggesting that the effect of RhoA on cell fate is specific to cell type, dose or duration of RhoA activation. It has been shown that blocking RhoA or Rho kinase activity through pharmacological inhibition can induce apoptosis in HUVEC (100), neuroblastoma (34) and Jurkat cells (126) suggesting a protective role for basal levels of RhoA activity. In contrast, expression of activated Gα12 or Gα13 induces apoptosis in HEK293, COS-7 and CHO cells through RhoA activation (4; 15). Ectopic expression of RhoA was also found to induce apoptosis in NIH3T3 cells implicating RhoA as a mediator of cell apoptosis (43). Prior to my thesis work, there were no published reports investigating the role of RhoA in cardiomyocyte survival or apoptosis.

I.F. Mechanisms of apoptosis

Apoptosis is defined as programmed cell death and is thought to be an evolutionarily conserved process present in all multicellular organisms. Apoptosis is critical to a variety of normal processes such as development and immunity, but also plays a role in disease states such as cancer where loss of apoptosis contributes to increased cell proliferation. An important role for apoptosis in the development of cardiomyopathy and heart failure has also been demonstrated (178). There are two mechanisms of apoptotic cell death; the death receptor or extrinsic pathway and the mitochondrial or intrinsic pathway (Figure 3). While distinct, there are also multiple levels of crosstalk between the two. The role of each pathway in executing apoptotic cell death is stimulus specific and the mitochondrial death pathway appears most relevant in cardiomyocytes.

Apoptotic signaling mediated by the intrinsic mitochondrial pathway is typically initiated by stress or toxic insult, i.e. oxidative stress, nutrient deprivation or agents that cause DNA damage, ultimately impacting mitochondrial function and/or integrity. Mitochondrial disruption results in the release of several known apoptogenic agents, among them cytochrome *c*. Cytochrome *c* facilitates the association of APAF-1 and procaspase-9, leading to cleavage and activation of caspase-9, which then cleaves and activates executioner caspases-3 and -7 causing degradation and cell death (64).

The B-cell lymphoma 2 (Bcl-2) family of proteins are known regulators of mitochondrial mediated apoptosis. The family consists of roughly 25 gene products, of which there are both pro- and anti-apoptotic members. The prototypical anti-apoptotic Bcl-2 family member is Bcl-2 itself, which is thought to prevent apoptosis

by binding to and preventing pro-apoptotic Bcl-2 proteins from translocating to and interacting with the outer mitochondrial membrane. Bcl-2 associated X protein (Bax) and Bcl-2 homologous antagonist killer (Bak) are two well studied pro-apoptotic Bcl-2 family members. Studies have demonstrated the ability of Bax and Bak to form oligomers at the outer mitochondrial membrane in response to apoptotic insult (6; 7). It is generally believed that this Bax-Bak complex inserts into the outer membrane forming a pore that mediates the release of cytochrome *c*, triggering apoptosis. The mitochondrial permeability transition pore (mPTP) is a 1.5 kD voltage-dependent channel which, when opened, triggers a loss in mitochondrial outer membrane potential, an influx of water and swelling of the mitochondrial matrix, and ultimately, rupture of the outer membrane and release of cytochrome *c* and other pro-apoptotic proteins into the cytosol (65). Whether or not Bax or Bak can interact with components of the mPTP to induce cytochrome *c* release is still uncertain.

Previous work has demonstrated that Bax is transcriptionally upregulated in response to apoptotic stimuli (61; 92). The transcription factor p53 regulates transcription of *BAX* leading to increased Bax protein expression (123). p53 is activated by a variety of stressors including DNA damage and oxidative stress, and once active, translocates from the cytosol to the nucleus where it initiates transcription. In healthy cells, p53 protein levels remain low due to proteosome-mediated degradation initiated by Mdm2. Phosphorylation of p53 disrupts Mdm2 association, allowing for increased nuclear p53 protein accumulation and increased gene transcription.

I.G. Introduction to PI3K-Akt signaling

Signaling events resulting in phosphoinositide 3-kinase (PI3K) activation and subsequent activation of Akt exert protective or pro-survival effects in a wide range of cell types including cardiomyocytes.

The phosphoinositide 3-kinases (PI3K) are a family of lipid kinases able to phosphorylate the 3' position on the inositol ring of phosphatidylinositol (PtdIns). The PI3K family is divided into classes based on substrate specificity. Class I PI3K is responsible for receptor-mediated increases in phosphatidylinositol (3,4,5)-trisphosphate (PIP_3), and will be the only class discussed here (for an excellent review of the PI3K family see (131)). Class I is further subdivided into Class IA and IB, both of which are expressed in the heart and vasculature. Class IA enzymes consist of a catalytic p110α, β, or δ subunit and an associated p85α or β regulatory subunit. The p85 regulatory subunits contain SH2 and SH3 domains that allow for their recruitment and binding to the phosphorylated YXXM motif present in receptor tyrosine kinases (RTKs). Class IA PI3K are activated downstream of RTKs and also by the βγ subunits of GPCRs. Class IB PI3K consists of the catalytic p110γ and the associated p101 regulatory subunit and is regulated by both βγ subunits and Ras. Both classes of PI3K, once activated, function at the plasma membrane to phosphorylate phosphatidylinositol (4,5)-bisphosphate (PIP_2) at the 3' position converting it to phosphatidylinositol (3,4,5)-trisphosphate (PIP_3).

Notably, the actions of PI3K are reversed by the lipid phosphatases PTEN (phosphatase and tensin homologue) and SHIP-1 and -2. PTEN acts specifically by dephosphorylating the 3' position of PIP_3 to produce phosphatidylinositol (4,5)-

bisphosphate, while SHIP-1 and SHIP-2 both hydrolyze PIP_3 at the 5' position producing phosphatidylinositol (3,4)-bisphosphate. By reducing PIP_3 levels at the plasma membrane, these phosphatases negatively regulate PI3K-Akt signaling.

Several genetically modified mouse models have been generated to investigate the *in vivo* role of PI3K isoforms in the heart. Global deletion of either p110α or p110β causes embryonic lethality at E9-10 and E3-7 respectively. Cardiac specific expression of a dn p110α construct caused a decrease in myocyte size, whereas cardiac specific overexpression of a constitutively activated p110α led to enlarged cardiomyocytes and cardiac hypertrophy (158). Studies using mice engineered to express a dominant negative p85α mutant (preventing p110α activation) selectively in the heart, had smaller cardiomyocytes and subsequently, smaller hearts. Cardiac specific expression of a dn p110γ construct showed no changes in overall heart or myocyte size (130). These mice, however, had increased contractility, whereas interfering with p110α signaling showed no change in contractile function. The basis for this unexpected observation was examined and shown to involve displacement of endogenous PI3K from β-adrenergic receptors (βAR) in the dn p110γ animals, leading to decreased receptor internalization and increased βAR signaling. Taken together, these studies suggest that PI3K Class IA signaling appears to mediate cardiomyocyte growth, whereas the Class IB isoform, p110γ, seems to regulate myocyte contractility. Global ablation of PTEN causes spontaneous cardiac hypertrophy, further implicating phosphatidylinositol signaling in cardiomyocyte growth (31).

The activation of PI3K and subsequent generation of PIP_3 at the plasma membrane creates a binding site for proteins containing a pleckstrin homology (PH)

domain. The pro-survival kinase Akt, as well as its upstream kinase, phosphoinositide dependent kinase 1 (PDK1), both contain PH domains necessary for plasma membrane association. Increased PIP_3 production causes recruitment of Akt to the membrane allowing PDK1 to phosphorylate Akt at threonine 308, partially activating the kinase and promoting further phosphorylation at serine 473, thereby rendering Akt fully active (Figure 4). The kinase(s) responsible for serine 473 phosphorylation of Akt remains controversial, however recent evidence suggests that mTORC2 is responsible (46). Recent work has also demonstrated the ability of a novel phosphatase, termed PHLPP (PH domain and leucine rich repeat protein phosphatase), to selectively dephosphorylate Akt at serine 473, thereby decreasing Akt activity and signaling (18; 57). Thus another possibility is that phosphorylation at this site is regulated in large part by this phosphatase.

Once activated, Akt is able to phosphorylate a plethora of substrates involved in various cellular processes including cell growth, proliferation and survival. A role for Akt signaling has been demonstrated in cardiac growth in several animal models, and in the heart, overexpression of Akt leads to a range of phenotypes based on the construct that is expressed. Work from the Rosenzweig laboratory has demonstrated that cardiac expression of myristolated Akt causes cardiac hypertrophy and protects the heart from ischemia/reperfusion injury (113). Expression of nuclear localized Akt in the heart also affords a protective advantage but does not induce cardiac hypertrophy suggesting that the localization of activated Akt is critical to its signaling and phenotype (159). Akt provides protection from apoptotic insult in cultured cardiomyocytes as well (112). Akt is thought to mediate cell survival in part by

regulating gene expression. Phosphorylation of the transcription factor FoxO1 by Akt leads to its cytosolic sequestration and prevents expression of pro-apoptotic factors (16; 32; 167). A role for Akt in the modulation of Glut-1, vascular endothelial growth factor (VEGF) and Bcl-2 expression has also been demonstrated (13; 84; 137). Akt signaling can regulate translational events as well via activation of mammalian target of rapamycin (mTOR) and subsequent phosphorylation of p70S6K (136). Akt also inhibits apoptosis by phosphorylating Bad and preventing downstream caspase activation (33). Akt-mediated phosphorylation of eNOS and GSK3α/β have also been shown to have protective effects in the cardiovasculature (56; 170). Further, Akt can down-regulate p53 activity through the ubiquitin E3 ligase, Mdm2, to prevent apoptotic cell death. Phosphorylation of Mdm2 by Akt causes its translocation to the nucleus allowing increased proteosomal dependent degradation of p53 (114). Recent work from our lab has also demonstrated an additional acute protective function of Akt via phosphorylation of hexokinase II at the mitochondria and subsequent inactivation of the mPTP, leading to increased cardiomyocyte survival (121).

I.H. Rationale and Significance

It is well established that RhoA can mediate a host of cellular outcomes in a variety of cell types. Specifically in cardiomyocytes, we and others have demonstrated a clear role for RhoA in mediating hypertrophic cell growth. However, the literature is strikingly lacking in terms of RhoA involvement in cell survival or apoptosis. The literature has demonstrated that cell attachment and engagement of cell surface integrins with the extracellular matrix impacts cell survival (50; 59; 60; 62;

116; 139). It is established that RhoA signaling affects the cytoskeletal architecture of the cell and could play a role in cell adhesion. Given this knowledge, it was hypothesized that increased RhoA activation could engage this pro-survival pathway. Further, because PI3K/Akt signaling is known to afford protection in cardiomyocytes, we asked whether RhoA activation could signal via this pathway to increase cardiomyocyte survival.

In contrast, based on results obtained from the cardiac specific RhoA transgenic mouse generated previously by our laboratory, which showed cardiac dilation and premature death due to heart failure (150), we also sought to investigate whether RhoA activation in the cardiomyocyte specifically could induce apoptosis. The importance of the mitochondrial death pathway in cardiomyocyte apoptosis led us to ask whether chronic RhoA activation could induce apoptosis via induction of mitochondrial dysfunction.

Determining what dictates the development of cardiac hypertrophy and heart failure, and defining the underlying signaling pathways, remains a challenge in cardiovascular research. The overall aim of this thesis is to determine whether RhoA signaling can modulate survival of cardiomyocytes and to elucidate the mechanisms responsible. In doing so, this work will serve to further our understanding of the pathways involved in the loss of cardiomyocytes in the heart, a process critical to the development of heart failure.

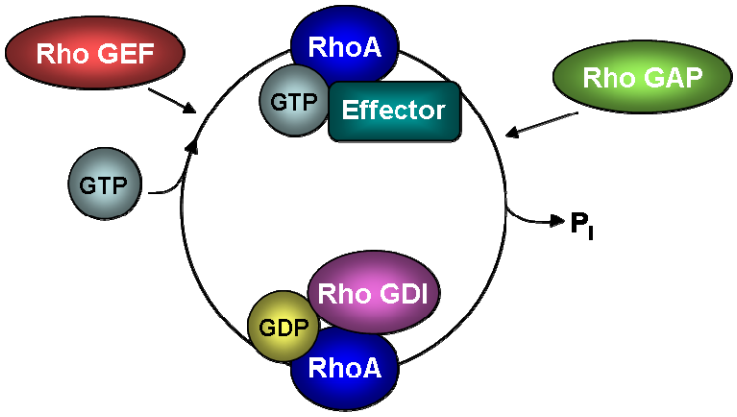

Figure 1. Schema illustrating the RhoA activation cycle. RhoA cycles between an active GTP-bound and an inactive GDP-bound moiety. Guanine exchange factors (GEFs) promote the exchange of GTP for GDP and activate RhoA. Activated RhoA can then interact with numerous effector proteins to initiate signaling. In contrast, GTPase activating proteins, or GAPs, catalyze the hydrolysis of GTP to GDP and inactivate RhoA. Rho guanine nucleotide dissociation inhibitors (RhoGDIs) bind to inactive RhoA, preventing membrane localization and thus its subsequent activation by GDP exchange.

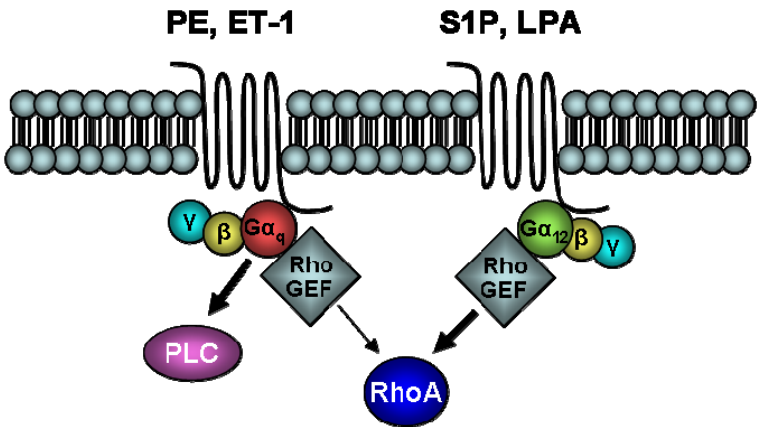

Figure 2. Agonist and associated GPCR signaling pathways mediating activation of RhoA. Ligands such as phenylephrine (PE) and endothelin (ET-1) bind GPCRs that couple strongly to Gαq heterotrimeric G-proteins. Signaling initiated by these agonists leads to robust hypertrophic responses in cultured NRVMs but relatively weak activation of RhoA. In contrast, the lysophospholipid agonists sphingosine 1-phosphate (S1P) and lysophosphatidic acid (LPA) bind receptors that couple to Gα12/13 heterotrimeric G-proteins and elicit robust activation of RhoA in cultured cardiomyocytes.

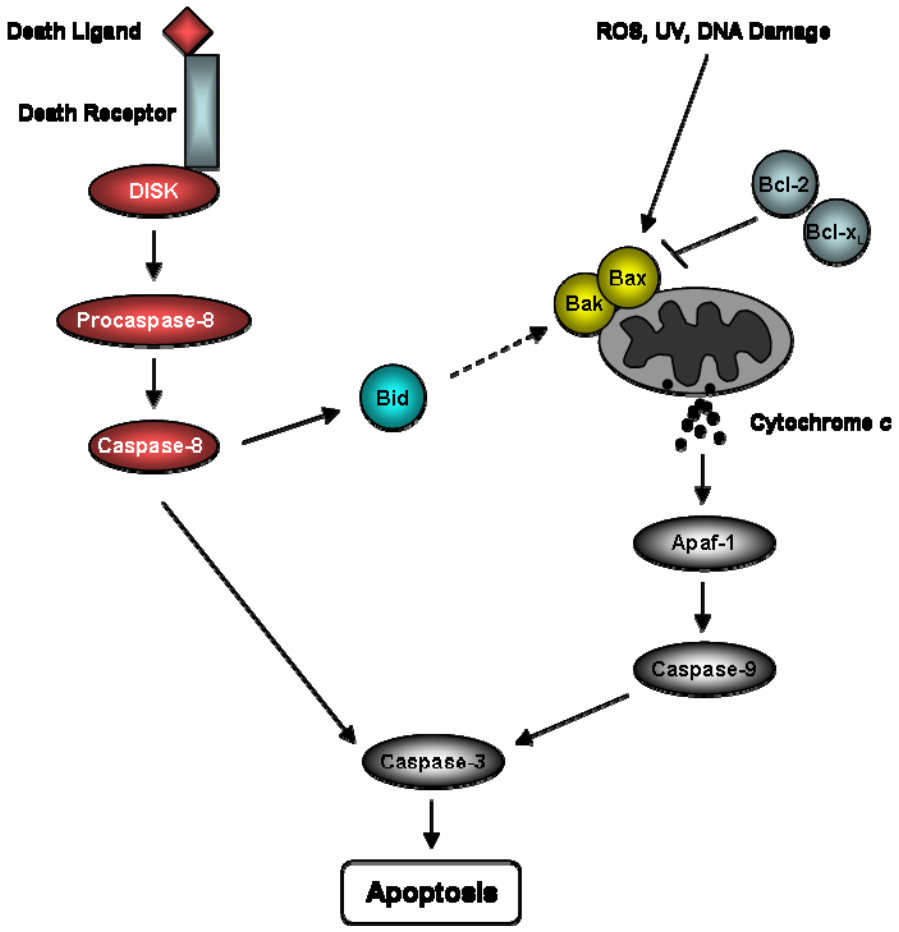

Figure 3. Apoptotic signaling cascades. The extrinsic pathway is initiated by the engagement of a transmembrane death receptor by an extracellular death ligand leading to death-inducing signaling complex (DISC) formation and autoactivation of caspase-8, the initiator caspase of the extrinsic pathway. The intrinsic pathway, also known as the mitochondrial pathway, is activated by stimuli that lead to mitochondrial outer membrane permeabilization and the release of apoptogenic factors including cytochrome *c*. Cytochrome *c* release recruits apoptotic protease-activating factor-1 (APAF-1) and forms the apoptosome, leading to caspase-9 activation. The Bcl-2 family protein BID can be activated by both pathways making it an important converging point in apoptotic signaling.

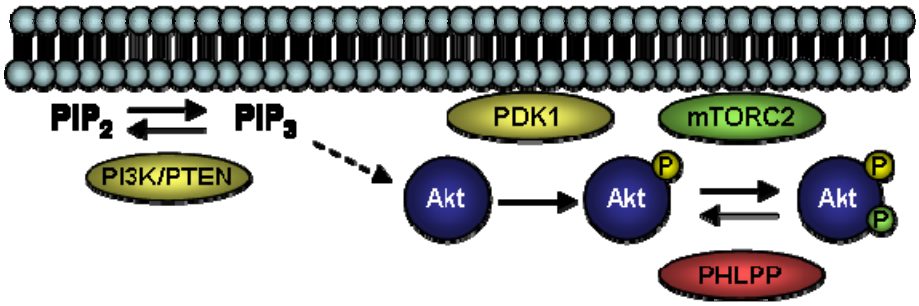

Figure 4. Regulation of Akt activation. Upon activation of PI3K and increased PIP_3 production, Akt is recruited to the plasma membrane via its PH domain, where it is phosphorylated by PDK1 at thr308 (yellow). Phosphorylation at ser473 (green) renders Akt fully active and recent work has demonstrated the involvement of mTORC2 in this phosphorylation event. Akt is selectively dephosphorylated at ser473 and inactivated by the phosphatase PHLPP. PTEN negatively regulates Akt activation by dephosphorylating PIP_3 to PIP_2.

II. Materials and Methods

II.A. Neonatal rat ventricular myocyte isolation

Neonatal rat ventricular myocytes were isolated and cultured as described previously (76). Briefly, hearts were excised from 1-2 day old Sprague-Dawley rat pups, atria removed and the remaining tissue was treated with trypsin overnight and rocked gently at 4°C. The following day, hearts were treated with collagenase for one hour at 37°C and then passed through a 70 μm strainer to remove undigested material. The cells were then pelleted using a clinical centrifuge and resuspended in medium 199 containing 15% fetal bovine serum (FBS) a total of two times. The resulting myocytes were plated onto either 3.5 cm dishes (at a density of $0.3x10^6$ cells per plate), 6 cm dishes ($0.8x10^6$ cells per plate), or 10 cm dishes ($1.5x10^6$ cells per plate), coated with 1% gelatin prior to plating. In each case, cells were incubated overnight in medium 199 supplemented with 15% FBS and antibiotics (100 units/ml penicillin and 100 μg/ml streptomycin), and then maintained in serum-free Dulbecco's modified Eagles medium (DMEM) for the duration of the experiments. This method of cell isolation results in a culture that is >95% myocytes; relatively free of contaminating fibroblasts and other cell types.

II.B. Generation of adenovirus

The (L63)RhoA adenovirus has been previously characterized (76). Briefly, the (L63)RhoA cDNA, encoding the amino acid substitution glutamate to leucine at

position 63, was excised from its pCMV backbone vector and subcloned into a pACCMV shuttle vector, between the cytomegalovirus enhancer/promoter and SV40 polyadenylation sequences. Recombinant adenovirus expressing (L63)RhoA was generated through homologous recombination in HEK293 cells via co-transfection of pACCMV-(L63)RhoA with pJM17 as described (1). The amplification of adenovirus was performed in HEK293 cells, and a CsCl gradient was used to prepare high titer adenovirus as described (1). Briefly, thirty two 15 cm plates were grown to confluence with HEK293 cells. These cells were infected using low concentrations of adenovirus for 1-3 days. When the cells were 30% lysed, the cells were scraped and the medium plus the cells were collected. Following 6 minutes of centrifugation on a clinical bench top centrifuge at the maximum setting, the medium was aspirated, and the cell pellet was resuspended in 5 ml PBS and frozen at -20°C. Before preparation of the CsCl gradient, the cell pellet was thawed in a 37°C water bath, refrozen, and thawed again. The pellet was spun down again in the clinical centrifuge and the resulting supernatant loaded onto a CsCl gradient. Following ultracentrifugation at 35,000K the purified viral band was harvested, run over desalting PD-10 columns, aliquotted, and stored at -80°C in a buffer containing glycerol and MgCl until needed.

II.C. Neonatal rat ventricular myocyte culture and infection

After the initial plating incubation in serum containing medium overnight, cells were washed and maintained in serum free medium for 2-3 hours before infection with adenoviruses at 2-10 multiplicity of infection (MOI) for 2 hours, or as indicated. Cells were subsequently washed and maintained in serum free medium for the remainder of

the experiment. The dominant negative mutant Rho kinase expressing adenovirus was a kind gift of Dr. Masahiko Hoshijima (UCSD). The adenovirus encoding a dominant negative mutant p53 was obtained from Dr. Lorrie Kirshenbaum (University of Manitoba). The FRNK expressing adenovirus was kindly provided by Dr. Robert Ross (UCSD).

II.D. Preparation of lysates for Western blot analysis

Cardiomyocytes were harvested in lysis buffer containing 20 mM Tris pH 7.6, 250 mM NaCl, 3 mM EDTA, 3 mM EGTA, 20 mM β-glycerophosphate and 0.5% Nonidet P-40 supplemented with protease and phosphatase inhibitors (100 μM Na_3VO_4, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 1 mM *p*-nitrophenyl phosphate, 1 mM phenylmethylsulfonyl fluoride, 1 mM NaF). Bradford analysis was performed to determine protein concentration. Equal amounts of protein (10-30 μg) were loaded onto SDS-PAGE, run, transferred to an Immobilon membrane, and the resulting blot probed using the following antibodies. The cleaved caspase-3, cleaved caspase-9, total Bax, phospho-Akt (T308 and S473), total Akt, phospho-ERK (p42/44), and p85 phosphorylated binding motif antibodies were purchased from Cell Signaling Technologies. The RhoA, FRNK, p85, total Src, total MYPT-1 and actin antibodies were purchased from Santa Cruz Biotechnology. The phospho-Src (tyrosine 416) antibody was purchased from Upstate Millipore. The Bak and total FAK antibodies were purchased from BD Biosciences. The Bcl-2 and Bcl-x_L antibodies were purchased from Transduction Laboratories. The mouse monoclonal anti-Bax (6A7) and phospho-FAK (tyrosine 397) antibodies were purchased from Calbiochem. The

phospho-MYPT-1 (Thr696) antibody was purchased from U.S. Biological. The HA-tag antibody was purchased from Roche Applied Sciences.

II.E. Immunofluorescence

Neonatal rat ventricular myocytes were plated on 3.5 cm dishes at a density of $0.3x10^6$ cells per plate. Following the same protocol as above, cardiomyocytes were maintained in serum free medium prior to adenovirus infection. Cells were incubated with adenovirus for 2 hours, washed, and cultured for 24 hours. Cells were washed twice with PBS and fixed with 3.7% paraformaldehyde (Sigma-Aldrich) at room temperature for 10 minutes. The cells were then permeabilized with 0.3% Triton X for 10 minutes at room temperature, washed two times with PBS, and blocked in 3% BSA for 1 hour at room temperature. Cardiomyocytes were then incubated in either anti-ANF rabbit polyclonal antibody (Peninsula Laboratories, Bachem) or anti-phospho-FAK (tyrosine 397) rabbit polyclonal antibody (BioSource) for 1 hour at room temperature or overnight at 4°C, respectively. Cells were then incubated with 10% normal goat serum for 30 minutes at room temperature before incubating with FITC anti-rabbit (Molecular Probes) and rhodamine-conjugated phalloidin (to visualize F-actin filaments) for 90 minutes at room temperature. Confocal imaging was carried out using a Bio-Rad MRC1024ES imaging system equipped with a Kr/Ar-ion laser fitted to an Olympus BX50WI inverted microscope with assistance from Drs. Matt Conklin and Nicholas C. Spitzer (UCSD).

II.F. Co-Immunoprecipitation of FAK-p85 complex

Neonatal rat ventricular myocytes were plated on 10 cm dishes and infected with control AdCMV or (L63)RhoA adenovirus for 2 hours and virus washed out. Cells were either treated with vehicle, 10 μM Y-27632 (Calbiochem) or 1 μM PF271 (gift of Dr. David Schlaepfer, UCSD) at time of washout. Twenty four hours after virus washout cells were washed with ice cold PBS twice, aspirated well and nutated with 200 μl RIPA lysis buffer at 4°C for 20-30 minutes. The lysates were then cleared and quantitated for total protein using the MicroBCA protocol (Pierce Biotechnology). 600-800 μg of protein from each treatment was incubated with 4 μg anti-FAK primary antibody (BD Transduction Laboratories) at 4°C overnight. Complexes were incubated with 100 μl of protein A/G sepharose beads (Santa Cruz Biotechnology) for 4 additional hours at 4°C before collection by centrifugation at 14,000 rpm for 1 minute. The beads were washed 3 times with ice cold PBS, once with lysis buffer, and then boiled with sample buffer for 10 minutes. Samples were resolved by SDS-PAGE and the immunoprecipitates were probed with a pan p85 antibody (Santa Cruz Biotechnology).

II.G. Precipitation of activated Bax

Neonatal rat ventricular myocytes were plated on 10 cm dishes and infected with control AdCMV or (L63)RhoA adenovirus for 2 hours and virus washed out. Cells were treated with vehicle or 10 μM Y-27632. 24 hours after virus washout, lysates were prepared in RIPA lysis buffer described above and 200 μg of total protein was precleared with Protein G-Sepharose for 30 minutes at 4°C. Samples were then incubated with 4 μg of anti-Bax monoclonal antibody (clone 6A7, Calbiochem) at 4°C

overnight. Immunocomplexes were precipitated with 100 µl of 50% slurry Protein G-Sepharose, the beads pelleted by centrifugation (14,000 rpm for 1 minute), washed three times with ice cold lysis buffer, and boiled in 2x sample buffer for 10 minutes to elute captured protein. Proteins were resolved by SDS-PAGE and probed with total Bax antibody.

II.H. Fractionation of mitochondria from neonatal rat ventricular myocytes

Fractionation was performed as described previously according to manufacturer's instructions (Cytosol/Mitochondria Fractionation Kit, Calbiochem). Briefly, neonatal rat ventricular myocytes were harvested in ice cold PBS, the cells collected by centrifugation (600 rpm for 5 minutes at 4°C), resuspended in 50 µl of Cytosol Extraction Buffer Mix, vortexed and incubated on ice for 10 minutes. Samples were then centrifuged at 2600 rpm for 10 minutes at 4°C. The resulting pellet was discarded and the supernatant was transferred to a new tube and spun at 9600 rpm for 30 minutes at 4°C to precipitate mitochondria. The supernatant was removed and analyzed as the cytosolic fraction and the pellet was resuspended in Mitochondrial Extraction Buffer Mix and analyzed as the mitochondrial fraction.

II.I. Real-time PCR for quantitative analysis of Bax mRNA

Real-time PCR was performed using pre-optimized TaqMan Gene Expression Assays to quantify Bax and GAPDH mRNA as described by the manufacturer

(Applied Biosystems). Briefly, neonatal rat ventricular myocytes were plated on 6 cm dishes and infected with the appropriate adenoviruses. Cells were then harvested either 12, 24 or 48 hours following virus washout. RNA was isolated using the RNeasy mini kit (Qiagen), and reverse transcribed to cDNA using Super Script III reverse transcriptase (Invitrogen). TaqMan Universal PCR Master Mix was used with pre-optimized TaqMan primers to generate raw data, which were quantitated using standard curves for Bax and GAPDH.

II.J. Fluorescence-activated cell sorting (FACS) analysis to detect mitochondrial membrane depolarization

As described previously by our lab (120), cells were loaded with 50 nM of the fluorescent mitochondrial potential-dependent indicator, tetramethylrhodamine ethyl ester (TMRE) (Molecular Probes) for 20 min at room temperature and collected by trypsinization (0.05% trypsin, 0.53 mM EDTA). The fluorescence intensity was monitored at 582 nm (FL-2 channel) by FACScan (BD Biosciences) (15,000 cells /sample).

II.K. Laddering assay for qualitative examination of apoptosis

Oligonucleosomal DNA fragmentation was analyzed by DNA laddering as previously described (1). Briefly, neonatal rat ventricular myocytes from 6 cm dishes were lysed and DNA isolated according to the manufacturer's instructions (Puregene DNA isolation kit, Gentra Systems). Reconstituted, purified genomic DNA was run

out on 2% agarose gels at 20 volts for 4-5 hours. The gels were then stained with ethidium bromide for 20 minutes, destained with MQ water for 10 minutes, and imaged under ultraviolet light using the AlphaInnotech imaging system.

II.L. Cell death ELISA (POD) assay for quantitative examination of apoptosis

DNA fragmentation indicative of apoptosis was assayed using the Cell Death Detection ELISA^{PLUS} (Roche Applied Science) according to manufacturer's instructions. Briefly, lysates were prepared as described above (see Western blot analysis) from neonatal rat ventricular myocytes cultured in 6 cm dishes. Lysates were cleared by centrifugation (14,000 rpm for 5 minutes) and 5 µl of the supernatant was diluted in 15 µl of lysis buffer. The immunoreagent, consisting of anti-histone-biotin and anti-DNA-POD diluted in the provided incubation buffer, was prepared and incubated with the diluted lysate in a streptavidin-coated 96 well microplate and rocked for 2 hours at room temperature, followed by 10 minutes in an orbital shaker. Wells were then washed three times with incubation buffer, 100 µl of 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) colorimetric substrate added per well using a multichannel pipet, and absorbance measured at 405 nm using a plate reader (TECAN GENios).

II.M. TUNEL staining of neonatal rat ventricular myocytes

Following viral infection of neonatal rat ventricular myocytes plated on 3.5 cm dishes, terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) was performed using DeadEnd™ Fluorometric TUNEL System (Promega) according to manufacturer's instructions. Briefly, cells were washed two times with PBS, fixed in 3.7% paraformaldehyde for 10 minutes at room temperature, and permeabilized in 0.2% Triton X-100 for 10 minutes at room temperature. Cells were labeled with fluorescein-12-dUTP for 1 hour at room temperature to visualize apoptotic nuclei and then incubated with rhodamine-conjugated phalloidin (Molecular Probes) for 1 hour at 37°C to visualize F-actin filaments.

II.N. RhoA-GTP binding domain (RBD) pulldown assay

The amount of activated RhoA was investigated by pulldown assay using the RhoA binding domain of the RhoA effector rhotekin to precipitate GTP-bound RhoA as described previously (142). Briefly, treated cells were rinsed with Tris-buffered saline and harvested in lysis buffer containing 50 mM Tris HCl, pH 7.4, 10% glycerol, 0.1% Triton X-100, 100 mM NaCl, 5 mM $MgCl_2$, 10 μg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride and 10 μg/ml leupeptin. The lysates were cleared by centrifugation (14,000 rpm, 2 min, 4°C) and then incubated with the sepharose-bound GST-rhotekin-RBD and gently rocked for 40 min at 4°C. The beads and precipitated proteins were washed four times in ice cold lysis buffer and then boiled in sample buffer and separated by SDS-PAGE. Proteins were transferred to Immobilon membranes and probed with a RhoA specific antibody (Santa Cruz Biotechnology). The precipitated RhoA was normalized to the RhoA present in the whole cell lysate.

II.O. Mouse breeding, tailing and genotyping

Briefly, tail samples were digested in lysis buffer (75 mM NaCl, 25 mM EDTA, 10 mM Tris pH 8.0, 1% SDS) and 0.4 mg/ml Proteinase K. Genomic DNA was precipitated with isopropanol and resuspended in TE buffer (10 mM Tris pH 8.0, 1 mM EDTA). To detect the RhoA transgene, PCR was performed as follows: 95°C for 30 seconds, 55°C for 30 seconds and 72°C for 30 seconds for 35 cycles with a final extension step at 72°C for 7 minutes. The following sequences were used to detect the 550 kb PCR product indicative of the RhoA transgene: 5'GCCCATCATCCTAGTTGGGAA3' and 5'GGTCATGCATGCCTGGAATC3'. To detect the tetracycline-responsive transactivator transgene (tTA), PCR was performed as follows: 95°C for 45 seconds, 65°C for 1 minute and 72°C for 2 minutes for 35 cycles with a final extension step at 72°C for 7 minutes. The following sequences were used to detect the 450 kb PCR product indicative of the tetracycline-responsive transactivator transgene: 5'AGCGCATTAGAGCTGCTTAATGAGGTC3' and 5' GTCGTAATAATGGCGGCATACTATC3'.

II.P. Biochemical analysis of mouse ventricular tissue

Mice were transferred from the vivarium to the laboratory. Each mouse was cervically dislocated and placed in supine position. An incision was made at the diaphragm and continued through the ribs to the sternum. While the heart was still beating, it was removed using forceps and the atria were cut away. The ventricles

were quickly rinsed in ice cold PBS to remove blood, snap frozen in liquid nitrogen and stored at -80°C until homogenized. Frozen hearts were homogenized in ice cold RIPA lysis buffer using a tissue homogenizer (Tissuemiser, Fisher Scientific) and 30-40 μg of protein was loaded onto SDS-PAGE for Western blot analysis as described above. To determine RhoA activation status, 500 μg of protein was used to perform the RBD pulldown assay as described above.

II.Q. Statistical Analysis

All results are reported as mean ± S.E. All data was analyzed using GraphPad Sigma-Plot software. Comparisons of two groups were accomplished using unpaired Student's *t* test. Experiments with more than two groups were compared by one-way analysis of variance (ANOVA) followed by the Tukey post-hoc test for comparison between groups.

III. Expression of constitutively active RhoA increases Akt phosphorylation and promotes cell survival

III.A. Abstract

RhoA is a small G-protein that has an established role in cell growth and in regulation of the actin cytoskeleton. Far less is known about whether RhoA can modulate cell fate. Here we demonstrate that RhoA activation affords a survival advantage, protecting cardiomyocytes from apoptotic insult induced by either hydrogen peroxide treatment or glucose deprivation. Under conditions where RhoA is protective, we observe Rho kinase dependent cytoskeletal rearrangement and activation of focal adhesion kinase (FAK). Activation of endogenous cardiomyocyte FAK leads to its increased association with the p85 regulatory subunit of phosphatidylinositol-3-kinase (PI3K), and to concomitant activation of Akt. The pathway by which RhoA mediates Akt activation is demonstrated to require Rho kinase, FAK and PI3K, but not Src, based on studies with pharmacological inhibitors (Y-27632, LY294002, PF271 and PP2) and inhibitory protein expression (FRNK). Inhibition of RhoA-mediated Akt activation at any of these steps including inhibition of FAK prevents RhoA from protecting cardiomyocytes against apoptotic insult. We conclude that RhoA-mediated effects on the cardiomyocyte cytoskeleton are able to activate FAK and thus engage PI3K and Akt signaling, affording a novel mechanism for protection from apoptosis.

III.B. Introduction

The small G-protein RhoA is a transducer of signals from the plasma membrane which mediates a range of cellular processes. There is abundant evidence supporting a requirement for RhoA signaling in regulating the cytoskeleton, cell cycle progression and gene expression (19; 83; 99; 144). There is, in contrast, far less known regarding the involvement of RhoA in cell survival or apoptosis. Some published reports implicate RhoA as a mediator of survival (34; 100; 125) while others link it to cell death pathways (4; 15; 43). Recently our lab demonstrated that prolonged expression of activated RhoA in cardiomyocytes upregulates the pro-apoptotic protein Bax and triggers a mitochondrial death pathway and apoptosis (35). Interestingly we noted that, initially, RhoA expression induced a classic hypertrophic response with associated changes in gene expression, cell enlargement, and organization of the actin cytoskeleton.

It is well established that the engagement of the cytoskeleton with cell surface integrins and the extracellular matrix influences cell survival (59; 60; 139). Detachment of cells can induce programmed cell death (50; 62; 116). Conversely, increased cell adhesion and extracellular matrix interactions promote cell survival through a variety of signaling mechanisms in which activation of focal adhesion kinase (FAK) plays a central role (51; 80; 181). FAK is a non-receptor tyrosine kinase that serves as a scaffold for multiple signaling cascades, stimulating activation of tyrosine kinases, MAP kinases and phosphatidylinositol-3-kinase (PI3K) (133). Integrin engagement is an established mechanism of FAK activation (87; 119). Additionally, FAK has been shown to be activated through a RhoA/Rho kinase

dependent pathway in response to agonist treatment, stretch, or mechanical stress in cardiomyocytes (72; 172) and other cell types (48; 93; 138; 160). However, the biological significance of FAK activation in cardiomyocyte survival, and the involvement of RhoA and the cytoskeleton in this process, have not been explored.

In this report we demonstrate that RhoA activation affords a survival advantage to cardiomyocytes and this occurs through FAK phosphorylation, association of FAK with PI3K and, consequently, activation of Akt. Akt is a well accepted mediator of survival, protecting the heart from apoptotic insult (52; 113), but the precise molecular events leading to its activation in the heart are not fully established. This is to our knowledge the first report demonstrating that cytoskeletal events, and the downstream cascade initiated by RhoA, activate Akt and mediate protection of cardiomyocytes.

III.C. Results

III.C.1. RhoA expression and activation in cardiomyocytes

Western blot analysis using a RhoA-specific primary antibody revealed that total RhoA levels increased maximally to a modest 2-3 fold increase in cells infected with 4 MOI of (L63)RhoA adenovirus by 12 hours (Figure 5a and b) compared to control infected cells, and remained maximal through 48 hours (not shown). The amount of activated RhoA was investigated by pulldown assay using the RhoA binding domain of the RhoA effector rhotekin to precipitate GTP-bound RhoA as described previously (148; 156). The level of GTP-bound RhoA in cells expressing

the activated protein was increased approximately 9-fold over control AdCMV infected cells. This increase was comparable to the increased RhoA activation observed in cardiomyocytes treated with the GPCR agonist sphingosine 1-phosphate (S1P; Figure 5c). Thus the cellular responses we observe occur in response to physiologically relevant, albeit sustained, increases in RhoA activity.

III.C.2. RhoA expression induces cytoskeletal reorganization and activates FAK

RhoA has been shown to mediate hypertrophic growth of cultured rat cardiomyocytes as indicated by increased cell size, cytoskeletal organization, and gene expression (9; 75; 94; 110; 149; 169; 183). RhoA has also been implicated in mediating activation of FAK in response to endothelin and mechanical stretch (72; 172). To confirm that RhoA signaling pathways increase cytoskeletal organization, and to directly determine whether FAK is a downstream RhoA target, we infected neonatal rat cardiomyocytes with constitutively active (L63)RhoA and visualized the cells using confocal microscopy. Cells were infected with control (AdCMV) or RhoA adenovirus for 2 hours, washed, and examined 6-24 hours later. By 24 hours RhoA expressing cells were noticeably larger, appeared more spread and showed increased actin filament organization relative to control infected cells. RhoA expressing cells also showed a robust increase in activated FAK which colocalized with actin filaments (Figure 6). Thus RhoA-induced cytoskeletal changes are temporally and spatially associated with FAK activation.

Based on the immunocytochemical results described above we sought to confirm and quantify the effect of RhoA on FAK activation using Western blot analysis. These studies demonstrated a maximal 3-fold increase in FAK phosphorylation at tyrosine 397 (indicative of FAK activation) following 24 hours of RhoA expression (Figure 7). No significant difference in total FAK expression was observed at any of these times. Since FAK associates with and activates the nonreceptor tyrosine kinase, Src, we asked whether Src phosphorylation was also increased in cells expressing RhoA. Notably, Western analysis carried out using phospho-antibody directed to the tyrosine 416 site (indicative of activation) showed no change in Src phosphorylation in RhoA infected cells versus control cells (Figure 7).

Rho kinase has been demonstrated to mediate many of the effects of RhoA on the cytoskeleton, thus we asked whether Rho kinase was involved in FAK activation. The ability of the Rho kinase selective inhibitor, Y-27632, to block FAK phosphorylation was examined. As shown by both immunofluorescence (Figure 6) and Western blot analysis (Figure 8), treatment with 10 μM Y-27632 significantly reduced RhoA-induced FAK phosphorylation at tyrosine 397. To examine the role of RhoA-induced cytoskeletal changes in FAK activation, cells were treated with cytochalasin D to disrupt actin polymerization. Treatment with 10 μM cytochalasin D completely abolished the RhoA-induced increase in FAK phosphorylation (Figure 8). Taken together these data provide evidence that Rho kinase, as well as cytoskeletal integrity, are required for RhoA-mediated FAK activation.

III.C.3. RhoA increases the association of endogenous FAK and p85

FAK serves as a protein scaffold that mediates activation of signaling molecules including PI3K (133). An interaction between heterologously expressed FAK and p85, a regulatory subunit of the catalytic p110 subunit of PI3K, has been shown to increase kinase activity in fibroblasts (11; 25; 26). The p85 subunit contains an SH2 domain known to bind the consensus sequence Y*XXM, where X is any amino acid and Y is a phosphorylated tyrosine residue (161). A sequence scan of FAK revealed three corresponding motifs conserved between mouse, rat and human. To test the possibility that RhoA increases phosphorylation of this p85 recognition motif in FAK, we immunoprecipitated FAK from control and RhoA infected cells and probed for this phosphorylated motif. RhoA expression markedly increased phosphorylation of this sequence in FAK and this increase was mediated by Rho kinase, as it was completely blocked by Y-27632 treatment (Figure 9). To test for an interaction between FAK and p85 in cardiomyocytes, we performed co-immunoprecipitation assays using a total FAK antibody. These experiments demonstrated increased association of endogenous FAK and p85 in cells expressing RhoA (Figure 10a and b). This increased association was completely abolished by treatment with either Y-27632 (10 μM) or PF271 (1 μM), a pharmacological inhibitor of FAK, indicating that both Rho kinase and FAK activity are required to mediate this interaction (Figure 10a).

III.C.4. RhoA expression activates Akt, which is mediated by PI3K

Based on our findings that RhoA increases the association of FAK with PI3K, we asked whether RhoA activates the well characterized cardioprotective kinase, Akt. Western blot analysis demonstrated that expression of RhoA increased Akt phosphorylation at serine 473, as well as threonine 308 (data not shown) inducing a nearly 4-fold increase versus control cells (Figure 11a and b). Treatment with the PI3K inhibitor, LY294002 (10 μM), completely abolished the increase in Akt phosphorylation indicating involvement of PI3K (Figure 11a and b).

III.C.5. RhoA increases Akt-mediated substrate phosphorylation

To determine whether RhoA increases the phosphorylation of Akt substrates in the cardiomyocyte, cells were infected with either (L63)RhoA or control (AdCMV) adenovirus and lysates prepared 24 hours after virus washout. Using a PAS (phospho-Akt substrate) antibody that recognizes proteins containing the phosphorylated Akt consensus sequence RXRXXS/T, we observed multiple bands that showed marked increase. These data suggest that Akt activity is upregulated, concomitant with its increased phosphorylation, in response to RhoA overexpression (Figure 12). Identification of these substrates and their potential role will require further study beyond the scope of this thesis work.

III.C.6. RhoA increases activated Akt present at mitochondria

Recent work from our lab has demonstrated that in cardiomyocytes treated with agonists that activate Akt, the amount of phosphorylated Akt present at

mitochondria is increased. This is associated with protection of cardiomyocytes from apoptosis mediated by phosphorylation of hexokinase II at an Akt phosphorylation site (121). To determine if increased RhoA expression also increases the amount of phosphorylated Akt present at mitochondria, cytosolic and mitochondrial fractions were prepared from cardiomyocytes after 24 hours of RhoA expression. VDAC and actinin specific antibodies were used to determine the purity and equal loading of each sample, respectively. The amount of phosphorylated Akt present at mitochondria was significantly increased compared to control infected cells (Figure 13). In follow up studies, it will be of interest to determine whether hexokinase II phosphorylation is also increased and if this RhoA-mediated event contributes to the protection described below.

III.C.7. RhoA does not alter PTEN expression

Recent evidence has suggested that RhoA can alter PTEN signaling to effect Akt activation in other cell types (101; 152). Western blot analysis was performed to determine whether increased RhoA expression leads to altered PTEN protein expression. In cardiomyocytes, these experiments, repeated several times, clearly demonstrate that PTEN protein levels are unchanged in RhoA infected cells compared to control infected cells (Figure 14). Thus altered PTEN expression is not responsible for mediating RhoA-induced Akt activation.

III.C.8. RhoA-induced activation of Akt is mediated by FAK but not Src

As shown in Figure 10, there is increased recruitment of the PI3K p85 subunit to FAK in RhoA infected cells. To demonstrate that the observed Akt activation results from the recruitment of PI3K by FAK we infected cells with adenovirus encoding the FAK-related non-kinase, FRNK. FRNK is an endogenously expressed truncated form of FAK that contains the C-terminal domain of FAK but lacks its kinase domain and therefore acts as a dominant inhibitory regulator (143). Expression of FRNK adenovirus prevented RhoA-induced FAK phosphorylation at tyrosine 397, evidence that the construct acted as predicted (Figure 15). Importantly, expression of FRNK fully inhibited the phosphorylation of Akt elicited by RhoA expression. Western blot analysis was also performed on cells treated with Y-27632 (10 μM), the FAK inhibitor, PF271 (1 μM), or the Src selective inhibitor, PP2 (1 μM), and levels of phosphorylated Akt determined. Figure 16 quantifies Akt activation in the absence or presence of these inhibitors or FRNK adenovirus (10 MOI). The blockade of Akt activation by Y-27632 is consistent with the data above showing that Rho kinase is required for FAK phosphorylation and for PI3K association with FAK (Figure 8 and 10a). The lack of inhibition by PP2 is consistent with our observation that Src is not activated by RhoA expression (Figure 7). These data implicate FAK, acting independently of Src, as a RhoA/Rho kinase dependent mediator of PI3K and Akt activation.

III.C.9. RhoA expression protects cells from apoptotic insult

To examine the possibility that RhoA signaling pathways mediate a protective response, control and RhoA infected cells were treated with 100 μM hydrogen peroxide for 16-18 hours. Myocytes infected with control adenovirus showed a roughly 5-fold increase in DNA fragmentation. This increase was significantly attenuated in cells expressing RhoA (Figure 17). To demonstrate the involvement of PI3K/Akt in RhoA-mediated protection, we examined the effect of LY294002. Inhibition of PI3K had no effect on DNA fragmentation in control infected cells (data not shown). However, the ability of RhoA to protect against DNA fragmentation elicited by hydrogen peroxide was fully blocked in the presence of LY294002 (Figure 17) indicating that Akt activated by RhoA serves a protective function.

The data above suggest that RhoA and Akt-mediated protection occurs in response to increased FAK phosphorylation. To prove that FAK is the mediator of cardiomyocyte protection we tested the ability of FRNK adenoviral expression and of pharmacological FAK inhibition to block RhoA-mediated protection. Blocking FAK through either means precluded RhoA from protecting cardiomyocytes from apoptosis induced by hydrogen peroxide or glucose deprivation (Figures 17 and 18). Thus, not only is FAK activated by RhoA/Rho kinase, but this response is critical for protection against apoptosis.

III.D. Discussion

RhoA acts as a transducer of signals from a subset of GPCRs, as well as mechanical stretch and integrin engagement (19; 83; 99). Canonical RhoA signaling pathways alter the cytoskeleton via activation of the RhoA effector, Rho kinase, leading to increased myosin phosphorylation and contraction or to actin stress fiber formation (104; 144). The ability of RhoA to stimulate actin myofilament rearrangement in neonatal rat cardiomyocytes has been established (9; 77; 94; 149). However, the relationship between RhoA-mediated changes in cytoskeletal structure and cell fate has not been explored. Because increased focal adhesion formation and cell attachment are known to promote cell survival, we hypothesized that there might be a role for RhoA in cardiomyocyte protection. Our interest was impelled by the observation that, whereas sustained RhoA activation leads to apoptosis, the early hypertrophic response of cardiomyocytes was accompanied by increased Akt activation (35), which we predicted could function as a survival factor and mediator of cell protection by RhoA signaling.

Several published papers implicate RhoA signaling in Akt regulation, but these reports suggest that RhoA activation negatively regulates Akt. Studies using neutrophils and fibroblasts described the ability of RhoA to increase activity of the PIP_3 phosphatase PTEN and negatively regulate PI3K/Akt activation (101; 152). Work with endothelial cells and aortic tissue suggested a similar conclusion, demonstrating that Akt activation was increased when RhoA and/or Rho kinase were inhibited (45; 179). In a similar vein, expression of activated RhoA in HUVECs was shown to prevent Akt activation and negatively regulate NO synthesis (118). The ability of RhoA to regulate Akt activation in a terminally differentiated or non-

migratory cell, such as the cardiomyocyte, had not, however, been investigated. Our studies contrast with those cited above, and are the first to demonstrate that RhoA can positively regulate Akt activity through cytoskeletal events.

RhoA has been previously implicated in cytoskeletal regulation and FAK activation in cardiomyocytes (72; 77; 172). Our work demonstrates that FAK can link RhoA signals to Akt activation. Interestingly, earlier reports demonstrated that FAK can physically interact with the regulatory p85 subunit of PI3K in fibroblasts (25), human glioblastoma cells (162), and also in the pressure overloaded heart (49). Our results demonstrate that this occurs in response to RhoA activation, which induces a robust increase in FAK phosphorylation at p85 binding sequences, and increases the association of endogenous p85 with FAK. To further implicate FAK in PI3K/Akt activation, we employed adenoviral overexpression of FAK-related non-kinase (FRNK), which has been demonstrated to reduce phosphorylation of FAK and its downstream substrates, and promote cell detachment (71; 143). Our studies are the first to demonstrate that FRNK not only prevents FAK activation, but blocks Akt activation and cardiomyocyte protection elicited by RhoA. Notably, while Src has been implicated in FAK-mediated activation of Akt in other cell types (119; 164), we find that Src is neither activated by RhoA nor required for RhoA and FAK-mediated Akt activation, suggesting cell type specificity of this signaling paradigm. These data provide a strong and heretofore undescribed link between FAK and Akt activation in the cardiomyocyte while further confirming FAK as a pro-survival signaling molecule.

Two models have been generated to study cardiomyocyte specific and inducible FAK ablation in the adult mouse (36; 134). Both groups implicate FAK in

the development of cardiac hypertrophy and suggest the possibility of altered ERK signaling in this response. These reports are consistent with the finding that FAK is involved in the hypertrophic response of cultured cardiomyocytes (41; 168). More recent work using siRNA to knock down FAK expression in the adult mouse heart further implicated FAK signaling in the development of pressure overload-induced hypertrophy (28). These studies did not however examine Akt activation or cell survival. Thus the question of whether FAK signaling confers cardiac protection *in vivo* has not been addressed. Our studies suggest that imposing ischemia/reperfusion injury or myocardial infarct on cardiac specific FAK null animals could reveal an additional role for FAK signaling and demonstrate that FAK-mediated activation of PI3K/Akt normally plays a protective role in the *in vivo* heart.

While we suggest that RhoA/Rho kinase are protective in cardiomyocytes, several published papers suggest a maladaptive role of RhoA/Rho kinase (ROCK) signaling in the cardiovascular system. For example, inhibition of Rho kinase with Y-27632 or fasudil (HA-1077) decreased fibrosis in response to myocardial infarct (69), and reduced infarct size following both short- and long-term ischemia reperfusion injury (12; 179). Moreover it has been reported that both ROCK1 null and ROCK1$^{+/-}$ haploinsufficient mice respond better to increased cardiovascular demand, exhibiting a more compensatory hypertrophy and less cardiac fibrosis (146; 184). Similarly, recent work from Shi *et al* demonstrate increased cardiac function in mice lacking ROCK1 in the Gαq model of dilated cardiomyopathy (157). While these studies suggest that RhoA/Rho kinase signaling is maladaptive rather than protective, the site of inhibition of RhoA/Rho kinase signaling may not be the cardiomyocyte. Indeed, a host of other

cell types including fibroblasts, endothelial cells and macrophages, present in the *in vivo* system, respond to RhoA with proliferative or inflammatory responses, which may explain the salutary response to RhoA/Rho kinase inhibition. We suggest that acute RhoA/Rho kinase signaling in cardiomyocytes affords protection via its profound effect on the cytoskeleton and consequent activation of FAK and Akt (Figure 19).

Our previous work is to our knowledge the only prior study to observe a role for RhoA in determining cardiomyocyte fate (35). We demonstrated that extended RhoA activation leads to upregulation of Bax and subsequent apoptosis. Interestingly, as we now demonstrate, shorter durations of RhoA activation provide a striking protective advantage from apoptotic insult. It is possible that the transition from cardiomyocyte survival to apoptosis reflects the transient nature of RhoA-induced FAK and Akt activation, ultimately shifting the balance from protection to cell death. We conclude that RhoA is a pleiotropic molecule that can modulate cell survival and apoptosis and, when activated acutely, affords protection of cardiomyocytes. It will be of interest to determine whether mechanical stretch or increased pressure, which activate RhoA, initially affords protection through a RhoA/FAK/Akt signaling scaffold.

III.E. Acknowledgment

This chapter, in part, is a reprint of the material from **Del Re, D.P.**, Miyamoto, S., and Brown, J.H. FAK as a RhoA-activatable signaling scaffold mediating cardiomyocyte

protection. (2008) In Revision. *J. Biol. Chem*. The dissertation author was the primary researcher and author of this manuscript. Dr. Joan Heller Brown directed and supervised the research which forms the basis for this chapter.

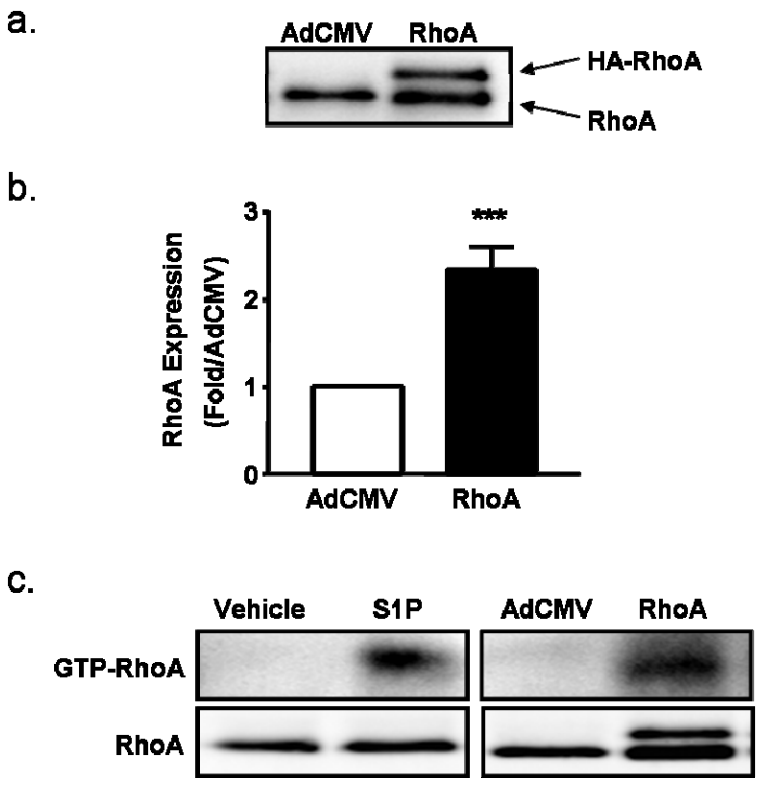

Figure 5. RhoA expression and activation in cardiomyocytes. Neonatal rat ventricular myocytes were infected with (L63)RhoA or AdCMV at 4 MOI. A. and B. Lysates were prepared 24 hours following virus washout and RhoA expression was determined by Western blot analysis. Values represent averages ± S.E. (n = 10). ***, $p<0.001$ versus AdCMV. C. Cardiomyocytes were treated with 5 μM sphingosine 1-phosphate (S1P) or vehicle for 5 minutes or infected with (L63)RhoA or control AdCMV adenovirus for 24 hours and harvested to determine RhoA activation. The RhoA binding domain of Rhotekin was used to selectively pulldown activated GTP-bound RhoA. Samples were analyzed by SDS-PAGE.

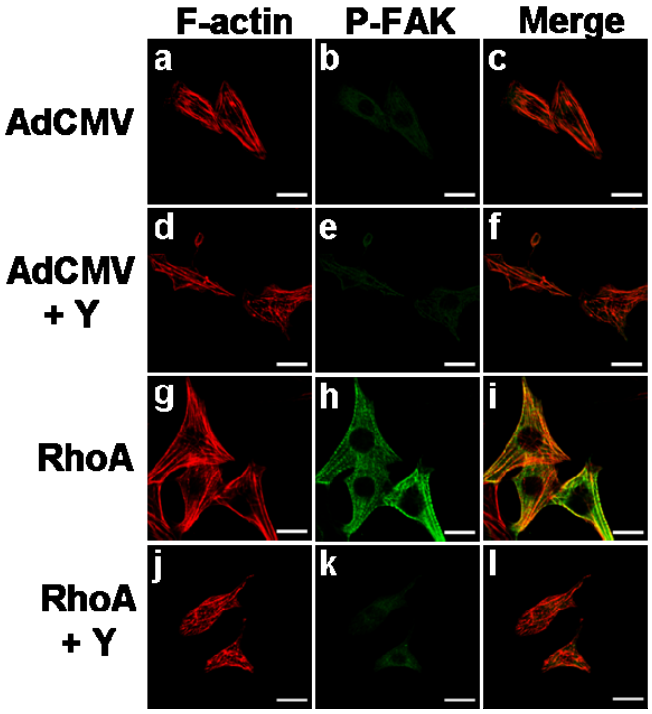

Figure 6. RhoA expression induces cytoskeletal reorganization and activates FAK. Neonatal rat ventricular myocytes were infected with constitutively activated (L63)RhoA adenovirus or control (AdCMV) adenovirus. Cells were treated with 10 μM Y-27632 or vehicle at virus addition, fixed and stained 24 hours after infection. Rhodamine-conjugated phalloidin was used to visualize actin filaments (a, d, g, j) and rabbit polyclonal anti-FAK (pY397) and FITC anti-rabbit were used to visualize phosphorylated FAK (b, e, h, k). Bar represents 20 μm.

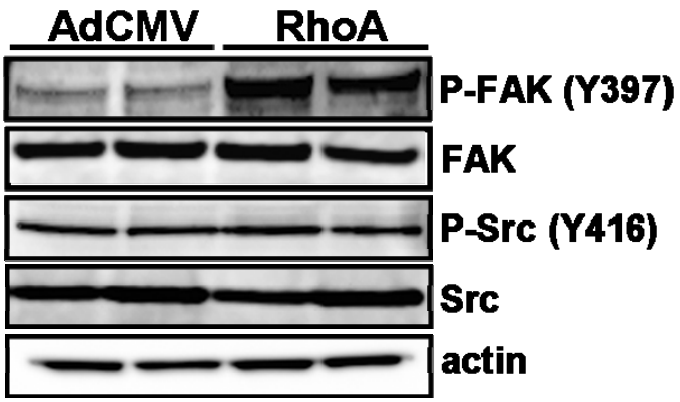

Figure 7. RhoA increases phosphorylation of FAK but not Src. Neonatal rat ventricular myocytes were infected with constitutively activated (L63)RhoA adenovirus or control (AdCMV) adenovirus. Lysates were prepared 24 hours after infection and levels of phosphorylated FAK (Y397), total FAK, phosphorylated Src (Y416) and total Src were determined by Western blot.

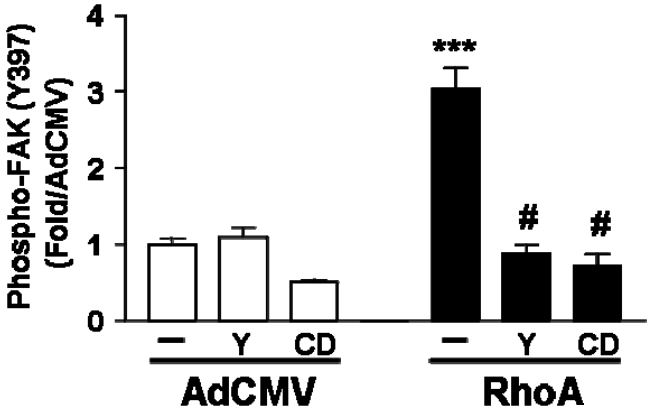

Figure 8. RhoA-induced FAK activation is dependent on cytoskeletal integrity. Neonatal rat ventricular myocytes were infected with constitutively activated (L63)RhoA adenovirus or control (AdCMV) adenovirus. Lysates were prepared from cardiomyocytes infected for 24 hours and treated with either 10 μM Y-27632 (at infection) or 10 μM cytochalasin D (treatment for 1 hour prior to cell collection). Values from Western blot analysis were quantitated by densitometry and are shown as averages ± S.E. (n = 3). ***, $p<0.001$ versus AdCMV. #, $p<0.001$ versus RhoA.

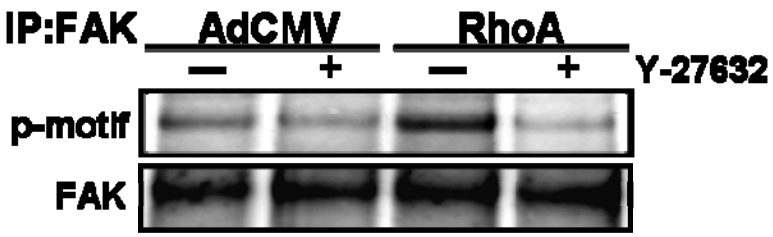

Figure 9. RhoA increases the phosphorylation of the p85 binding motif present in FAK. Neonatal rat ventricular myocytes were infected with (L63)RhoA or control (AdCMV) adenovirus for 2 hours, washed, and harvested at 24 hours. Cells were treated with 10 μM Y-27632 or vehicle at time of infection and lysates prepared. Total FAK protein was immunoprecipitated using mouse monoclonal anti-FAK antibody, separated by SDS-PAGE and probed using a selective p85 recognition phospho-motif antibody.

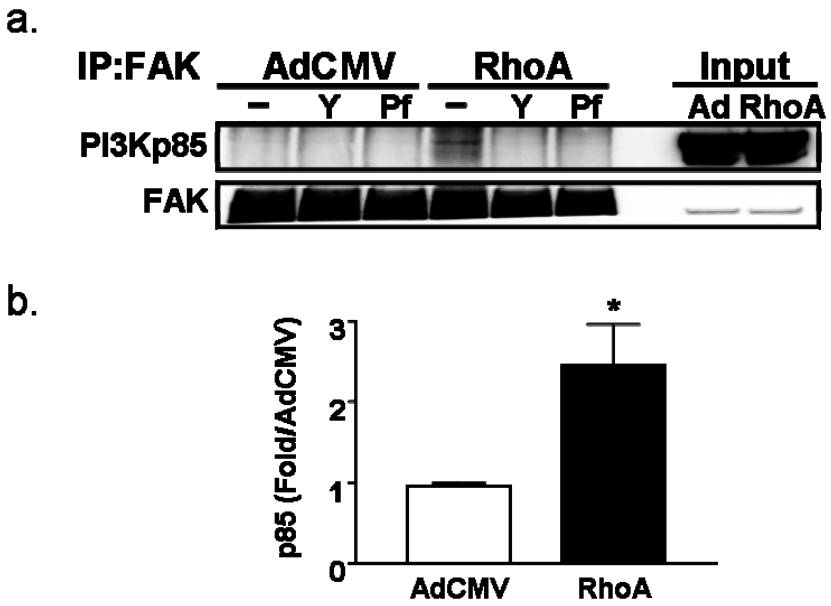

Figure 10. RhoA increases the association of endogenous FAK and p85. A. and B. Neonatal rat ventricular myocytes were infected with (L63)RhoA or control (AdCMV) adenovirus for 2 hours, washed, and harvested at 24 hours. A. Cells were treated with 10 μM Y-27632, 1 μM PF271 or vehicle at time of infection. Co-immunoprecipitation was performed using mouse monoclonal anti-FAK antibody, complexes separated by SDS-PAGE, and probed with a pan p85 antibody to recognize endogenous p85 protein. B. Values from Western blot analysis were quantitated by densitometry and are shown as averages ± S.E. (n = 3). *, $p<0.05$ versus AdCMV.

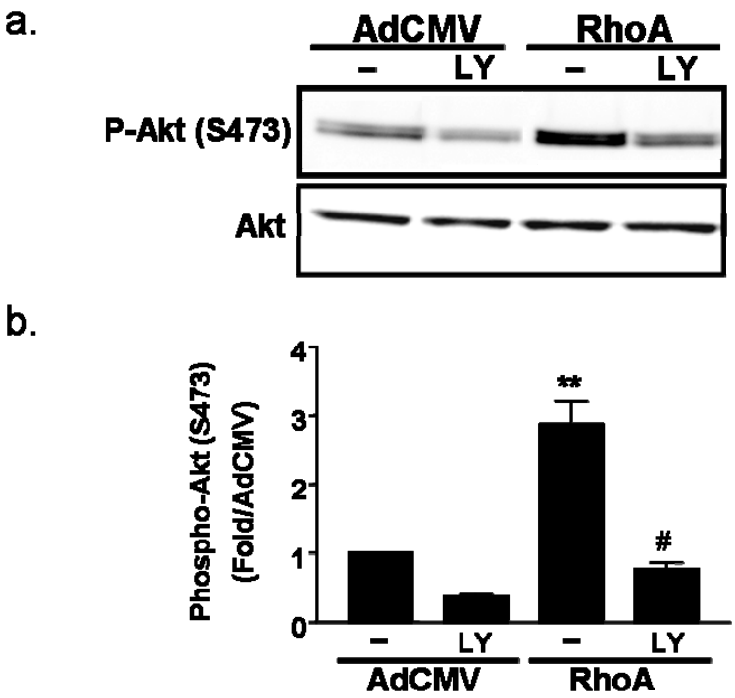

Figure 11. RhoA expression activates Akt, which is mediated by PI3K. A and B. Neonatal rat ventricular myocytes were infected with (L63)RhoA or control (AdCMV) adenovirus in the presence or absence of 10 μM LY294002 for 24 hours. Lysates were prepared and levels of phosphorylated Akt (S473) were determined by Western blot analysis. A. Representative blot showing LY294002 inhibits Akt phosphorylation. B. Quantified densitometry results. Values represent averages ± S.E. (n = 5). **, $p<0.01$ versus AdCMV. #, $p<0.001$ versus RhoA.

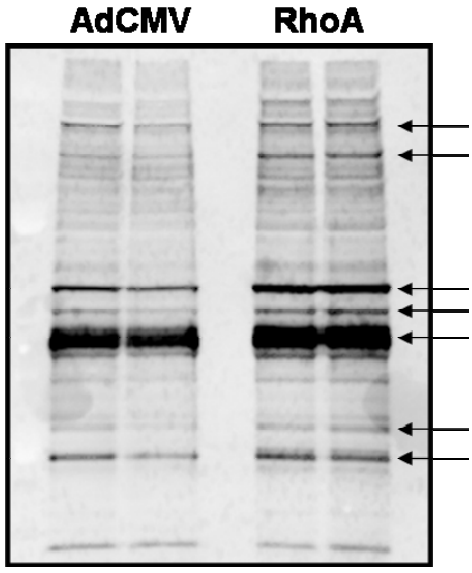

Figure 12. RhoA increases the phosphorylation of Akt substrates. Neonatal rat ventricular myocytes were infected with (L63)RhoA or control (AdCMV) adenovirus and lysates prepared 24 hours later. The levels of phosphorylated Akt substrates were determined by Western blot analysis using a selective antibody that recognizes a unique phosphorylated Akt substrate consensus sequence. Arrows designate proteins showing increased Akt dependent phosphorylation.

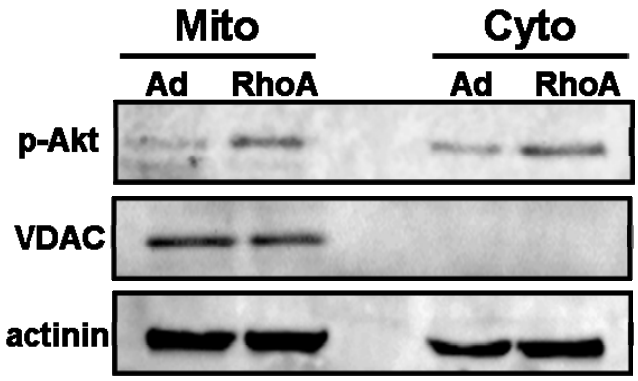

Figure 13. RhoA increases phosphorylated Akt present at mitochondria.
Neonatal rat ventricular myocytes were infected with (L63)RhoA or control (AdCMV) adenovirus for 2 hours, washed, and harvested at 24 hours. Cell lysates were obtained and cytosolic and mitochondrial fractions were prepared as described in methods. Western blot analysis was performed to determine phosphorylated Akt (S473) levels. An antibody selective to VDAC was used as specific marker of the mitochondrial fraction.

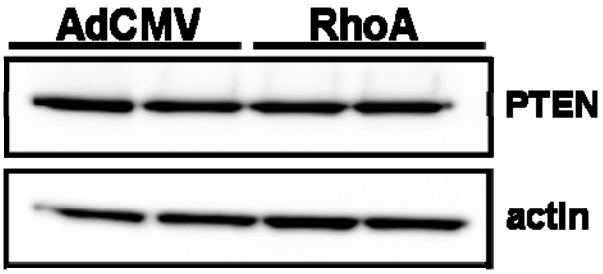

Figure 14. RhoA does not alter PTEN protein expression. Neonatal rat ventricular myocytes were infected with (L63)RhoA or control (AdCMV) adenovirus for 2 hours, washed, and harvested at 24 hours. Western blot analysis was performed to determine PTEN protein levels.

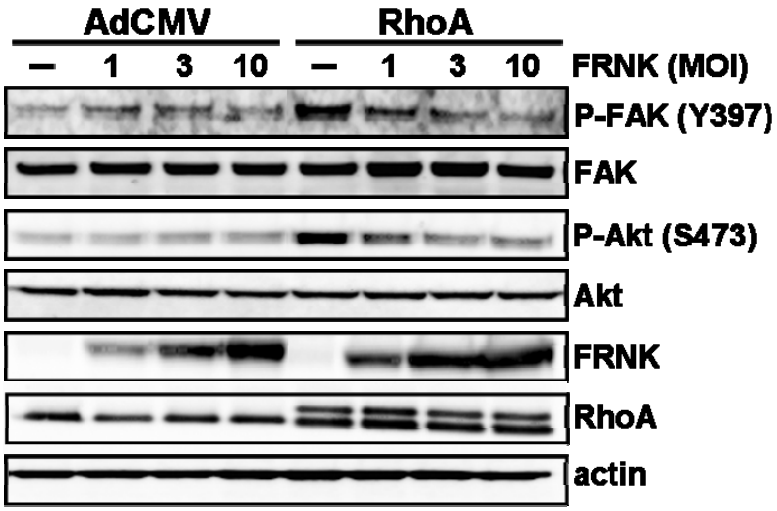

Figure 15. RhoA-induced activation of Akt is inhibited by FRNK. Neonatal rat ventricular myocytes were infected with (L63)RhoA or control (AdCMV) adenovirus and co-infected with increasing amounts of FRNK adenovirus. Lysates were prepared 24 hours after infection and Western blot analysis performed.

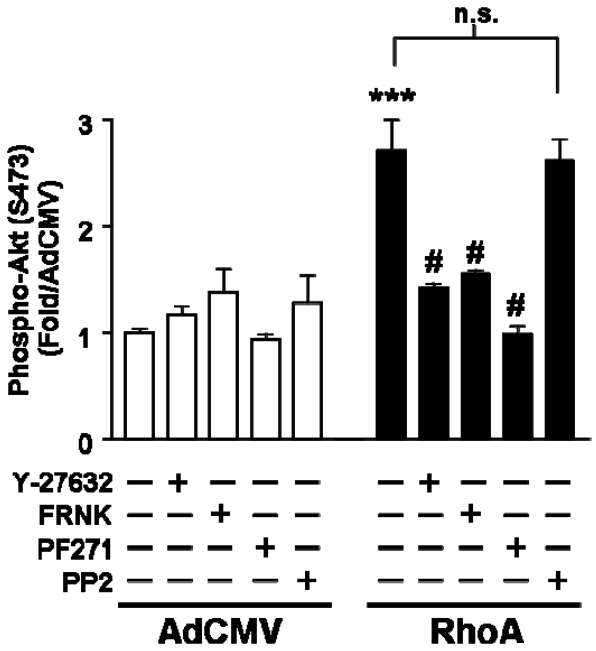

Figure 16. RhoA-induced Akt activation is mediated by Rho kinase and FAK, but not Src. Neonatal rat ventricular myocytes were infected with (L63)RhoA or control (AdCMV) adenovirus for 2 hours, washed, and harvested at 24 hours. Cells were either co-infected with FRNK adenovirus (10 MOI), or treated with 10 μM Y-27632, 1 μM PF271, 1 μM PP2 or vehicle. Western blot analysis was performed to determine phospho-Akt levels. Values from Western blot analysis were quantitated by densitometry and are shown as averages ± S.E. (n = 3). ***, $p<0.001$ versus AdCMV. #, $p<0.001$ versus RhoA. n.s., not significant.

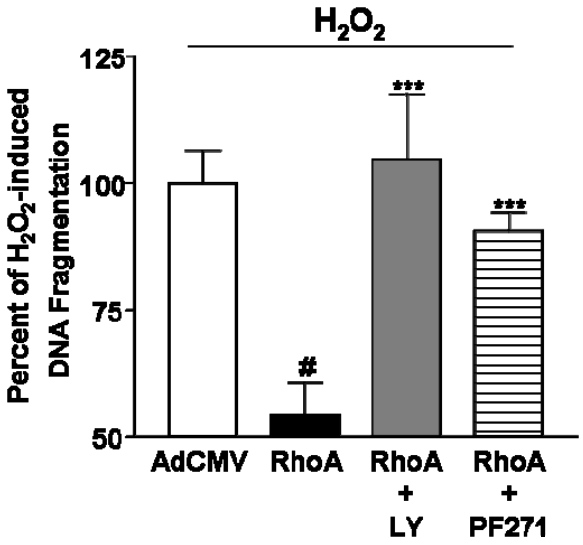

Figure 17. RhoA expression protects cells from apoptotic insult. Neonatal rat ventricular myocytes were infected with (L63)RhoA or control (AdCMV) adenovirus for 2 hours. Following virus removal, cells were pretreated with 10 μM LY, 1 μM PF271 or vehicle for 30 minutes prior to treatment with 100 μM H_2O_2 for 16-18 hours. Lysates were prepared and nucleosomal fragmentation was quantified by POD assay as described in methods. Values represent averages ± S.E. (n = 5). #, $p<0.001$ versus AdCMV + H_2O_2. ***, $p<0.001$ versus RhoA + H_2O_2. †, $p<0.05$ versus AdCMV + 2-DOG. %, $p<0.05$ versus RhoA + 2-DOG.

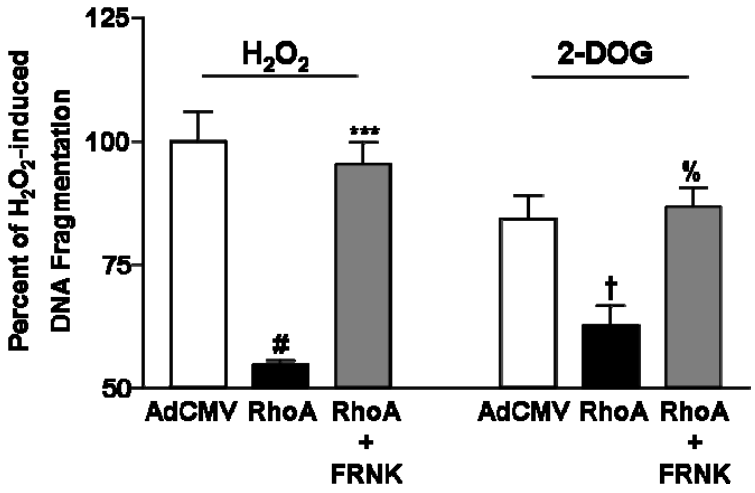

Figure 18. RhoA-mediated protection is dependent on FAK. Neonatal rat ventricular myocytes were infected with (L63)RhoA or control (AdCMV) adenovirus and co-infected with FRNK (10 MOI) or control virus for 2 hours. Following virus removal, cells were treated with 100 μM H_2O_2 for 16-18 hours or cultured in low glucose medium containing 4 mM 2-DOG for 30 hours. Lysates were prepared and nucleosomal fragmentation was quantified by POD assay as described in methods. Values represent averages ± S.E. (n = 5). #, $p<0.001$ versus AdCMV + H_2O_2. ***, $p<0.001$ versus RhoA + H_2O_2. †, $p<0.05$ versus AdCMV + 2-DOG. %, $p<0.05$ versus RhoA + 2-DOG.

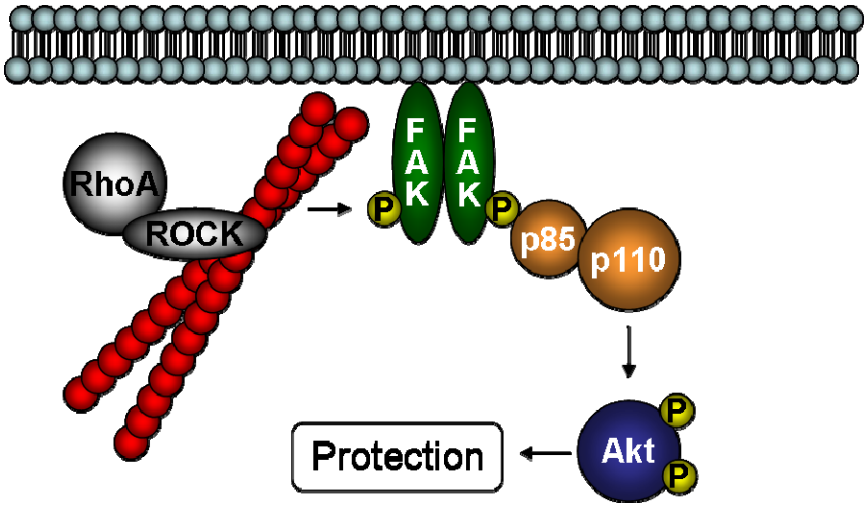

Figure 19. Proposed schema illustrating RhoA-mediated protection. Increased RhoA activation signals through its effector Rho kinase to induce cytoskeletal rearrangement, mediating the activation of FAK. FAK binds and recruits PI3K via its regulatory subunit, p85, leading to increased Akt activation and cardiomyocyte protection.

IV. Chronic RhoA activation up-regulates Bax and induces cardiomyocyte apoptosis

IV.A. Abstract

The small G-protein RhoA regulates the actin cytoskeleton and its involvement in cell proliferation has also been established. In contrast little is known about whether RhoA participates in cell survival or apoptosis. In cardiomyocytes *in vitro* RhoA induces hypertrophic cell growth and gene expression. *In vivo*, however, RhoA expression leads to development of heart failure (150), a condition widely associated with cardiomyocyte apoptosis. We demonstrate here that adenoviral overexpression of activated RhoA in cardiomyocytes induces hypertrophy which transitions over time to apoptosis, as evidenced by caspase activation and nucleosomal DNA fragmentation. Interestingly, this correlates with the RhoA-mediated transient activation of two pro-survival kinases, FAK and Akt. The Rho kinase inhibitors Y-27632 and HA-1077, and expression of a dominant negative Rho kinase, block the RhoA-induced apoptotic responses. Caspase-9, but not caspase-8, is activated and its inhibition prevents DNA fragmentation, consistent with involvement of a mitochondrial death pathway. Interestingly RhoA expression induces a 3-4 fold upregulation of the pro-apoptotic Bcl-2 family protein Bax. RhoA also increases levels of activated Bax and the amount of Bax protein localized at mitochondria. Bax mRNA is increased by RhoA, indicating transcriptional regulation, and the ability of a dominant negative (dn) p53 mutant to block Bax upregulation implicates p53 in this response. The involvement of Bax in RhoA-induced apoptosis was examined by treatment with a Bax inhibitory

peptide, which was found to significantly attenuate DNA fragmentation and caspase-9 and -3 activation. The dn p53 also prevents RhoA-induced apoptosis. We conclude that RhoA /Rho kinase activation upregulates Bax through p53 to induce a mitochondrial death pathway and cardiomyocyte apoptosis.

IV.B. Introduction

The small G-protein RhoA is a transducer of signals from G-protein coupled receptors (GPCRs) and the extracellular matrix, influencing cell morphology as well as gene expression (83). Signals elicited by GPCR activation or integrin engagement induce hypertrophic growth of cardiomyocytes characterized by increases in cell size and by altered gene expression and morphology (19). The involvement of RhoA in hypertrophy of neonatal rat ventricular myocytes has been established (9; 75; 77; 94; 110; 149; 169; 183)(75). Interestingly, however, transgenic mice overexpressing RhoA in the myocardium showed only a modest degree of cardiac hypertrophy and rapidly developed a dilated cardiomyopathy and lethal heart failure (150). At the time that our laboratory generated and characterized the RhoA transgenic mouse line a role for cardiomyocyte apoptosis in the development of heart failure was not established. In light of the currently recognized importance of apoptosis in the transition from hypertrophy to heart failure, we set out to evaluate the possibility that the response to sustained RhoA expression might reflect the ability of RhoA to stimulate cardiomyocyte apoptosis.

There is limited data concerning involvement of RhoA in apoptosis and cell survival. The available literature is also conflicting, suggesting that cell fate in

response to RhoA is cell type specific. Most of the published data indicates that RhoA can serve a protective function (34; 100; 125). Thus inhibiting RhoA /Rho kinase signaling by treatment with C3 exoenzyme (which ribosylates RhoA) or with the Rho kinase inhibitors Y-27632 and HA-1077 has been shown to lead to apoptosis in HUVEC (100), neuroblastoma (34) and Jurkat cells (125), consistent with the notion that RhoA is protective. In contrast to the apparent pro-survival effects of RhoA signaling in these cell types, overexpression of wild-type or activated (L63)RhoA was shown to induce apoptosis in NIH3T3 cells (43). RhoA was also suggested to mediate the apoptotic response to heterologously expressed and constitutively activated Gα12 and Gα13 in COS-7, CHO and HEK293 cells (4; 15). The mechanisms by which RhoA can promote cell death have not been elucidated. While RhoA-mediated apoptosis was shown to be Bcl-2 sensitive, the events antagonized by overexpression of this anti-apoptotic Bcl-2 family protein were not identified (4).

Bcl-2 family proteins are known to affect cell survival by regulating the permeability of mitochondria. Current thinking holds that the ratio of pro- to anti-apoptotic Bcl-2 family proteins can determine cell fate. The pro-survival proteins Bcl-2 and Bcl-x_L bind to and sequester pro-apoptotic factors, such as Bax and Bak, preventing their ability to increase mitochondrial permeability (64). Activation of Bax, which occurs in response to a variety of apoptotic stimuli, leads to its oligomerization and translocation to mitochondria where it induces cytochrome *c* release (6; 7; 85; 90; 95; 180). The transcription factor p53 has been shown to directly regulate Bax expression and can induce apoptosis via activation of the mitochondrial death pathway (140).

The studies reported here are the first to examine RhoA involvement in cardiomyocyte apoptosis and our findings demonstrate that RhoA, acting through its effector Rho kinase, can induce cardiomyocyte programmed cell death. Our work further reveals that RhoA-induced caspase activation and DNA fragmentation are initiated by an early Rho kinase dependent increase in Bax expression which is mediated by the transcription factor p53 and executed through Bax effects on the mitochondria.

IV.C. Results

IV.C.1. RhoA expression induces transient activation of FAK and Akt

The previous chapter demonstrated the ability of RhoA to induce FAK activation, increased association between FAK and PI3K, and subsequent activation of Akt. The kinetics of both FAK and Akt activation initiated by RhoA was examined in neonatal rat ventricular myocytes infected with (L63)RhoA or control AdCMV at 4 MOI. A maximal 3-fold increase in FAK phosphorylation at tyrosine 397 (indicative of FAK activation) was observed following 24 hours of RhoA expression, with lesser but significant increases at earlier (12 hours) and later (48 hours) times (Figure 20a). Akt phosphorylation was likewise increased significantly within 12 hours of RhoA infection, was maximal at 24 hours, and then declined by 48 hours post infection (Figure 20b) paralleling the time course of FAK activation. The observation that neither Akt nor FAK activation was sustained beyond 24 hours suggested that the

protective advantage afforded by RhoA at earlier times, and characterized in the previous chapter, might dissipate with chronic RhoA activation.

IV.C.2. Timecourse of hypertrophy and apoptosis in RhoA infected cardiomyocytes

To explore the effect of the diminished protective signals afforded by RhoA at longer times of expression, we examined phenotypic changes in cardiomyocytes expressing exogenous RhoA. The involvement of RhoA in cardiomyocyte hypertrophy has been well documented, and the ability of activated RhoA to induce myofilament organization and atrial natriuretic factor (ANF) gene expression has been shown previously by our lab (76). Marked hypertrophy, evidenced by increases in cell size and actin myofilament organization, was observed at 24 hours (Figure 21a). Increased ANF expression was also clearly evident at this time (Figure 21a). Paralleled increases in cell size and FAK phosphorylation were shown in the previous chapter (Figure 6).

Interestingly, cardiomyocytes infected with (L63)RhoA adenovirus and maintained in culture for 48 hours showed clear apoptotic cell death by TUNEL staining (Figure 21b). No TUNEL positive nuclei were detected at earlier times (0-24 hours). POD and DNA laddering assays also failed to reveal signs of apoptosis until 36 hours or later (data not shown) again correlating with the transient activation of FAK and Akt and, presumably, the protective effect of RhoA. Thus RhoA signaling

initially elicits both a protective and hypertrophic response, while more chronic, long-term RhoA activation is sufficient to induce cardiomyocyte apoptosis.

IV.C.3. RhoA expression induces cardiomyocyte apoptosis

To confirm that RhoA indeed is an apoptotic signal at longer times, cardiomyocytes were again infected with adenovirus encoding (L63)RhoA or control AdCMV. Expression of RhoA induced DNA fragmentation as assessed by DNA laddering. This response, a hallmark of apoptosis, was completely blocked when cells were concomitantly treated with the non-selective caspase inhibitor Idun1965 (Figure 22a). Quantitative analysis of this response by the ELISA based POD assay showed a 3-fold increase in DNA fragmentation elicited by RhoA expression, which was fully inhibited by Idun1965 (Figure 22b).

To further characterize the RhoA-induced apoptotic response we infected cardiomyocytes with a range of concentrations of activated RhoA adenovirus and quantified DNA fragmentation by POD assay. At a dose half that used initially (2 MOI), no significant increase in DNA fragmentation was detected. However, when the initial dose was doubled, the apoptotic response nearly doubled to roughly 5-fold over control (Figure 23a). Activation of caspase-3 is a hallmark of apoptotic cell death and caspase-3 cleavage is indicative of its activation. As determined by Western blot analysis, capsase-3 cleavage also increased dose dependently in response to increased RhoA expression providing further evidence of RhoA-induced cardiomyocyte apoptosis (Figure 23b).

IV.C.4. Rho kinase mediates RhoA-induced cardiomyocyte apoptosis

To begin to elucidate the mechanism for RhoA-induced apoptosis, we examined the possibility that the RhoA effector Rho kinase was involved. Rho kinase activity was assessed by examining phosphorylation of the myosin binding subunit of myosin light chain phosphatase, MYPT-1, a well established Rho kinase specific substrate. Western blot analysis showed increased MYPT-1 phosphorylation in cells expressing RhoA and this was blocked by the Rho kinase inhibitor Y-27632 and adenoviral expression of a Rho kinase mutant shown previously to have strong dominant negative activity (5) (Figure 24). When cardiomyocytes were treated with Y-27632, or another Rho kinase inhibitor HA-1077, the time of RhoA infection and again after virus washout there was significant protection from RhoA-induced DNA fragmentation (Figure 25a). Comparable protection was also observed with expression of the dominant negative Rho kinase (Figure 25a). We also examined the involvement of Rho kinase in RhoA-induced caspase-3 activation. Myocytes infected with activated RhoA and treated with Y-27632 were assayed for caspase-3 cleavage at 48 hours by Western blot analysis. As shown in Figure 25b, Y-27632 treatment completely prevented RhoA-induced caspase-3 cleavage, providing further evidence that Rho kinase mediates this apoptotic response.

IV.C.5. RhoA induces apoptosis through caspase-9 activation

We next determined which initiator caspases upstream of caspase-3 were activated by RhoA. Caspase-8 activation is typically associated with the extrinsic, or

death receptor pathway of apoptosis, while caspase-9 is involved in the mitochondrial apoptotic death pathway and is activated following mitochondrial disruption and cytochrome *c* release. We examined caspase-9 activation by Western blot analysis and found a significant increase in the cleaved caspase-9 product in RhoA infected compared to control cells at 48 hours (Figure 26a). This increase in caspase-9 cleavage was fully abrogated by treatment with Y-27632 indicating that both RhoA and Rho kinase are upstream of caspase-9 activation. Western analysis of cleaved caspase-8 showed no increase following RhoA overexpression (data not shown). Caspase-8 and caspase-9 peptide inhibitors were then used to selectively block the function of each respective caspase. Inhibition of caspase-8 provided no protection against RhoA-induced DNA fragmentation whereas treatment with the caspase-9 inhibitor afforded complete protection (Figure 26b). These data indicate that caspase-9 mediates RhoA-induced apoptosis and imply involvement of the mitochondrial death pathway.

IV.C.6. RhoA does not signal through mPTP to elicit cardiomyocyte apoptosis

A well characterized mechanism of cytochrome *c* release and caspase-9 activation is through the mitochondrial permeability transition pore (mPTP). Opening of the mPTP leads to an influx of ions into the mitochondrial matrix resulting in loss of mitochondrial membrane potential, swelling and rupture of the outer mitochondrial membrane, and release of cytochrome *c*. To test whether the mPTP was involved in

the RhoA-induced apoptotic response we used a known mPTP blocker, bongkrekic acid. Treatment with bongkrekic acid did not prevent DNA fragmentation induced by RhoA overexpression (Figure 27). Mitochondrial membrane potential was also monitored in response to RhoA infection. The mitochondrial membrane potential sensitive dye TMRE was used to stain for intact mitochondria and cells were then sorted by FACS analysis, as described in our previous work (120). No change in mitochondrial membrane potential was observed in cells infected with RhoA compared to control infected cells (Figure 28). This contrasts with the marked loss of mitochondrial membrane potential in cells infected with constitutively activated Gαq (Figure 28) as shown previously (120). These data argue against involvement of an mPTP-dependent mechanism in RhoA-mediated caspase activation and apoptosis.

IV.C.7. RhoA induces Rho kinase dependent upregulation of Bax

Previous reports have provided evidence that the pro-apoptotic Bcl-2 family protein Bax can translocate to the outer mitochondrial membrane. At the outer mitochondrial membrane, Bax, perhaps in concert with the pro-apoptotic protein Bak, can form a pore which allows the release of cytochrome *c* from the intermembrane space and activation of downstream caspases. To test the hypothesis that RhoA increased the ratio of pro-apoptotic to anti-apoptotic Bcl-2 family proteins we performed Western blot analysis for Bax, Bak, Bcl-2 and Bcl-x_L proteins. Levels of Bax protein were increased approximately 4-fold over control following 48 hours of expression of activated RhoA, while levels of Bcl-2 and Bcl-x_L, pro-survival mediators, were unchanged. We also detected no change in Bak protein expression

(Figure 29a). The RhoA-induced increase in Bax protein was completely abrogated by treatment with Y-27632 and expression of the dominant negative Rho kinase construct (data not shown), suggesting upstream Rho kinase involvement (Figure 29b). Bax mRNA was also measured in response to RhoA expression. Using real-time PCR we determined that Bax message was increased approximately 2-fold at 24 hours and 2.5-fold at 48 hours, suggesting transcriptional activation of this gene (Figure 30).

IV.C.8. RhoA expression increases Bax activation

To investigate whether RhoA expression increases levels of activated Bax protein, we used the mouse monoclonal anti-Bax (6A7) antibody, which recognizes only the active conformation of Bax, to immunoprecipitate Bax from cell lysates. There was a marked increase in activated Bax in cells expressing RhoA compared to control cells (Figure 31) and this increase was inhibited by treatment with the Rho kinase inhibitor Y-27632. Thus RhoA expression and Rho kinase activation not only induce upregulation of Bax mRNA and protein expression, but also increase Bax activation.

IV.C.9. RhoA expression increases Bax association with mitochondria

Activated Bax has been shown to translocate to mitochondria (79; 180). Cell fractionation experiments were carried out to determine whether RhoA expression increased the amount of mitochondrial associated Bax. Cells infected with RhoA were

collected, lysed and fractionated to prepare mitochondrial and cytosolic fractions. Fractions were subjected to Western blot analysis with VDAC and RhoGDI antibodies to confirm the purity of the mitochondrial and cytosolic fractions respectively (Figure 32). The Bax antibody was then used to detect total endogenous Bax protein in the cytosolic and mitochondrial fractions. RhoA expression led to significant increases in Bax protein in the mitochondrial fraction when compared to control AdCMV infected cells.

IV.C.10. RhoA-induced Bax upregulation and apoptosis are mediated by p53

The transcription factor p53 is a known inducer of apoptosis and can activate transcription of various pro-apoptotic factors including Bax (122-124; 140). To further investigate the upregulation of Bax we tested the effect of blocking p53 function on Bax protein expression. Adenoviral expression of a mutant p53 construct, shown previously to act as a dominant negative *in vivo* as well as *in vitro* (68), significantly inhibited RhoA-induced Bax protein upregulation (Figure 33a). Further, blocking p53 function with the dominant negative construct markedly inhibited RhoA-induced DNA fragmentation (Figure 33b).

IV.C.11. Bax inhibition prevents RhoA-induced caspase activation and apoptosis

To determine the functional relevance of increased Bax protein in RhoA-induced apoptosis we employed a commercially available peptide that was previously shown to bind directly to Bax, prevent its mitochondrial translocation, and inhibit Bax-mediated apoptosis (154). Treatment with the Bax inhibitory peptide at 100 μM and 200 μM concentrations reduced RhoA-induced DNA fragmentation, as assessed by POD analysis, by 50% and 60% respectively, whereas treatment with the control peptide afforded no protection (Figure 34a). RhoA-induced activation of caspase-9 and caspase-3 were also significantly attenuated by treatment with the Bax inhibitory peptide (Figure 34b and c).

IV.C.12. Expression of Bcl-2 and Bcl-x_L prevent RhoA-induced apoptosis

As further evidence for a causal role of Bax in RhoA-mediated apoptosis, we tested the effect of adenoviral expression of Bcl-2 and Bcl-x_L, two known antagonists of Bax-mediated apoptosis (47; 166). Expression of either Bcl-2 or Bcl-x_L was able to nearly completely abolish RhoA-induced cell death (Figure 35).

IV.C.13. Bax upregulation is an early RhoA /Rho kinase-mediated event that is sufficient to cause apoptosis

To better understand the role of Bax in RhoA signaling we examined the kinetics of Bax protein expression. A roughly 1.5-fold increase in Bax protein, albeit not statistically significant, was seen as early as 12 hours after virus washout. By 24

hours Bax protein levels were more than two-fold greater than control and a 4-fold increase was apparent at 48 hours (Figure 36). These data indicate that Bax upregulation is an early response to RhoA activation. As demonstrated above (Figure 25) Rho kinase inhibitors prevent RhoA-induced DNA fragmentation and caspase activation. Interestingly, however, when addition of the Rho kinase inhibitor was delayed until 12 hours after virus washout (time denoted by arrow on Figure 36) it failed to protect against development of apoptosis (Figure 37a). Delayed addition of the Rho kinase inhibitor also failed to prevent the RhoA-induced increase in Bax expression (Figure 37b). These data suggest that increased Bax expression is initiated within a 12 hour time window and is necessary to achieve RhoA and Rho kinase-induced apoptosis.

IV.C.14. Phenylephrine treatment accentuates RhoA-induced Bax upregulation but attenuates RhoA-induced apoptosis

Cardiomyocyte hypertrophy initially develops as a compensatory mechanism to increased cardiac demand but often transitions to heart failure, a condition for which the importance of apoptotic cell loss has been established. To determine whether enhanced RhoA expression could contribute to this process we tested the effect of activated RhoA on hypertrophied cardiomyocytes. Phenylephrine (PE), although not an efficacious activator of RhoA, is a robust hypertrophic agonist in neonatal rat ventricular myocytes (96). To test the effect of hypertrophy on the ability of RhoA to induce Bax upregulation and cardiomyocyte apoptosis, we pretreated

myocytes with PE for 24 hours and then infected the cells with control AdCMV or RhoA adenovirus. Our data show that PE pretreatment, which induces hypertrophy, synergistically increases RhoA-induced Bax upregulation (Figure 38a). Interestingly, myocytes that were initially hypertrophied were partially protected against RhoA-induced cell death (Figure 38b). PE has been shown previously to activate numerous signaling pathways known to afford protection in cardiac myocytes. As shown in Panel c of Figure 38, a 36 hour PE treatment increased phosphorylation of two known protective molecules, ERK (p42, p44) and Akt, which may account for the ability of PE treatment to attenuate RhoA-induced apoptosis.

IV.C.15. Inhibition of Akt activation exaggerates RhoA-induced apoptosis

We showed in the previous chapter that RhoA mediates Akt activation. We reasoned that this protective RhoA signaling pathway opposes RhoA-induced apoptosis. To test the role of Akt in apoptotic signaling, we treated both control and RhoA infected cardiomyocytes with vehicle or 10 μM LY to inhibit PI3K/Akt activation. The ability of RhoA to induce apoptosis was significantly increased at both 4 and 8 MOI in cells in which Akt activation was inhibited by LY treatment (Figure 39). These data suggest that there is in fact a balance between the pro-survival and pro-apoptotic signaling pathways initiating from RhoA, and that this balance can be manipulated through pharmacological inhibition.

IV.D. Discussion

We previously demonstrated that expression of RhoA in cardiomyocytes induces cellular hypertrophy and hypertrophic gene expression (77). These observations are confirmed by the increases in cell size and ANF expression shown here following 24 hours of RhoA expression. Although an initial hypertrophic response was observed, cardiomyocytes expressing RhoA for 36- 48 hours became frankly apoptotic, as indicated by TUNEL staining, DNA laddering and fragmentation, and caspase activation. All of these responses were blocked by treatment with either of two Rho kinase inhibitors, Y-27632 and HA-1077, as well as expression of dominant negative Rho kinase. Remarkably, while DNA fragmentation and caspase activation were not evident for 36-48 hours, a critical event occurs during the first 12 hour window. Thus when addition of Rho kinase inhibitors is delayed until 12 hours after virus washout DNA fragmentation is not prevented. The effects of Rho kinase that ultimately lead to apoptosis must therefore be elicited at the time of hypertrophy to initiate the subsequent sequence of apoptotic events.

There are currently two major paradigms for apoptotic cell death; the receptor-mediated "extrinsic" pathway and the mitochondrial mediated "intrinsic" pathway. The mitochondrial death pathway is initiated by cellular signals that ultimately impact upon the mitochondria leading to the release of cytochrome *c* and caspase activation (64). Our finding that caspase-9 is activated downstream of RhoA /Rho kinase and that a caspase-9 selective inhibitor is able to block RhoA-induced DNA fragmentation, implicates a mitochondrial death pathway in RhoA-induced apoptosis. The lack of caspase-8 activation and a lack of protection by inhibition of caspase-8 further support

the notion that a mitochondrial rather than a death receptor (extrinsic) pathway is involved. Our finding that expression of activated RhoA induces cytochrome *c* release (data not shown) provides additional support for a mitochondrial pathway. There are currently two known mechanisms of cytochrome *c* release from mitochondria, the first involving the opening of the mPTP, the second dependent on Bcl-2 family-mediated pore formation. Cells expressing RhoA for up to 48 hours showed no loss of inner mitochondrial membrane potential as assessed using the mitochondrial membrane potential sensitive dye, TMRE, and FACS analysis. This contrasted with data obtained in parallel using cells infected with constitutively activated Gαq, which dramatically decreased mitochondrial membrane potential (1; 120). Bongkrekic acid, an mPTP blocker previously shown to protect against Gαq-induced apoptosis (1), did not prevent RhoA-induced apoptosis, further suggesting that the mPTP was not involved.

An alternative mechanism of mitochondrial mediated caspase activation and apoptosis involves altered expression or activity of Bcl-2 family proteins. There is general agreement that a balance between pro- and anti-apoptotic Bcl-2 family proteins can directly regulate the mitochondria and determine cell fate (30). An initial screen found that the amount of Bax protein present in the cell was increased approximately 4-fold over control levels in response to activated RhoA. In contrast, Bcl-2, Bcl-x_L and Bak protein levels were unaffected by expression of RhoA. The timecourse of Bax expression revealed a trend toward increased protein at 12 hours, a significant two-fold increase by 24 hours and maximal expression at 48 hours. Bax mRNA levels were also significantly increased at 24 hours. Rho kinase was

implicated in Bax upregulation since the increase in Bax protein expression was inhibited by Y-27632 and dominant negative Rho kinase. Remarkably, while inhibition of Rho kinase with Y-27632 could fully abrogate RhoA-induced upregulation of Bax protein, this response was not prevented when Y-27632 addition was delayed until 12 hours after virus washout. These data suggest that Bax upregulation is an early RhoA /Rho kinase-mediated event that, once initiated, is sufficient to lead to apoptosis at later times.

Two published studies demonstrated that RhoA-mediated apoptosis was Bcl-2 sensitive (4; 15), but the apoptotic pathway inhibited by Bcl-2 was not elucidated. Pro-survival Bcl-2 family proteins are known to antagonize Bax-mediated mitochondrial membrane permeabilization (166), cytochrome *c* release and cell death (47). Thus our finding that RhoA-induced apoptosis involves the upregulation of Bax is consistent with, and can explain the protective effect of, increased Bcl-2 expression observed in these earlier studies. Further, we demonstrate that adenoviral expression of either Bcl-2 or Bcl-x_L prevents RhoA-induced cardiomyocyte apoptosis, presumably by antagonizing Bax function.

Activation and mitochondrial translocation of Bax appear to be required for its apoptotic activity. Bax is present in the cytosol of unstimulated cells and following its activation and change in conformation induced by apoptotic insult (129), it translocates to mitochondria (79; 180). Death signals cause Bax to oligomerize at the outer mitochondrial membrane (6; 7; 42) where this complex can form a pore allowing the release of cytochrome *c* and other apoptogenic factors into the cytosol (85; 95). We therefore also examined Bax activation and association with mitochondria. Our

data provide evidence that in addition to upregulating expression of Bax, RhoA expression leads to an increase in activated Bax and to an increase in Bax protein associated with the mitochondria. Bax translocation has not been extensively documented in cardiomyocytes, although recent evidence suggests that this can occur in neonatal rat ventricular myocytes in response to simulated ischemia/reperfusion (I/R) both acutely (3 hours) and chronically (36 hours) (20; 20; 78). Further, the ability of Bax ablation to prevent decreased cardiac function induced by I/R in mice demonstrates its role in cardiac injury (76). Importantly, our studies using the Bax inhibitor peptide provide evidence that Bax function is critical to subsequent caspase activation, DNA fragmentation and apoptosis. Our finding that sustained RhoA activation (48 hours) can also increase mitochondrial distribution of Bax, in combination with previous data demonstrating increased RhoA activity following myocardial infarction (86; 153), suggests that RhoA activation could play a role in I/R-induced cell death.

The tumor suppressor p53 has a well documented role in activating gene expression leading to apoptosis. p53 regulated pro-apoptotic target genes include Puma, Noxa, Bid and Bax (122-124; 175)(123). Our finding that RhoA increased Bax mRNA and our studies using a dominant negative mutant of p53, which acts to inhibit endogenous p53 function, (68) implicate p53 in RhoA-induced increases in Bax protein and DNA fragmentation. These data suggest that p53 mediates the apoptotic response and provides a link between RhoA /Rho kinase and Bax upregulation.

There is a growing literature indicating that inhibiting RhoA /Rho kinase has salutary effects in cardiovascular disease. It has been reported that RhoA /Rho kinase

expression and activity are upregulated in diabetes (117) hypertension (86; 153), and myocardial infarction (12; 69). Recent reports in the literature suggest that treatment with the Rho kinase inhibitors Y-27632 and fasudil (HA-1077) affords protective cardiac effects *in vivo*. Rho kinase inhibition was able to decrease myocardial fibrosis in response to infarct in the rat (179). Inhibition of Rho kinase with Y-27632 was also shown to reduce infarct size and apoptosis resulting from 30 minutes ischemia followed by 150 minutes (rat) or 24 hours (mouse) of reperfusion (12; 179). Further, Rikitake *et al* (146) found decreased fibrosis in Rho kinase (ROCK-1) haploinsufficient mice following myocardial infarct and pressure overload. These data all demonstrate salutary effects of blocking Rho kinase activity, although it remains unclear whether the beneficial effect of blocking Rho kinase in these *in vivo* models reflects the inhibition of Rho kinase activity in the cardiomyocyte or in other peripheral cell types such as fibroblasts, neutrophils or endothelial cells. However, a recent report by Chang *et al* describes the importance of caspase-3 cleavage in activation of ROCK-1 and apoptosis in cardiomyocytes (21). We demonstrate here that sustained RhoA and Rho kinase activity in cardiomyocytes can lead to caspase-3 activation and apoptosis. Taken together, these findings suggest that Rho kinase can function both upstream and downstream of caspase-3 to induce cardiomyocyte apoptosis. Further, they provide an explanation for the salutary cardiac effects of Rho kinase inhibitors *in vivo*.

Based on our findings we propose that RhoA activation in cardiomyocytes signals through its effector Rho kinase to induce p53-mediated increases in Bax mRNA, total and activated Bax protein, and Bax protein present at mitochondria.

Why this upregulation of Bax expression occurs early and does not induce apoptosis until later times is still unclear. It is possible that other genes are concomitantly upregulated, possibly pro-survival genes that allow the cell to balance the apoptotic actions of Bax with survival pathways. Interestingly, in this regard, we find that the hypertrophic agonist PE acts synergistically with RhoA to induce Bax upregulation but does not exacerbate RhoA-induced apoptosis. Indeed PE attenuates RhoA-induced apoptosis probably due to its ability to activate protective molecules such as ERK and Akt. This paradigm suggests a balance between pro-survival and pro-apoptotic signaling events in the hypertrophic cardiomyocyte. This balance seems to occur in RhoA infected cells as well, since our studies also demonstrate that RhoA expression activates the survival molecule Akt at early times. Over time, however, Bax upregulation appears to be sufficient to shift the balance in Bcl-2 family protein expression, induce mitochondrial permeabilization, initiate activation of caspase-9 and -3, and ultimately induce cardiomyocyte DNA fragmentation and cell death (schema Figure 40). We have generated inducible lines of cardiac specific RhoA transgenic mice as model systems that allow us to temporally control RhoA expression and signaling in the cardiomyocyte *in vivo*. This avoids the lethality observed with our previous RhoA transgenic lines (150) and provides a means to further explore the salutary and deleterious cardiac effects of RhoA in a physiologic context.

IV.E. Acknowledgment

This chapter, in part, is a reprint of a manuscript by **Del Re, D.P.**, Miyamoto, S., and Brown, J.H. RhoA/Rho kinase up-regulate Bax to activate a mitochondrial death pathway and induce cardiomyocyte apoptosis. *J. Biol. Chem.* 2007 Mar 16;282(11):8069-78. The dissertation author was the primary researcher and author of this manuscript. Dr. Joan Heller Brown directed and supervised the research which forms the basis for this chapter.

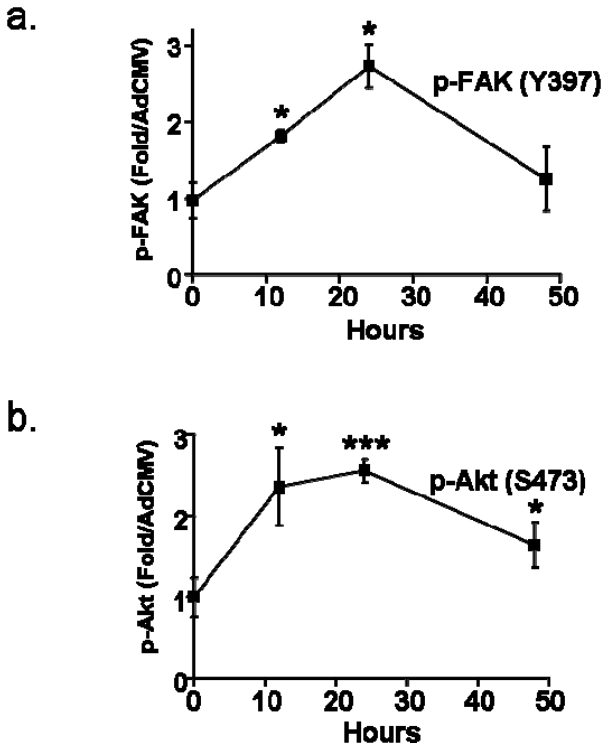

Figure 20. RhoA-induced activation of FAK and Akt is transient. A. and B. Neonatal rat ventricular myocytes were infected with (L63)RhoA or control (AdCMV) adenovirus for 2 hours, washed, and harvested at 0, 12, 24 and 48 hours. Cell lysates were prepared and Western blot analysis was performed to determine levels of phosphorylated FAK (Y397) and phosphorylated Akt (S473). Values are represented as averages ± S.E. (n = 3). *, $p<0.05$ versus time matched AdCMV. ***, $p<0.001$ versus time matched AdCMV.

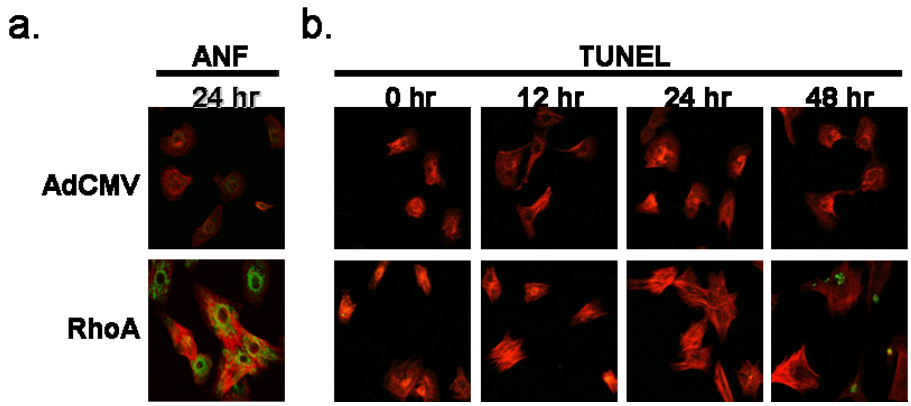

Figure 21. Timecourse of hypertrophy and apoptosis in RhoA infected cardiomyocytes. Neonatal rat ventricular myocytes were infected with (L63)RhoA or control AdCMV adenovirus. Cells were washed and fixed at 0, 12, 24 and 48 hours following virus washout. A. Cardiomyocytes were probed with anti-atrial natriuretic factor (ANF) primary antibody followed by FITC conjugated secondary antibody. Actin filaments were visualized using rhodamine conjugated phalloidin. B. Cells were stained with rhodamine conjugated phalloidin and apoptotic nuclei were identified by TUNEL as described in methods.

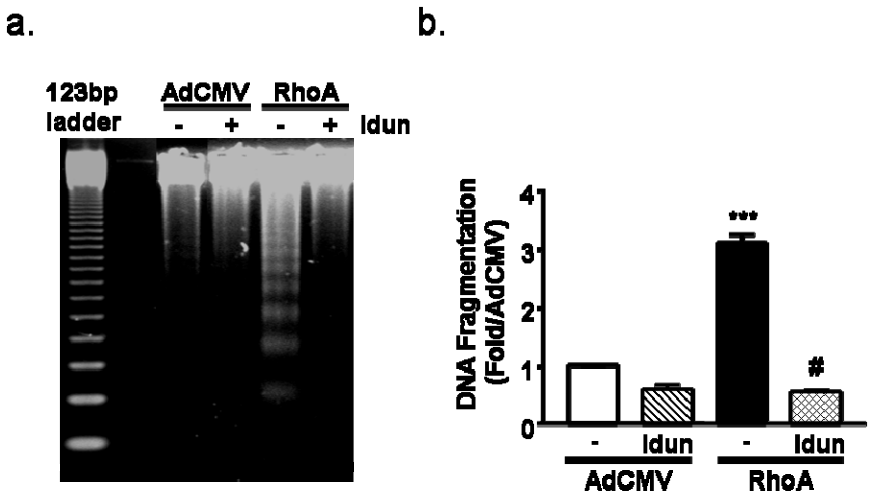

Figure 22. RhoA expression induces cardiomyocyte apoptosis. A. and B. Neonatal rat ventricular myocytes were infected with (L63)RhoA adenovirus or control AdCMV adenovirus at 4 MOI. Cells were treated with 10 μM Idun1965 (Idun), a non-selective caspase inhibitor, or vehicle as described in methods. A. Genomic DNA was harvested 48 hours following virus washout and laddering assay performed. B. Lysates were prepared 48 hours following virus washout and nucleosomal fragmentation was quantified by the ELISA-based POD assay. Values are represented as averages ± S.E. (n = 4). ***, $p<0.001$ versus AdCMV. #, $p<0.001$ versus RhoA.

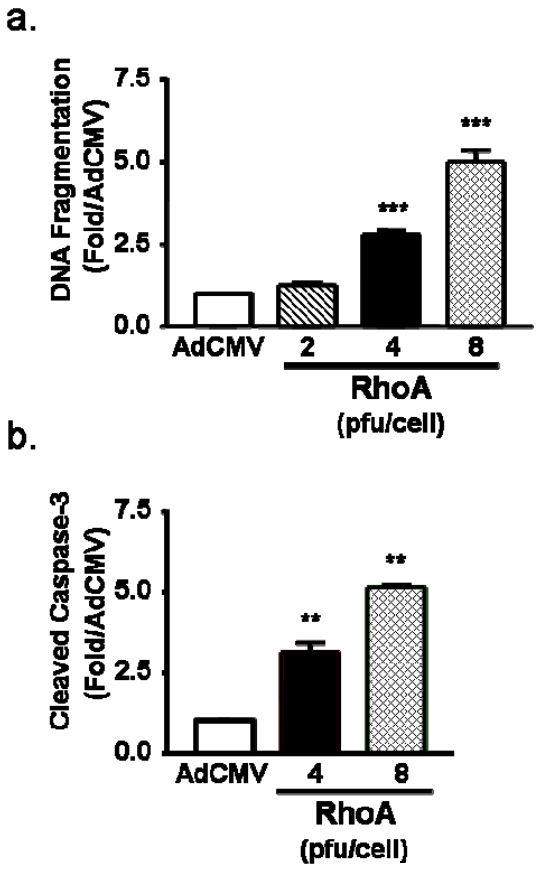

Figure 23. RhoA-induced cardiomyocyte apoptosis is dose dependent. A. and B. Neonatal rat ventricular myocytes were infected with (L63)RhoA adenovirus or control AdCMV adenovirus at increasing titers. A. Lysates were prepared 48 hours following virus washout. Nucleosomal fragmentation was quantified by POD assay. Values represent averages ± S.E. (n = 5). ***, $p<0.001$ versus AdCMV. B. Lysates were prepared 48 hours following virus washout. SDS-PAGE was performed and Western blot analysis quantified by densitometry. Values represent averages ± S.E. (n = 3). **, $p<0.01$ versus AdCMV.

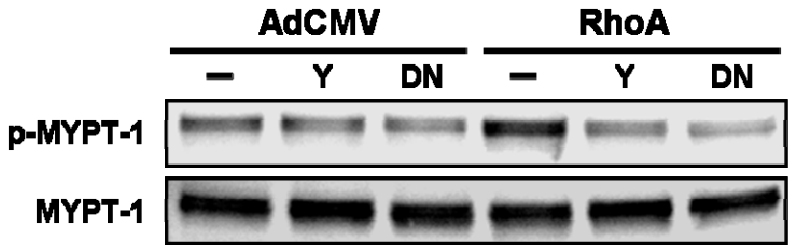

Figure 24. RhoA expression increases Rho kinase activity. Neonatal rat ventricular myocytes were infected with L63RhoA, dominant negative Rho kinase (DN) at 500 MOI, or control AdCMV adenovirus and treated with 10 μM Y-27632 (Y), 10 μM HA-1077 (HA) or vehicle as described in methods. Cell lysates were obtained 48 hours following virus washout. Western blot analysis was performed using specific antibodies to determine the level of phosphorylated (Thr696) MYPT-1 and total MYPT-1 respectively.

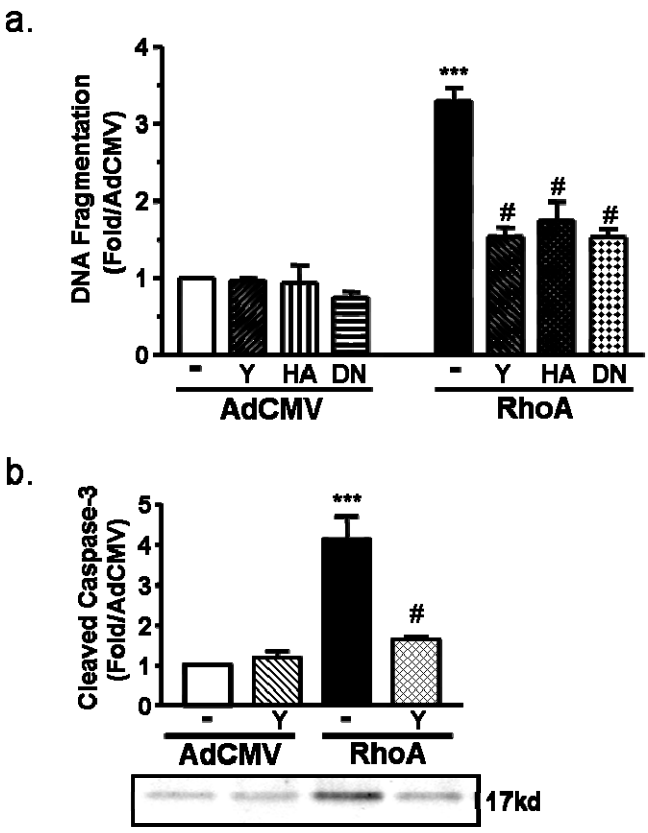

Figure 25. RhoA-induced apoptosis is mediated by Rho kinase. Neonatal rat ventricular myocytes were infected with L63RhoA, dominant negative Rho kinase (DN) at 500 MOI, or control AdCMV adenovirus and treated with 10 μM Y-27632 (Y), 10 μM HA-1077 (HA) or vehicle as described in methods. Cell lysates were obtained 48 hours following virus washout. A. The POD assay was used to quantify DNA fragmentation. Values represent averages ± S.E. (n = 5). ***, $p<0.001$ versus AdCMV. #, $p<0.001$ versus RhoA. B. Western blot analysis was performed to detect levels of cleaved caspase-3 using a selective antibody that only recognizes the 17 kD fragment. Values represent averages ± S.E. (n = 4). ***, $p<0.001$ versus AdCMV. #, $p<0.001$ versus RhoA.

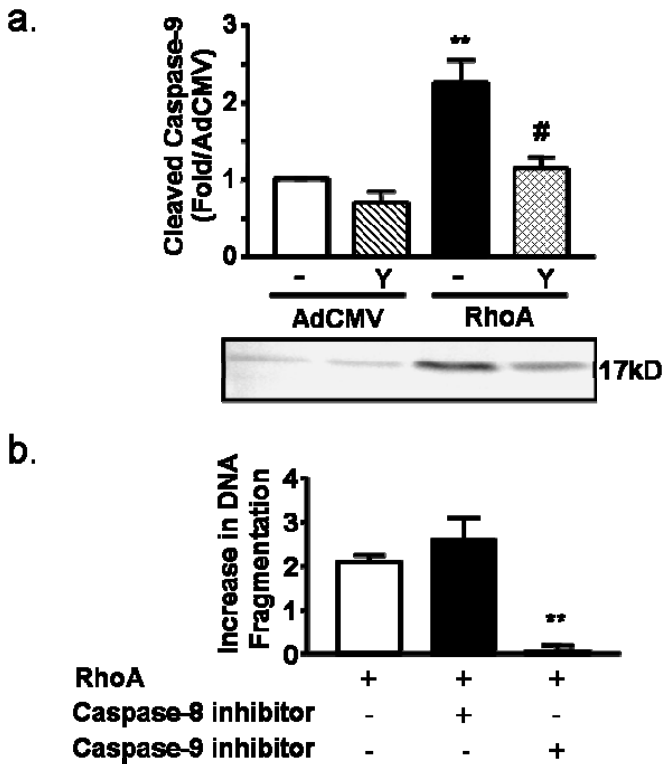

Figure 26. RhoA induces apoptosis through caspase-9 activation. Neonatal rat ventricular myocytes were infected with (L63)RhoA or control AdCMV adenovirus. A. Cells were treated with 10 μM Y-27632 (Y) or vehicle and lysates were obtained 48 hours after virus washout. Western blot analysis was performed to detect the 17 kD fragment of cleaved caspase-9. Values were quantified by densitometry and represent averages ± S.E. (n = 4). **, $p<0.01$ versus AdCMV. #, $p<0.01$ versus RhoA. B. Cardiomyocytes were treated with a caspase-8 or a caspase-9 selective inhibitor or vehicle. Lysates were prepared 48 hours following virus washout and POD assay performed to quantify DNA fragmentation. Values represent averages ± S.E. (n = 4). **, $p<0.01$ versus RhoA.

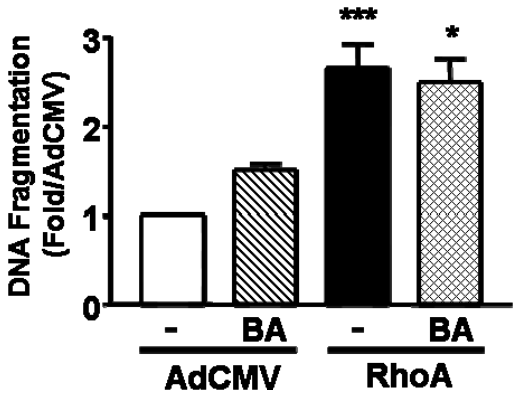

Figure 27. Bongkrekic acid treatment does not prevent RhoA-induced apoptosis. Neonatal rat ventricular myocytes were infected with (L63)RhoA or control AdCMV adenovirus. Cardiomyocytes were treated with 50 μM bongkrekic acid (BA) or vehicle and lysates prepared 48 hours following virus washout. POD assay was performed and DNA fragmentation quantified. Values represent averages ± S.E. (n = 4). ***, $p<0.001$ versus AdCMV. *, $p<0.01$ versus AdCMV.

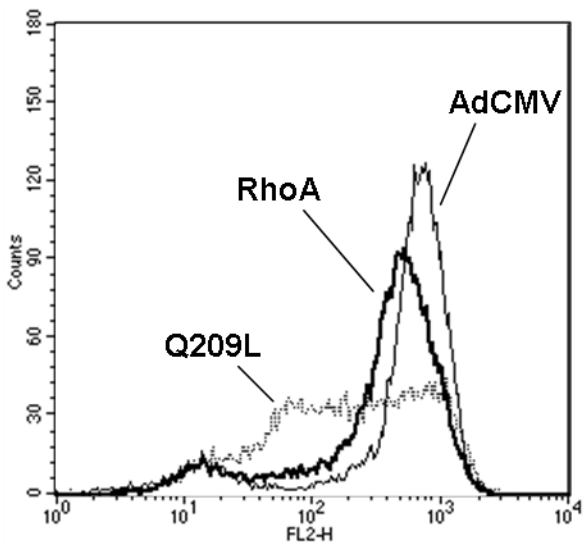

Figure 28. RhoA expression does not induce mitochondrial membrane depolarization. Neonatal rat ventricular myocytes were infected with (L63)RhoA, AdCMV or activated Gαq (Q209L) adenovirus. 48 hours after virus washout, cells were loaded with 50 nM TMRE for 20 minutes, collected by trypsinization, and subjected to FACS analysis. Data are from a representative experiment replicated three times.

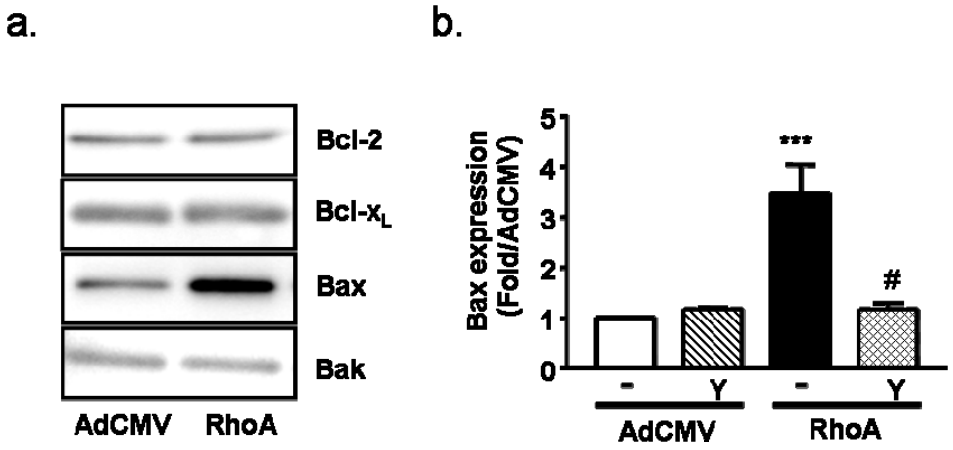

Figure 29. RhoA induces Rho kinase dependent upregulation of Bax. Neonatal rat ventricular myocytes were infected with (L63)RhoA or control AdCMV adenovirus. A. Western blot analysis was performed on lysates collected 48 hours after virus washout to determine Bcl-2 family protein expression. B. Cells were treated with 10 μM Y-27632 (Y) or vehicle at infection and Western blot analysis performed. Values represent averages ± S.E. (n = 4). ***, $p<0.001$ versus AdCMV. #, $p<0.01$ versus RhoA.

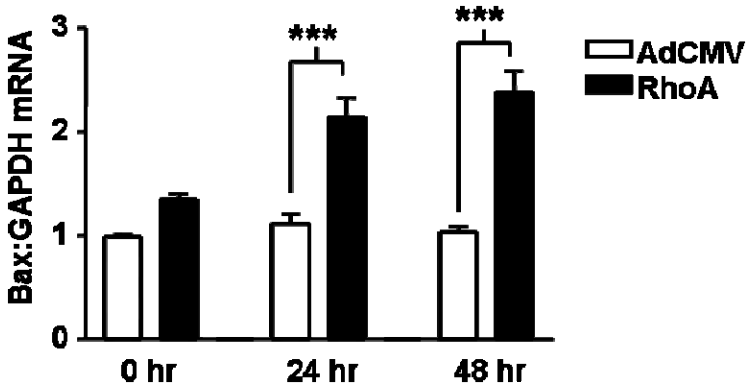

Figure 30. Timecourse of RhoA-induced increase in Bax mRNA expression. RNA was prepared from (L63)RhoA and control AdCMV infected neonatal rat ventricular myocytes at 0, 24 and 48 hours following virus washout. Real-time quantitative PCR was performed. Bax mRNA was normalized to GAPDH mRNA. Values represent averages ± S.E. (n = 9). ***, $p<0.001$ versus time matched AdCMV.

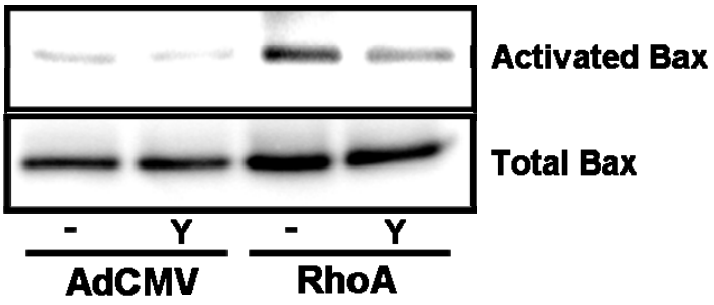

Figure 31. RhoA increases activated Bax. Neonatal rat ventricular myocytes were infected with (L63)RhoA or control AdCMV adenovirus. Cardiomyocytes were treated with 10 μM Y-27632 (Y) or vehicle and lysates prepared 48 hours after virus washout. Anti-Bax monoclonal antibody (clone 6A7) was used to selectively immunoprecipitate the activated conformation of Bax. Western blot analysis was performed on the resulting whole cell lysate and IP samples.

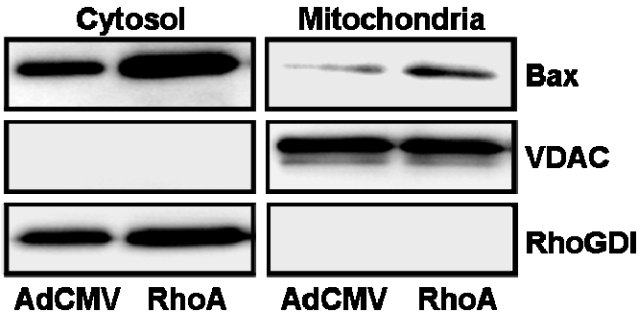

Figure 32. RhoA expression increases Bax association with mitochondria. Neonatal rat ventricular myocytes were infected with (L63)RhoA or control AdCMV adenovirus. 48 hours following virus washout, cell lysates were obtained and cytosolic and mitochondrial fractions were prepared as described in methods. Western blot analysis was performed to determine Bax protein levels. Antibodies to VDAC and RhoGDI were used to identify these proteins for use as specific markers of mitochondrial and cytosolic fractions.

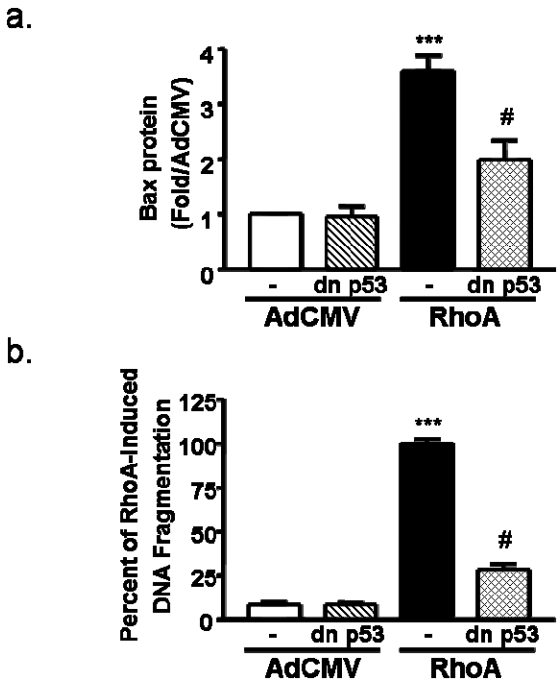

Figure 33. RhoA-induced Bax upregulation and apoptosis are mediated by p53. Neonatal rat ventricular myocytes were co-infected with (L63)RhoA or control AdCMV adenovirus and a dominant negative p53 adenovirus (8 MOI). Cardiomyocytes were harvested 48 hours following virus washout and lysates prepared. A. Western blot analysis was performed to detect Bax protein. Values are averages ± S.E. (n = 7). ***, $p<0.001$ versus AdCMV. #, $p<0.001$ versus RhoA. B. POD analysis was performed to quantify DNA fragmentation. Values are averages ± S.E. (n =9). ***, $p<0.001$ versus AdCMV. #, $p<0.001$ versus RhoA.

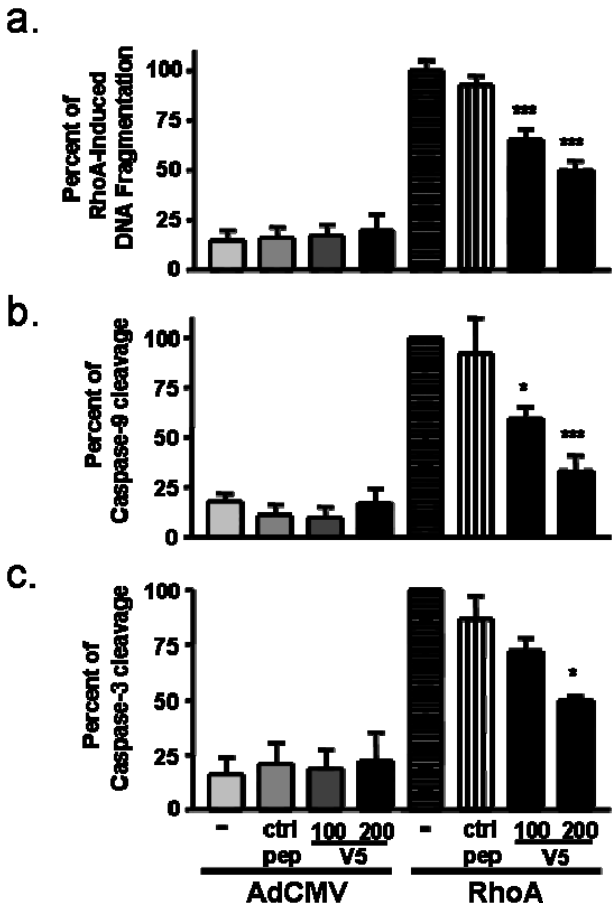

Figure 34. Bax inhibition prevents RhoA-induced caspase activation and apoptosis. Neonatal rat ventricular myocytes were infected with (L63)RhoA or control AdCMV adenovirus. Cells were treated with 100 or 200 μM V5 Bax inhibitory peptide (V5), 200 μM control peptide (ctrl pep) or vehicle. Lysates were prepared 48 hours after virus washout. A. POD assay was performed to quantify DNA fragmentation. Values are representative of averages ± S.E. (n = 8). B. and C. Western blot analysis was performed to determine levels of cleaved caspase-9 and cleaved caspase-3 respectively. Values represent averages ± S.E. (n = 3). ***, $p<0.001$ versus RhoA. *, $p<0.05$ versus RhoA.

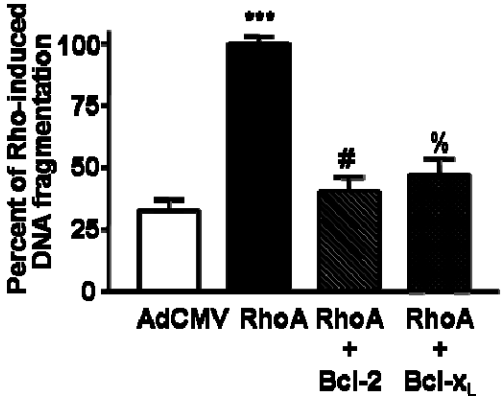

Figure 35. Expression of Bcl-2 or Bcl-x_L prevents RhoA-induced apoptosis. Neonatal rat ventricular myocytes were infected with (L63)RhoA or control AdCMV adenovirus and co-infected with Bcl-2 (200 MOI) or Bcl-x_L (200 MOI) adenovirus. Lysates were prepared 48 hours after virus washout and POD assay was performed to quantify DNA fragmentation. Values are representative of averages ± S.E. (n = 3). ***, $p<0.001$ versus AdCMV. #, $p<0.001$ versus RhoA. %, $p<0.01$ versus RhoA.

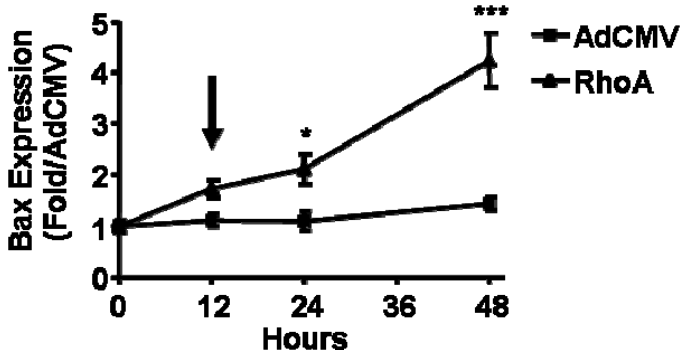

Figure 36. Timecourse of RhoA-induced Bax upregulation. Neonatal rat ventricular myocytes were infected with (L63)RhoA or control AdCMV adenovirus. Cell lysates were prepared at 0, 12, 24, and 48 hours following virus washout and subjected to Western blot analysis to detect Bax expression levels. Values are averages ± S.E. (n = 8). ***, $p<0.001$ versus AdCMV 48 hour. *, $p<0.05$ versus AdCMV 24 hour.

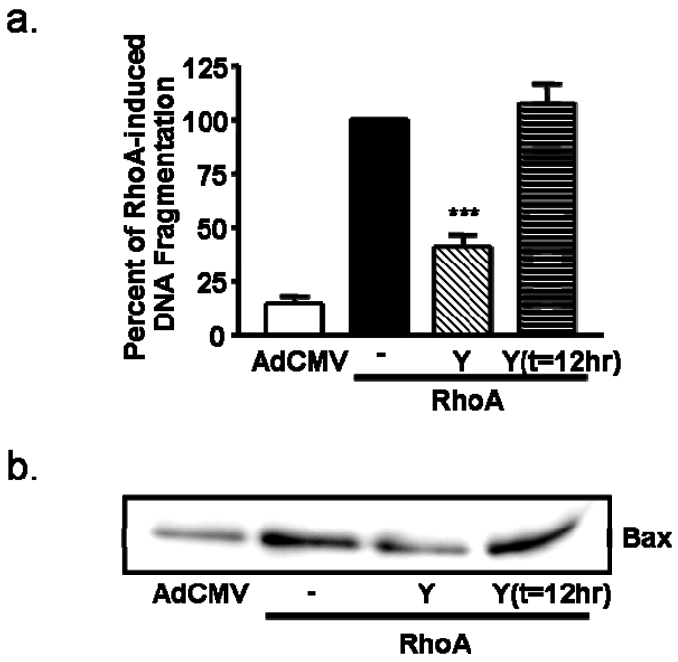

Figure 37. Bax upregulation is an early RhoA /Rho kinase-mediated event that is sufficient to cause apoptosis. Neonatal rat ventricular myocytes were infected with (L63)RhoA or control AdCMV adenovirus. A. and B. Cardiomyocytes were treated with vehicle or 10 μM Y-27632 (Y) as in previous experiments, or treatment with 10 μM Y-27632 was delayed 12 hours (to the time noted by arrow in Figure 34)(Y[t=12hr]). A. Cells were lysed 48 hours following virus washout and POD assay performed to quantify DNA fragmentation. Values represent averages ± S.E. (n = 4). ***, $p<0.001$ versus RhoA. B. Western blot analysis was performed to determine Bax protein expression in cells treated as described in A.

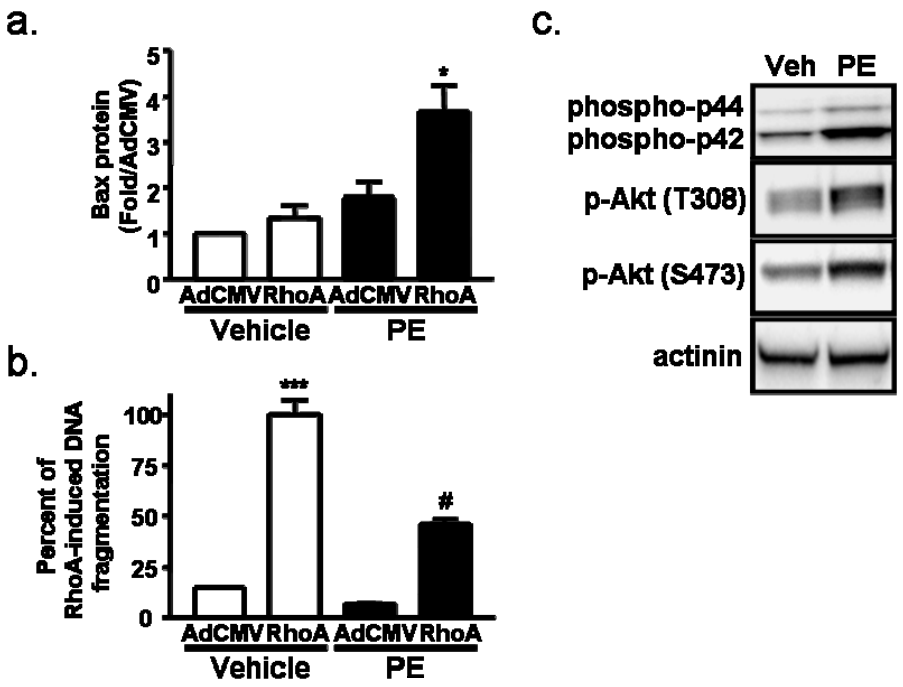

Figure 38. Phenylephrine treatment accentuates RhoA-induced Bax upregulation but attenuates RhoA-induced apoptosis. A. and B. Neonatal rat ventricular myocytes were treated with 100 μM phenylephrine (PE) or vehicle for 24 hours prior to infection with (L63)RhoA or control AdCMV adenovirus. A. Cardiomyocytes were harvested 12 hours following virus washout and lysates prepared. Western blot analysis was performed to detect Bax protein. Values are averages ± S.E. (n = 3). *, $p<0.05$ versus vehicle AdCMV. B. Cardiomyocytes were harvested 48 hours following virus washout and POD analysis was performed to quantify DNA fragmentation. Values are averages ± S.E. (n =3). ***, $p<0.001$ versus vehicle AdCMV. #, $p<0.001$ versus vehicle RhoA. C. Cardiomyocytes were treated with 100 μM phenylephrine or vehicle for 48 hours and lysates prepared. Western blot analysis was performed to detect phospho-p42/44, phospho-Akt (T308 and S473) and α-actinin.

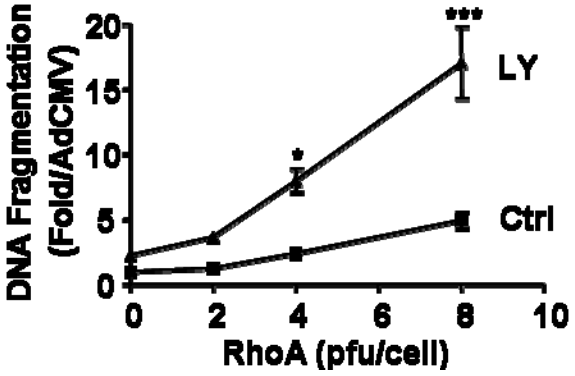

Figure 39. Inhibition of PI3K/ Akt signaling exaggerates RhoA-induced apoptosis. Neonatal rat ventricular myocytes were infected with (L63)RhoA adenovirus or control AdCMV adenovirus at increasing titers (0-8 MOI) and treated with 10 μM LY249002 (LY) or vehicle (Ctrl). Lysates were prepared 48 hours following virus washout. Nucleosomal fragmentation was quantified by POD assay. Values represent averages ± S.E. (n = 3). ***, $p<0.001$ versus RhoA (8 MOI) + vehicle. *, $p<0.05$ versus RhoA (4 MOI) + vehicle.

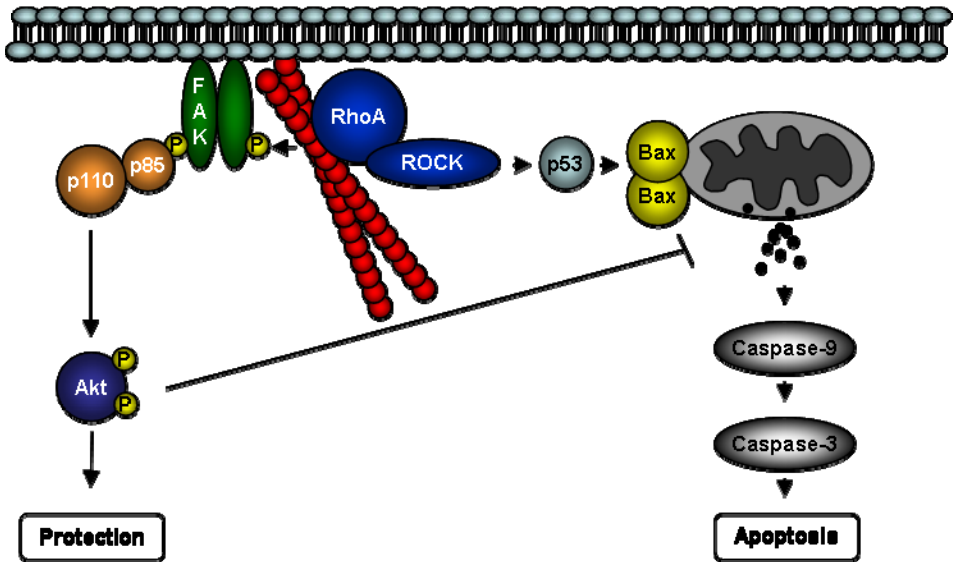

Figure 40. Summary schema illustrating RhoA signaling pathways that mediate both cardiomyocyte protection and apoptosis. RhoA activation signals through its effector Rho kinase (ROCK) to induce cytoskeletal rearrangement, mediating the activation of FAK. FAK binds and recruits PI3K via its regulatory subunit, p85, leading to increased Akt activation and cardiomyocyte protection. In contrast, chronic activation of RhoA /Rho kinase leads to p53-mediated upregulation of Bax expression and activation. Bax translocates to, and permeabilizes the mitochondria, allowing the release of apoptogenic mediators and subsequent caspase-9 and caspase-3 activation, culminating in cardiomyocyte apoptosis.

V. Examination of cardiac specific RhoA signaling pathways *in vivo*

V.A. Abstract

Examination of the effect of increased RhoA expression and activation in cultured cardiomyocytes has revealed that, depending on the extent of RhoA activity, either protective or apoptotic signaling pathways can be initiated. These *in vitro* findings led us to question whether there were similar pleiotropic effects of RhoA signaling *in vivo*. To enable investigations of this important problem we set out to develop an inducible line of cardiac specific RhoA transgenic mice. Initial data obtained with the first line we generated from this bi-transgenic system demonstrates successful transgene expression and suggests that it will be a useful *in vivo* model to study RhoA signaling in the heart. As another approach to examine the importance of RhoA signaling in cardiomyocyte survival *in vivo*, we have begun to examine the pathways of endogenous RhoA activation in the heart. The Langendorff method was used to assess whether ischemia/reperfusion (I/R) injury or ischemic preconditioning (IPC) affect RhoA activation in the isolated heart. We determined in preliminary studies that global I/R activates RhoA, and that IPC, while apparently having no basal effect on RhoA activation, completely abolished I/R-induced activation of RhoA. It will be of interest to further elucidate the mechanism of RhoA activation in response to these interventions, as well as to continue to evaluate the impact of increased RhoA expression and activity on cardiomyocyte survival in the *in vivo* heart.

V.B. Introduction

Little is known about the regulation of RhoA activation in the heart. One previous report showed that short term transverse aortic constriction (TAC) upregulates RhoA/Rho kinase association and Rho kinase activity in the heart (171). However, our preliminary results demonstrate, conversely, a reduction in activated RhoA in response to acute TAC (10 min – 3 hours) (Figure 41). Considerable data exists demonstrating a role for Rho kinase (presumably activated by RhoA) in the maladaptive responses to chronic TAC (135; 146; 184), hypertension (55; 153), myocardial infarction (MI) (69; 146), and *in vivo* I/R (12). These data are consistent with evidence suggesting that the activity of RhoA/Rho kinase is increased in a hypertensive rat model (86; 153), as well as in response to MI (69) and I/R injury (12) in mice. It is possible that the observed increase in RhoA/Rho kinase activity in these models is initially a compensatory response to cardiac insult. However, sustained RhoA/Rho kinase signaling appears to be maladaptive and may contribute to an exacerbated outcome.

To better understand the effect of increased RhoA expression and activation in the heart, both basally and during increased cardiac stress, we collaborated with Dr. Gerald Dorn's laboratory (University of Cincinnati, now Washington University) to generate a line of inducible transgenic mice, engineered to express activated (L63)RhoA specifically in cardiomyocytes. The transgene is under control of an attenuated αMHC promoter that has been reengineered to incorporate seven Tet operon repeats (151). Under normal conditions, the transgenic mice should not express the RhoA transgene (unless the system is "leaky"). However, when crossed

with the tetracycline-responsive transactivator (tTA) transgenic mouse line, cardiomyocytes now express the tTA protein, which binds the Tet operons in the attenuated αMHC promoter so that the RhoA transgene is expressed. To silence gene expression, e.g. to avoid developmental effects, doxycycline (a stable analog of tetracycline) is incorporated into the diet of pregnant females and resulting pups. While present, doxycycline is able to bind to and sequester tTA protein and prevent its actions on the attenuated αMHC promoter. Removal of the drug allows tTA-DNA binding and subsequent transgene expression (Figure 42).

V.C. Results

Initial protein expression screens of the 5 positive (southern blot and PCR) founder lines revealed at least one positive line (two lines have yet to be tested). Using this line we have demonstrated that pups positive for both transgenes (RhoA and tTA; designated TG), and are not fed doxycycline, express the RhoA transgene, whereas littermate controls (WT), which contain only the RhoA transgene or the tTA transgene, do not express. Western blot analysis performed from heart homogenate of 4 week old mice shows roughly a two-fold increase in RhoA protein expression in the TG versus WT mice (Figure 43). The amount of activated RhoA was also examined using the same heart homogenate. RBD pulldown assay revealed a 2-3 fold increase in the amount of GTP-RhoA present in the transgenic animals compared to littermate controls (Figure 43). Preliminary transgene expression screening was performed in animals never exposed to doxycycline to ensure both arms of the system were functioning as predicted. A previous round of founders (four lines) generated two

years prior to this group all failed to express the RhoA transgene in the absence of doxycycline. Hence this second cohort was generated with our collaborators and has proven more successful than the first with at least one positive line observed so far.

Experiments to test the inducibility of the system and to further characterize the transgene expression and basal phenotype of this line are ongoing. To test the responsiveness of the tet-off system, pregnant females from the positive founder line identified thus far have been placed on a diet that incorporates doxycycline and will remain on this diet until the pups are weaned (approximately 4 weeks). The pups will then be placed on a normal diet and transgene detected by Western blot analysis two to four weeks following doxycycline withdrawal. Currently, both TG and WT mice from the positive founder line are being maintained in the absence of doxycycline so that RhoA transgene expression/activation, as well as basal phenotype (hypertrophy, heart failure) and possible downstream signaling targets, can be evaluated over time.

Studies mentioned previously in the introduction indicate that RhoA/Rho kinase contribute to I/R damage, implying that this pathway is activated during I/R. To determine whether I/R injury in the adult mouse heart is in fact able to activate RhoA, we employed the Langendorff method of retrograde perfusion to simulate global ischemia followed by reperfusion. Control hearts were continuously perfused for 40 minutes. Ischemic hearts underwent stopped-flow global ischemia for 30 minutes. The I/R group was subjected to 30 minutes of ischemia followed by 10 minutes of reperfusion. Hearts were removed and immediately snap frozen in liquid nitrogen. Heart homogenates were analyzed for RhoA activation using the RBD pulldown assay, and Western blot analysis was performed to determine protein

expression and phosphorylation levels. We found RhoA activation to be modestly increased in hearts exposed to ischemia only, but markedly upregulated in response to I/R compared to control perfused hearts (Figure 44). Our data also show that Akt phosphorylation was increased in the I/R treated hearts, consistent with previous reports.

To further investigate a possible role for RhoA activation in response to global I/R, we asked whether RhoA was also activated by ischemic preconditioning (IPC). To simulate IPC, hearts were treated with three 5 minute ischemic periods, each separated by 5 minutes of reperfusion. Following the final 5 minute reperfusion, hearts were harvested and RhoA activity measured by RBD pulldown assay. No difference in the level of RhoA activation was observed in response to this IPC regimen compared to control perfused hearts (Figure 45). However, when hearts were exposed to IPC prior to 30 minutes ischemia and 10 minutes of reperfusion, the observed RhoA activation in response to I/R was completely abolished (Figure 45). IPC had no effect on the level of total RhoA protein in these experiments.

V.D. Discussion

Our findings here demonstrate that global I/R in the isolated mouse heart leads to robust activation of RhoA. The mechanism of RhoA activation in the isolated perfused heart is yet to be determined. One possible explanation is the increased force/pressure generated by the reperfusion process itself. There are published data from our lab and others demonstrating the ability of mechanical stretch to increase

RhoA activation in cultured cardiomyocytes (2; 88). Perhaps increased mechanical force can elicit similar activation of RhoA in adult cardiomyocytes *in vivo* as well.

It is also possible that autocrine and/or paracrine responses are responsible for increased RhoA activation following I/R. It has been demonstrated previously that I/R injury induces multiple signaling pathways (40; 70; 128). It is possible that agonists such as S1P or LPA, known activators of RhoA, are released by endothelial cells, fibroblasts or even cardiomyocytes themselves, free to bind their respective receptors and elicit RhoA activation. Clearly, this preliminary data provides more questions than answers and much work remains to elucidate the molecular mechanism responsible for RhoA activation post I/R.

Interestingly, we find that following I/R insult in the adult mouse heart, increased activation of RhoA correlates with increased Akt phosphorylation. Activation of Akt during global I/R has been demonstrated previously (173; 182), but in light of our Chapter 2 findings describing a mechanism by which RhoA can induce Akt activation, it will be of interest to determine whether there is any relationship between *in vivo* RhoA activation and Akt phosphorylation observed following I/R in the heart. Whether or not this increase in RhoA activation is part of an endogenous attempt to preserve heart survival and function remains to be seen. It is possible that activation of RhoA by I/R serves a deleterious purpose and may be responsible, in part, for the harmful consequences of I/R injury. On the other hand, the data using IPC suggest that RhoA activation and downstream signaling could afford protection and may no longer be required due to initiation of other protective signaling pathways.

Initial characterization of the newly generated line of tet-responsive cardiac specific RhoA transgenic mice has begun. We have demonstrated here that these mice show moderate increases in RhoA expression and increased RhoA activation in heart tissue at 4 weeks of age. These mice are currently being aged to determine what, if any, basal phenotype is present. Future experiments will also subject these transgenic mice to cardiac interventions known to cause increased apoptosis, fibrosis and decreased function, i.e. myocardial infarction, *in vivo* I/R and chronic TAC. This will be an invaluable system to determine whether increased RhoA expression and activity in the hearts of these mice either predispose them to improved or exacerbated outcomes in response to these interventions.

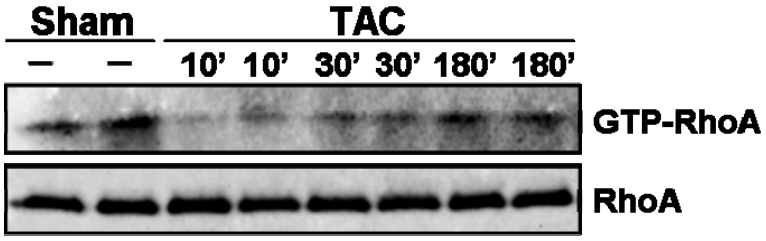

Figure 41. Transverse aortic constriction inhibits RhoA activation. Adult male mice were subjected to transverse aortic constriction (TAC) for 10, 30 or 180 minutes or sham operated. Hearts were snap frozen and homogenized. Activation of RhoA was determined by RBD pulldown assay.

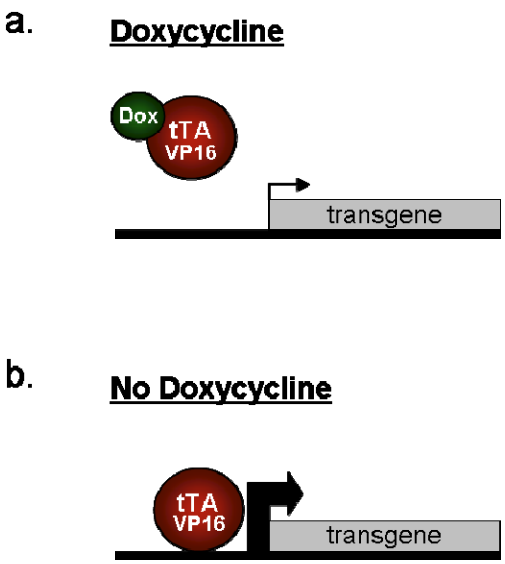

Figure 42. The "tetracycline (Tet) off" inducible system. Doxycycline, a stable analog of tetracycline, is used to silence transgene expression. A. When doxycycline (Dox) is present (either supplemented in food or water, or administered by injection), it binds the tet-responsive transactivator protein (tTA) preventing its interaction with the transgenic promoter, thus inhibiting gene expression. B. This repression is released via the removal of Dox, thereby allowing tTA-promoter interaction and transgene expression.

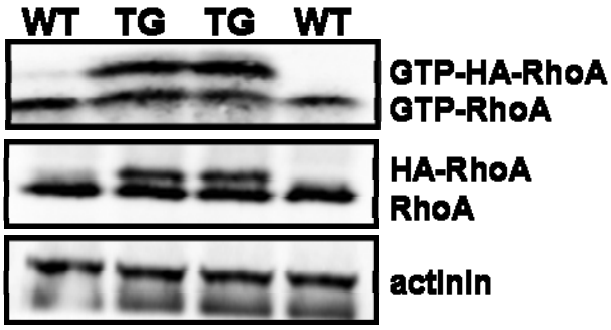

Figure 43. Cardiac expression and activation of RhoA in the transgenic mouse. Two transgenic (TG) and two wild-type (WT) littermates from the previously described positive line were sacrificed at 4 weeks of age. Ventricle tissue was removed, snap frozen and homogenized. RhoA activation was determined by RBD pulldown assay. Total RhoA expression was measured by Western blot analysis. The RhoA transgene contains an HA-tag and runs slightly larger on SDS-PAGE.

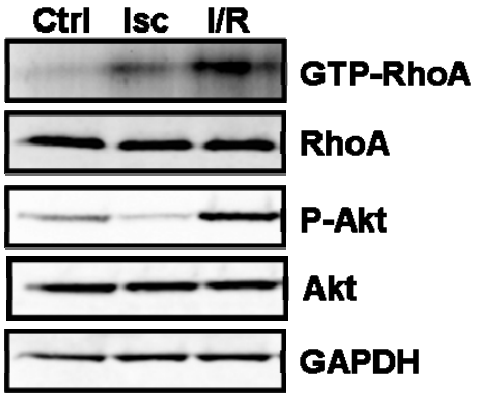

Figure 44. RhoA is activated by global ischemia/ reperfusion in the heart. Isolated adult mouse hearts were subjected to global ischemia/ reperfusion (I/R) injury using the Langendorff method of retrograde perfusion. Following 40 minutes of perfusion (Ctrl), 30 minutes of ischemia (Isc), or 30 minutes of ischemia and 10 minutes of reperfusion (I/R), hearts were snap frozen and homogenized. Activation of RhoA was determined by RBD pulldown assay. Akt phosphorylation was measured by Western blot analysis.

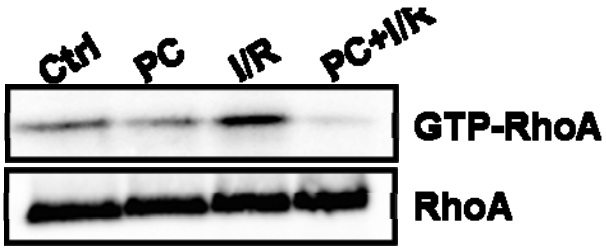

Figure 45. Preconditioning abolishes I/R-induced RhoA activation. Isolated adult mouse hearts were subjected to a preconditioning (PC) regimen of three 5 minute ischemic periods followed by 5 minutes of reperfusion prior to global ischemia/reperfusion (I/R) injury using the Langendorff method of retrograde perfusion. Immediately following the final reperfusion, hearts were snap frozen and homogenized. Activation of RhoA was determined by RBD pulldown assay.

VI. Discussion

Significant progress in understanding the signaling pathways and cellular responses mediated by the small GTPase RhoA has been made since the discovery of these proteins less than twenty years ago. The seminal paper of Ridley and Hall was the first to describe a role for RhoA in cytoskeletal organization (145). Subsequent work has demonstrated RhoA involvement in a wide range of cellular responses including cell proliferation and division, cell migration, gene expression and cell growth. Our laboratory and others have shown previously that RhoA mediates hypertrophic cell growth of terminally differentiated primary neonatal rat cardiomyocytes. A transgenic mouse model of cardiac specific RhoA overexpression generated by our laboratory subsequently revealed the possibility that RhoA signaling pathways might regulate cardiomyocyte survival. Prior to this thesis work, very little was known regarding the ability of RhoA to modulate cell survival in any cell type.

The studies described in this thesis have demonstrated that increased expression and activation of RhoA is sufficient to engage a protective signaling pathway via FAK activation, leading to recruitment of PI3K and subsequent activation of the protective kinase Akt. We have shown that this response is dependent on the ability of RhoA to induce cytoskeletal reorganization through its effector Rho kinase, presumably increasing points of contact between the cardiomyocyte and the extracellular matrix, leading to FAK aggregation and activation. Interestingly,

however, my work has also demonstrated that this protective response is transient and diminishes concomitant with downregulation of FAK and Akt activation. The observed decrease in phosphorylation of these protective kinases and the accompanying loss of cardioprotection cannot be explained simply by decreased RhoA expression or activation, as both parameters remain elevated and maximal throughout the duration of all experimental protocols tested (up to 48 hours). The molecular mechanisms responsible for the inability to sustain FAK and Akt activation, in the face of the continued presence of activated RhoA, remain to be elucidated. Determination of the pathways that turn off FAK and Akt signaling, and may, ultimately contribute to the transition from protection to apoptosis could provide insight into ways to modulate and prolong Akt activation, hopefully translating to better protected cardiomyocytes.

As demonstrated by experiments included in this dissertation, RhoA-mediated protection of cardiomyocytes from apoptotic insult is dependent on PI3K/Akt signaling. This is not surprising considering the evidence demonstrating the ability of Akt to activate protective events leading to cardiomyocyte survival. There are, however, many possible downstream targets of Akt that can mediate cell protection, amongst which are the Akt targets GSK3α/β, eNOS, FoxO1, and Bad. My work has suggested several potential Akt targets whose phosphorylation is increased in response to RhoA. It is beyond the scope of this thesis work to identify and further investigate the role of these potential targets in mediating cardiomyocyte protection, however future studies will aim to do so and could offer further insight into the mechanism of RhoA-mediated protection via FAK-Akt signaling.

The finding that RhoA increases the amount of phosphorylated Akt present at mitochondria offers additional insight into the possible protective actions of Akt in this system. Recently published work from our laboratory demonstrated the ability of Akt to afford cardiomyocyte protection through specific phosphorylation events at the mitochondria. Future work will also test whether this same protective paradigm holds true for RhoA-mediated cell survival.

This dissertation also defined the molecular pathway involved in RhoA-induced cardiomyocyte apoptosis. Upregulation of the pro-apoptotic Bcl-2 family member Bax was demonstrated to be critical to the mitochondrial death pathway initiated by prolonged RhoA activation. Interestingly, although Bax was increased at early times in response to RhoA expression (increased by 12 hours and significantly upregulated at 24 hours), resulting apoptosis was not detected until later times. This work also shows that pharmacological inhibition of PI3K/Akt signaling was able to robustly exaggerate the ability of RhoA to induce apoptosis. Taken together, these data suggest that there is a balance present between the pro-survival and pro-apoptotic signaling pathways initiated by RhoA expression, and that blocking one can alter the other. It is still unknown how pro-survival signaling (via Akt) is able to delay the onset of Bax-mediated apoptosis and this is another area of interest and further research.

In elucidating the mechanism of RhoA-induced cardiomyocyte apoptosis, we discovered that Bax upregulation is dependent on the transcription factor p53. This finding is consistent with the literature describing the ability of p53 to upregulate transcription of the *BAX* gene, and that Bax can mediate p53-induced apoptosis. The

mechanism, however, by which the RhoA effector Rho kinase signals to activate p53 and initiates Bax transcription has yet to be determined. There are no reports in the literature describing the ability of Rho kinase to modulate p53-mediated transcription. Because p53 signaling is involved in a host of human diseases, it is of considerable interest to determine if this regulation is through direct phosphorylation or indirect means, and if so, which intermediates are involved. This could potentially shed new light on a novel mechanism by which p53 is regulated. Whether this signaling pathway for RhoA-mediated Bax upregulation and apoptosis functions in other cell types or is limited to cardiomyocytes also needs to be examined.

A large portion of this dissertation work was performed in primary isolated neonatal rat ventricular myocytes. This is a tractable system that allows for both pharmacological intervention and exogenous overexpression to study signal transduction pathways *in vitro*. However, the use of any cell culture system has its limitations and a more physiologic system could offer more insight into the study of human disease. To this end, we, in collaboration with Dr. Gerald Dorn's laboratory (Washington University), generated an inducible line of cardiac specific RhoA transgenic mice using the tetracycline-responsive system developed by Dr. Jeffrey Robbins (Cincinnati Children's Hospital). Our laboratory's previous studies with cardiac specific RhoA transgenic lines demonstrated a profound lethal cardiomyopathy suggestive of increased cardiomyocyte cell death (150), but these lines could not be maintained for further breeding and study. Our aim in creating the inducible RhoA transgenics is to test whether our findings in the primary cell culture system will be translated *in vivo*. This is the first inducible model of increased RhoA

expression to be created and will be of great value to better understand RhoA signaling in the heart in a physiological context.

There is a growing literature describing the beneficial effects of blocking Rho kinase, the best characterized downstream mediator of RhoA signaling, in the cardiovascular system. Studies using pharmacological inhibitors of Rho kinase demonstrate improved cardiac survival and function in response to I/R, MI and TAC in both mice and rats. Genetic evidence supports these findings as both homozygous and heterozygous ROCK1 null animals both respond better to cardiac insult. These data, however, are based on studies in which there is global Rho kinase inhibition/ablation. RhoA/Rho kinase are known to play an important role in maintaining vascular tone as well as endothelial cell function, thus it is possible that the salutary effects of blocking Rho kinase signaling are due to actions at secondary targets. Examination of RhoA signaling specifically in the cardiomyocyte *in vivo* has yet to be undertaken. This is precisely what our transgenic model will allow for, making it a powerful tool to tease apart the possible role for RhoA in mediating cardiomyocyte survival in the *in vivo* heart.

There is a great deal that remains to be discovered still regarding the regulation of RhoA in the heart. Data presented here demonstrate increased RhoA activation in response to global I/R. Whether this induction occurs following I/R *in vivo* is not known, however pharmacological inhibition of Rho kinase following *in vivo* I/R appears beneficial suggesting that RhoA/Rho kinase are activated by I/R and that this response is maladaptive. Possible agonists that may activate RhoA in the heart include S1P and LPA. These signaling intermediates are generally thought to be

protective, and work from our laboratory has demonstrated that genetic ablation of S1P receptors increases infarct damage following *in vivo* I/R. Whether RhoA signaling plays a role in this protective mechanism has yet to be investigated. Previous work has shown that mechanical stretch of cultured cardiomyocytes and short durations of TAC (pressure overload) *in vivo* can activate RhoA. Although my data do not confirm this TAC-induced RhoA activation, it will be interesting to determine whether longer intervals of TAC can affect RhoA activity, and further, whether RhoA signaling pathways are involved in the eventual decompensation to heart failure caused by long-term TAC. Ultimately, a better understanding of RhoA signaling *in vivo* and its role in either the prevention or progression of I/R-induced cardiomyocyte loss and the development of heart failure will be paramount in identifying and pursuing RhoA as a possible therapeutic target in this disease.

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