ALPHA₁-ADRENERGIC RECEPTOR ACTIVATION MIMICS ISCHEMIC POSTCONDITIONING IN CARDIAC MYOCYTES

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## ABBREVIATIONS

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<tr>
<td>3-MA</td>
<td>3-methyladenine</td>
</tr>
<tr>
<td>5-HD</td>
<td>5-hydroxydecanoate</td>
</tr>
<tr>
<td>ACS</td>
<td>acute coronary syndrome</td>
</tr>
<tr>
<td>ANF</td>
<td>atrial natriuretic factor</td>
</tr>
<tr>
<td>AR</td>
<td>adrenergic receptor</td>
</tr>
<tr>
<td>ARVM</td>
<td>adult rat ventricular myocytes</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>CM</td>
<td>cardiac myocyte</td>
</tr>
<tr>
<td>CQ</td>
<td>chloroquine</td>
</tr>
<tr>
<td>DAG</td>
<td>diacylglycerol</td>
</tr>
<tr>
<td>DAPI</td>
<td>4',6,-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>ER</td>
<td>endoplasmic reticulum</td>
</tr>
<tr>
<td>ETC</td>
<td>electron transport chain</td>
</tr>
<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
</tr>
<tr>
<td>FITC</td>
<td>fluorescein isothiocyanate</td>
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<tr>
<td>GPCR</td>
<td>G protein- coupled receptor</td>
</tr>
<tr>
<td>HBSS</td>
<td>Hank’s balanced salt solution</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<td>--------------</td>
<td>-----------</td>
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<tr>
<td>INT</td>
<td>iodonitrotetrazolium</td>
</tr>
<tr>
<td>IP₃</td>
<td>inositol triphosphate</td>
</tr>
<tr>
<td>I/R</td>
<td>ischemia and reperfusion</td>
</tr>
<tr>
<td>Iso</td>
<td>isoproterenol</td>
</tr>
<tr>
<td>LDH</td>
<td>lactate dehydrogenase</td>
</tr>
<tr>
<td>MI</td>
<td>myocardial infarction</td>
</tr>
<tr>
<td>mKATP</td>
<td>mitochondrial ATP-sensitive potassium channel</td>
</tr>
<tr>
<td>MPTP</td>
<td>mitochondrial permeability transition pore</td>
</tr>
<tr>
<td>NHE</td>
<td>Na⁺/H⁺ exchanger</td>
</tr>
<tr>
<td>N-I</td>
<td>non-ischemic</td>
</tr>
<tr>
<td>mTOR</td>
<td>mammalian target of rapamycin</td>
</tr>
<tr>
<td>LC3-II</td>
<td>microtubule associated protein 1 light chain 3</td>
</tr>
<tr>
<td>NAD</td>
<td>β-nicotinamide adenine dinucleotide</td>
</tr>
<tr>
<td>NADH</td>
<td>nicotinamide adenine dehydrogenase</td>
</tr>
<tr>
<td>NE</td>
<td>norepinephrine</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PCI</td>
<td>percutaneous coronary intervention</td>
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<tr>
<td>PFA</td>
<td>paraformaldehyde</td>
</tr>
<tr>
<td>Phe</td>
<td>phenylephrine</td>
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<tr>
<td>PIP₂</td>
<td>phosphatidylinositol 4,5-bisphosphate</td>
</tr>
<tr>
<td>PKC</td>
<td>protein kinase C</td>
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<tr>
<td>PKG</td>
<td>protein kinase G</td>
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PLC  phospholipase C
PMS  phenazine methosulfate
Pnt  phentolamine
PostC  postconditioning
Prop  propranolol
PI  propidium iodide
Pz  prazosin
RISK  reperfusion injury signaling kinase
ROS  reactive oxygen species
Rp  rapamycin
RT-PCR  Reverse transcriptase polymerase chain reaction
SAFE  survivor activating factor enhancement
STAT-3  signal transducer and activator of transcription-3
TAE  Tris-Acetate-EDTA buffer
TBS  Tris-buffered saline
TNFα  tumor necrosis factor alpha
TUNEL  terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling

x
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I dedicate this dissertation to Patricia & Edward McDonnell. I love and miss you both.
CHAPTER ONE

INTRODUCTION

Heart disease

Heart disease is the leading cause of death in the United States, responsible for 1 in every 3 deaths (Go et al. 2014). The major types of heart disease are acute coronary syndrome (ACS) and myocardial infarction (MI). ACS is caused by atherosclerosis of the coronary arteries, a thickening of the arterial walls due to an accumulation of cholesterol and inflammatory cells, resulting in plaque formation. Plaques lead to a stiffening of the arteries and narrowing of the vessels, causing a reduction in blood flow which results in reduced oxygen delivery to the myocardium. These plaques can rupture, leading to the blockage of a downstream vessel. The complete blockage of coronary blood flow results in an MI, which is characterized by extreme ischemia of the infarcted area leading to hypoxic damage and subsequent cardiac myocyte death. In both atherosclerotic coronary narrowing and MI there is a reduction in oxygen and nutrients reaching the affected areas of the myocardium. Ischemic insult of the heart leads to contractile arrest within minutes (Tennant and Wiggers 1935) and ultimately, depending upon the length and severity of the ischemic episode, an area of irreversible damage called an infarction. This myocardial damage capitulates into a reduction of contractile function that is permanent due to minimal cardiac myocyte regeneration.
In addition to direct ischemic damage, there is additional insult to the myocardium during reperfusion when blood flow is reestablished. The reintroduction of oxygen, nutrients, and physiological pH during reperfusion precipitates a number of responses that can further damage myocardial tissue through arrhythmia, hypercontracture, stunning, and cell death (Hearse et al. 1973). A main goal of therapeutic intervention during and after MI is to decrease the area of infarct by decreasing ischemia/reperfusion (I/R) injury, or cell death incurred during ischemia and reperfusion (Yellon et al. 1984). Post-ischemic interventions, collectively known as postconditioning (PostC), have proven successful in decreasing the final infarct size (Zhao et al. 2003, Tsang et al. 2004). This implies exacerbation of ischemic injury during the reperfusion period and represents a therapeutic opportunity to preserve existing cardiac tissue. Pharmacological intervention could be applied to activate cardioprotective pathways in cardiac myocytes (CMs). The focus of this dissertation is to investigate the role of α1-adrenergic receptors (α1-ARs) in the ischemic heart and to propose α1-AR stimulation as a PostC treatment.

**Cardiac myocytes**

The contraction of the heart is produced by the highly specialized CMs. While CMs make up most of the myocardial volume, they actually only make up ~30-60% of total adult cardiac cell number depending upon the species (Banerjee et al. 2007). CMs are of mesodermal origin and during the first postnatal week in mice there is extensive myocyte hyperplasia as the heart grows (Brodsky et al. 1980). Following this stage,
proliferation comes to a halt and cardiac growth is then mediated by hypertrophic growth of the existing cells (Brodsky et al. 1980). Once CMs cease to proliferate they are terminally differentiated and maintain limited to no ability to divide (Beltrami et al. 2001). Upon cardiac injury resulting in CM cell death, the cells are not able to repopulate the damaged area. To maintain the integrity of the cardiac wall fibroblasts migrate to the zone of injury and secrete large amounts of collagen forming a fibrous scar (Skosey et al. 1972). Known as fibrosis, this physiological solution serves to strengthen the myocardial wall. As the damaged heart continues to function with fewer myocytes to sustain it, pathological hypertrophy occurs as compensation for the decrease in contractile function. CMs are the main functional unit of the myocardium and in an instance of injury even low rates of apoptosis or necrosis can have large impacts on cardiac function. This situation is further complicated due to the terminally differentiated state of CMs that confine their injury response to an increase in size rather than in number.

**Alpha<sub>1</sub>-adrenergic receptors**

**Alpha<sub>1</sub>-adrenergic receptor signaling**

Alpha<sub>1</sub>-adrenergic receptors (α1-ARs) are class I type G-protein coupled receptors that are predominantly expressed on the cell surface and are activated by the endogenous catecholamines norepinephrine and epinephrine. The α<sub>1</sub>-AR subfamily is comprised of three subtypes -the α<sub>1A</sub>, α<sub>1B</sub>, and α<sub>1D</sub> adrenergic receptors, of which α<sub>1A</sub> and α<sub>1B</sub> are expressed on most mammalian CMs (Jensen et al. 2013). Upon agonist binding as shown in Figure 1, these receptors couple primarily to the Gq/11 Gα protein leading to the
activation of phospholipase C (PLC) β which cleaves phosphatidylinositol 4,5-bisphosphate (PIP₂) into diacylglycerol (DAG) and inositol triphosphate (IP₃). DAG activates protein kinase C (PKC) which increases calcium (Ca²⁺) sensitivity. Also, IP₃ binds to and activates the IP₃ receptors on the endoplasmic reticulum (ER) causing Ca²⁺ release into the cytosol. Although this is the classical pathway for α₁-ARs, it has become clear that there are subtype specific effects that include activating multiple molecules in different pathways that include, among others, phospholipase A₂, p21-ras, PI3-kinase, mitogen-activated protein kinase (MAPK), and RhoA (Perez et al. 1993). There is also an established link between α₁-ARs and cardiac hypertrophy that is modulated mainly through the PLC-MAPK (Ramirez et al. 1997) and Rho (Sah et al. 1996) pathways in CMs.

Cardiac α₁-adrenergic receptors

Functional α₁-ARs have been found in multiple tissue types including the heart and smooth muscle of the vasculature. They are stimulated in conjunction with sympathetic activation in which their main effect is vasoconstriction. Stimulation of cardiac α₁-ARs has been shown to increase both contractility and heart rate (Endoh and Blinks 1988). They have been linked with cardiac growth during development, as well as hypertrophic growth induced by pressure overload (Simpson 1983). At a cellular level, α₁-AR stimulation has been shown to decrease cardiac myocyte apoptosis in response to hypoxia and β-AR stimulation (Iwai-Kanai et al. 1999, Zhu et al. 2000). Additionally, α₁-AR stimulation before an ischemic event, or pharmacological preconditioning, has been
5

Figure 1. Alpha\textsubscript{1}-adrenergic receptor signaling pathway. Alpha\textsubscript{1}-ARs are G-protein coupled receptors that signal primarily through the G\textsubscript{q/11} G\textalpha\ protein. This leads to activation of phospholipase C (PLC) which cleaves phosphatidylinositol 4,5-bisphosphate (PIP\textsubscript{2}) into diacylglycerol (DAG) and inositol triphosphate (IP\textsubscript{3}). DAG activates protein kinase C (PKC) which increases calcium (Ca\textsuperscript{2+}) sensitivity and IP\textsubscript{3} binds to and activates the IP\textsubscript{3} receptors on the sarcoplasmic reticulum (SR) causing Ca\textsuperscript{2+} release into the cytosol. Additional molecules not shown are also known to be modulated by alpha\textsubscript{1}-ARs.
shown to decrease myocardial damage in response to I/R in several different experimental models. In adult rat ventricular myocytes (ARVMs) pre-ischemic stimulation of the $\alpha_{1B}$-AR was shown to be beneficial by decreasing mitochondrial calcium overload, an effect secondary to increased opening of the mitochondrial KATP channel and stimulation of PKCε (Gao et al. 2007). Preconditioning has also been studied using a transgenic mouse model expressing cardiac specific constitutively active $\alpha_{1A}$ receptor in which a Langendorff perfusion was performed along with global ischemia and reperfusion (Rorabaugh et al. 2005). Hearts expressing constitutively active $\alpha_{1A}$-ARs recovered from 30 minutes of ischemia that was comparable to wild type hearts exposed to an ischemic preconditioning protocol. On the other hand, constitutively active $\alpha_{1B}$-AR hearts exposed to 30 minutes of ischemia did not similarly recover, suggesting that the $\alpha_{1A}$-AR subtype plays a role in $\alpha_{1}$-AR-mediated preconditioning. Also, inhibition of $\alpha_{1}$-ARs has also been shown to prevent benefits gained from ischemic preconditioning (Hu and Nattel 1995).

CMs express mRNA of all three $\alpha_{1}$-AR subtypes, however, only the $\alpha_{1A}$ and $\alpha_{1B}$ subtypes exhibit functional ligand binding. Still present in the cardiovascular system, the $\alpha_{1D}$ subtype is present in much larger amounts than the $\alpha_{1A}$ or $\alpha_{1B}$ subtypes in the coronary vasculature (Jensen et al. 2009). Stimulation of the $\alpha_{1D}$-AR is thus credited with vasoconstriction of the coronary arteries causing decreased myocardial blood flow. In humans, $\alpha_{1}$-AR stimulation has been shown to increase coronary vasoconstriction in patients with atherosclerotic plaques (Baumgart et al. 1999), making $\alpha_{1}$-AR inhibition a valid therapeutic avenue. Although $\alpha_{1}$-AR blockade has been proposed in order to
alleviate coronary vasoconstriction following MI, complete $\alpha_1$ blockade may cause additional unwanted effects. Interestingly, a clinical trial investigating general $\alpha_1$-AR blockade as a treatment for hypertension was terminated early because of increased incidence of cardiac events and heart failure (ALLHAT Collaborative Research Group 2000). This result of increased heart failure with $\alpha_1$-AR blockade indicates that some $\alpha_1$-AR signaling may be required for normal cardiac function in humans. Additionally, it may support the notion of beneficial $\alpha_{1A}$ and $\alpha_{1B}$ cardioprotection. Unfortunately, a lack of adequate pharmacological agents has hindered attempts to differentiate subtype specific effects in vivo. Much of what is known of $\alpha_1$-AR subtype specific physiological effects has been gleaned from experiments using transgenic mouse models. For example, $\alpha_{1A}/\alpha_{1B}$ double knockout mice display an increase in cardiac failure (O’Connell et al. 2006) that is not seen in any of the single subtype knockout mice. This result again suggests a necessity for both cardiac $\alpha_{1A}$ and $\alpha_{1B}$-AR signaling. Given these studies, it seems that it would be ideal to stimulate only the $\alpha_{1A}$ and $\alpha_{1B}$-ARs to induce protection of the myocardium, while avoiding $\alpha_{1D}$ subtype-mediated coronary vasoconstriction.

$\alpha_1$-ARs on CMs are a possible and promising target for cardioprotection. Not only are $\alpha_1$-ARs expressed on CMs, but they appear to be regulated differently than $\beta$-adrenergic receptors ($\beta$-ARs) with differences in desensitization as well as changes in receptor density. While receptor desensitization is still not completely understood, desensitization of $\alpha_1$-ARs has not been shown to impact $\alpha_1$-AR-mediated effects on the myocardium. It is thought that differential expression of G protein-coupled receptor kinases (GRKs) might be responsible for differences in $\alpha_1$ and $\beta$-AR rates of
desensitization (O’Connell et al. 2014). GRK2 levels are increased in heart failure (Huang et al. 2011) which may contribute to increased β1-AR desensitization while levels of GRK 3 which has been shown to interact with α1-ARs (Diviani et al. 1996) remains stable. Alpha1-ARs have been shown to increase in number in response to ischemia, which becomes especially important because β-ARs do not increase in number and in some cases there is a decrease in β-ARs during ischemia (Corr et al. 1981, Froldi et al. 1994). In addition, long-term sympathetic activation such as is present in human heart failure has been associated with increased α1-ARs and decreased β-ARs (Bristow et al. 1982, Jensen et al. 2009). Alpha1-AR stimulated increases in cardiac contractility are typically much less than those caused by β-AR stimulation. However, in the failing myocardium an increased ratio of α1-ARs to β-ARs leads to a greater reliance on α1-ARs for changes in contractility. These findings suggest that α1-ARs may serve as an evolutionary reserve for the heart in times of crisis. Supporting this idea, trabecula isolated from failing human hearts showed no difference in inotropic response between stimulation of α1-ARs and β-ARs (Skomedal et al. 1997). This is a stark difference from the healthy heart in which β-AR stimulation would cause a much larger response. These changes of α1 and β-AR number and/or sensitivity in pathological circumstances present a unique opportunity for cardioprotective intervention by α1-AR stimulation.

Cardiac ischemia and reperfusion

Ischemia can cause deleterious effects on the cell including decreased pH, sodium imbalance, and calcium overload (Neely et al. 1973). In normal myocardium, fatty acid
oxidation fuels oxidative phosphorylation in which oxygen is the final electron acceptor. In the absence of nutrients or oxygen in the ischemic myocardium, cardiac myocyte ATP production switches from oxidative phosphorylation to the less productive anaerobic glycolysis (Neely and Morgan 1974). Glycolysis results in ATP, NADH, and pyruvate; the resultant pyruvate is then converted to lactic acid and H⁺ by lactate dehydrogenase contributing to a reduction in intracellular pH. The Na⁺/H⁺ exchanger functions passively in response to excess intracellular H⁺ and consequently expels intracellular H⁺ while increasing intracellular Na⁺ (Frelin et al. 1984). Unfortunately, ATP produced by glycolysis alone is insufficient to sustain the high metabolic rate required for cardiac myocyte function. As ATP levels fall, cellular ion gradients are further disrupted by the inactivation of ATP-dependent ion pumps. Decreased activity of the Na⁺/K⁺-ATPase results in an even larger rise in intracellular Na⁺ causing cellular swelling (Godin et al. 1980). Inadequate ATP levels also give rise to intracellular calcium overload because of inactivation of the ATP-dependent calcium exporter as well as inactivation of calcium reuptake into the sarcoplasmic reticulum (Marban et al. 1987). Excess cytosolic calcium allows activation of calcium-dependent proteases, such as calpain, which induce membrane damage and may contribute to necrotic cell death. In addition to ion imbalance, a lack of oxygen causes production of reactive oxygen species (ROS). ROS are primarily produced by the electron transport chain (ETC) within mitochondria which endures oxidative damage due to its proximity to ROS production. Intracellular ROS produce negative effects by damaging proteins, mitochondrial DNA, and sometimes nuclear DNA. Most importantly, ROS induce plasma membrane damage. Ischemia can
induce cardiac myocyte death by necrosis or apoptosis. Cellular swelling because of ion imbalance paired with plasma membranes weakened by calcium activated proteases and oxidative stress can induce necrosis. On the other hand, calcium overload can lead to induction of apoptosis as well as increasing both apoptosis and necrosis if there is loss of mitochondrial membrane integrity. The extent of damage inflicted by ischemic insult while unpredictable, is mainly governed by the length of ischemia.

Reperfusion brings about a rapid return to physiological pH, oxygen, and nutrients, but leads to additional damage beyond that of ischemia. Again, cellular ion balance is central, and upon reperfusion the H⁺ gradient causes additional sodium overload via the Na⁺/H⁺ exchanger (Tani and Neely 1989). Disintegration of the cellular sodium gradient yields further changes in passive ion transport, most importantly reversing the action of the Na⁺/Ca²⁺ antiporter and contributes to calcium overload (Haigney et al. 1992). Additionally, sudden reintroduction of oxygen during reperfusion initiates a surge in production of ROS; levels of ROS reach their highest amount within minutes and continue to be elevated for up to 3 hours (Bolli et al. 1988). ROS production during reperfusion is thought to be caused by dysfunction of the ETC which has been damaged during ischemia. Despite oxidative damage, introduction of sufficient resources triggers the ETC to resume in order to increase ATP by oxidative phosphorylation. Recovery begins as ATP is produced and cellular ion pumps resume function. The Na⁺ gradient begins to return to physiological levels because of the Na⁺/K ATPase and intracellular Ca²⁺ is again regulated by plasma membrane calcium export and sarcoplasmic reticulum calcium reuptake (Marban et al. 1987). Recovery of cellular
processes after ischemia can occur gradually, however, it is dependent on the severity of ischemic insult which determines the extent of irreversible injury.

The intracellular changes brought about by both ischemia and reperfusion can lead to cellular damage and death. Ion imbalance resulting in cellular swelling can induce plasma membrane rupture and necrosis. Increased levels of intracellular and intramitochondrial calcium may result in calcium overload and the release of mitochondrial contents. The ETC is damaged during ischemia and produces large amounts of ROS during reperfusion. Oxidative damage and lipid peroxidation induced by this burst of ROS again damage cellular proteins and DNA, as well as lipids of the plasma membrane. Furthermore, reperfusion and return to neutral pH can cause opening of the Mitochondrial Permeability Transition Pore (MPTP) (Cohen et al. 2008). During ischemia, MPTP opening is inhibited by low pH and is therefore less likely to open than during reperfusion. MPTP pore opening allows movement of molecules between cytosol and mitochondrial matrix (Griffiths and Halestrap 1995). Opening of this pore causes dissipation of the mitochondrial membrane potential followed by swelling that eventually overwhelms the outer mitochondrial membrane and releases proapoptotic factors into the cytosol (Halestrap et al. 1998) inducing either apoptosis or necrosis. In some cases, ATP levels are not sufficient to support apoptotic cell death and necrosis ensues (Baines 2009). The maintenance of cellular ion gradients, decreasing the release of ROS, and prevention of MPTP opening are all possible targets for preventing myocardial cell death in I/R.
Cardiac postconditioning

Ischemic postconditioning

Cardiac PostC is any intervention applied following an ischemic episode with the goal of cardioprotection, or decreasing damage to the myocardium. The concept of applying myocardium-protecting treatment during reperfusion arose due to two developments: 1.) the acceptance of reperfusion as necessary for myocardial salvation (Ferdinandy et al. 2007), and 2.) the discovery that reperfusion indeed causes further injury (Hearse et al. 1973). Interest in the field was strengthened with the discovery of the protective effect elicited by ischemic PostC by Zhao et al. in 2003. Ischemic PostC is the application of repeated short episodes of ischemia and reperfusion following an extended period of ischemia (Figure 2), which results in decreased infarct size (Zhao et al. 2003). The mechanism(s) involved in this protective effect have not yet been fully elucidated, but investigations into both physiological and molecular avenues have led to the discovery of several possibilities. These include delay in pH normalization, mKATP channel opening, decreased ROS production, and stimulation of cardioprotective signaling pathways that decrease MPTP opening.

The mechanisms regulating ischemic PostC are complex and have been shown to remedy several damaging complications of reperfusion. It has been proposed that ischemic PostC decreases infarct size by slowing the restoration of physiological pH. Multiple short episodes of ischemia applied following a longer episode of ischemia will more slowly introduce the changes of reperfusion. By decreasing the rate at which
Figure 2. Schematic representation of ischemic postconditioning and pharmacological postconditioning. Postconditioning is cardioprotection applied following an ischemic insult. Ischemic postconditioning involves shorter episodes of ischemia (I) and reperfusion (R) after an ischemic insult. Pharmacological postconditioning is treatment with pharmacological agents shortly before or at the onset of reperfusion.
intracellular pH returns to normal, there is decreased cell death that is attributed to MPTP inhibition and prevention of calcium overload. Low intracellular pH is a known inhibitor of MPTP opening, thus it is possible that ischemic postconditioning’s beneficial effect is through maintaining a more acidic pH during early reperfusion (Cohen et al. 2007). Also, keeping extracellular pH low changes the dynamic of ion exchange. With less H⁺ moving outward through the Na⁺/H⁺ exchanger (NHE), less sodium is transported inward lessening the sodium gradient that drives reverse mode activity of the Na⁺/Ca²⁺ exchanger (Kitakaze et al. 1988). This produces a more favorable cellular environment in which calcium overload is prevented. Supporting the involvement of pH in ischemic PostC, reperfusion with acidic buffer has been shown to decrease infarct size in hearts exposed to global ischemia (Cohen et al. 2007). Alterations in pH, however, may not fully account for the beneficial effects of ischemic PostC and other mechanisms are also likely involved.

The mitochondrial ATP-sensitive potassium channel (mKATP) has been shown to be involved in ischemic PostC. Inhibition of mKATP opening with 5-hydroxydecanoate (5-HD) prevents cardioprotection with ischemic PostC indicating an important role for opening of the mKATP channel (Yang et al. 2004). Further, diazoxide, a pharmacological mKATP opener, has been shown to be protective during ischemia and reperfusion (Murata et al. 2001). Opening of the mKATP allows flow of potassium into the mitochondrial matrix resulting in depolarization. Depolarization slows the passive entrance of calcium which prevents mitochondrial calcium overload and preserves mitochondrial membrane integrity (Murata et al. 2001). It has been suggested that just the
maintenance of mitochondrial matrix and inner membrane space are sufficient to induce cardioprotection (Laclau et al. 2001). Opening of the mKATP has also been implicated in production of ROS (Forbes et al. 2001) that might be important for stimulation of cardioprotective signaling pathways.

Ischemic PostC has been theorized to be protective both by interrupting and decreasing the production of ROS normally produced during reperfusion. ROS cause oxidative damage to DNA, lipid membranes, and multiple proteins throughout the cell and contribute to cardiac dysfunction in vivo (Bolli et al. 1988). In addition to decreasing tissue exposure to damaging ROS (Zhao et al. 2003), there is some evidence that small bursts of ROS produced during the transient reperfusion episodes of ischemic PostC actually activate protective signaling pathways. Transient reperfusion releases ROS while transient ischemia then allows time for activation of molecular changes known as the reperfusion injury signaling kinase (RISK) pathway (Cohen et al. 2008). Exploration of the molecular mechanisms behind these processes is extensive and has produced multiple pathways that may provide some protection against CM death.

*Molecular players in ischemic postconditioning*

While regulation and effects of molecular events of cardioprotection are not yet understood, ischemic PostC activates multiple cardioprotective signaling pathways. Current interventions are thought to converge upon inhibiting MPTP opening, and are mediated in some way by one or a combination of delayed intracellular pH normalization, mKATP channel opening, or decreased ROS production. These events stimulate and may
even be regulated by signaling molecules among which several cardioprotective pathways have emerged (Figure 3). Studies in the myocardium or in isolated CMs have implicated the reperfusion injury salvation kinase (RISK), survivor activating factor enhancement (SAFE), PKC, and cGMP/PKG pathways in regulating PostC cardioprotective mechanisms.

The reperfusion injury salvation kinase (RISK) pathway encompasses several molecular cascades that have been shown to be active in the myocardium during ischemic pre- and postconditioning. The RISK pathway involves Akt (Tsang et al. 2004) and ERK1/2 (Yang et al. 2004) activation as shown in Figure 3 leading to the activation of various downstream effectors such as p70S6K, BAD (Jonassen et al. 2001), endothelial nitric oxide synthase (eNOS) (Bell and Yellon 2003), PKG, and GSK3β. GSK-3β inhibition has been shown to decrease MPTP opening (Gomez et al. 2008), and it is thought that other members stimulators of the RISK pathway also decrease cardiac damage by inhibition of MPTP opening.

The survivor activating factor enhancement (SAFE) pathway is activated by stimulation of the tumor necrosis factor alpha (TNFα) receptor 2 with low concentrations of TNFα. This causes activation of the transcription factor signal transducer and activator of transcription-3 (STAT-3). Inhibition of either component leads to inhibition of cardioprotection by ischemic PostC (Lacerda et al. 2009). SAFE signaling is thought to block activation of the MPTP (Figure 3).

Activation of multiple PKC isoforms has been studied in I/R injury (Figure 3). Both PKCα and PKCε have been shown to be important to ischemic PostC. PKCε is
Figure 3. Postconditioning signaling pathways for cardioprotection. PKG has been shown to induce protection through inhibition of the Na⁺/H⁺ exchanger (NHE). Because the NHE functions to normalize pH during reperfusion, its inhibition serves to delay pH normalization which is cardioprotective. PKG has also been shown to block function of GSK3β through phosphorylation. GSK3β has been associated with opening of the MPTP. Opioid receptors (OpR) are GPCRs which when activated post-ischemically have been shown to inhibit GSK3β and open mKATP channels. Additional GPCRs thought to be involved in ischemic postconditioning include adenosine receptors (AdR), bradykinin receptors (B₂), and possibly alpha₁-adrenergic receptors (α₁-AR). These have been shown to signal through the reperfusion injury salvation kinase (RISK) pathway which includes activation of Akt, ERK1/2, and PKC. RISK activation stimulates opening of the mKATP channel through PKC and is thought to function through multiple other factors downstream of Akt and ERK1/2. Finally, in the survivor activating factor enhancement (SAFE) pathway, activation of the tumor necrosis factor alpha receptor 2 (TNFαR2) leads to activation of a transcription factor called signal transducer and activator of transcription-3 (STAT-3). STAT-3 is thought to be protective through inhibition of MPTP opening.
thought to decrease cell death by opening the mKATP during reperfusion (Lemoine et al. 2010). On the other hand, inhibition of PKCδ during reperfusion has been shown to decrease cell death (Murriel et al. 2004).

PKG activation has been shown to induce PostC through inhibition of the Na+/H+ exchanger which in turn delays pH normalization upon reperfusion (Inserte et al. 2011). PostC with PKG was shown to be dependent on nitric oxide (NO), while in preconditioning-mediated cardioprotection, PKG was shown to be activated via PI3K/Akt (Hu et al. 2011). PKG has also been implicated in stimulating opening of sarcolemmal KATP channels, phosphorylation of GSK3β, and phosphorylation of multiple cellular components such as phospholamban. However, these effects of PKG activation have not been evaluated for involvement in cardiac PostC.

 Activators of cardiac postconditioning

In the study of PostC, various humoral factors have been implicated in the activation of signaling through GPCRs among others in order to prevent cardiac myocyte cell death. While it is not known exactly how these pathways are activated, it is postulated that in ischemic PostC they are stimulated by an unknown endogenous factor that has accumulated and is subsequently reintroduced during the multiple episodes of reperfusion (Hausenloy and Yellon 2007). Possible candidates thought to initiate this signaling in vivo include adenosine, bradykinin, and opioids (Figure 3). Additionally, exogenous pharmacological agents are able to modulate events during early reperfusion to decrease injury. It is important to note that pharmacological mediation of PostC
pathways is a main goal in the field of cardioprotection and a large part of this dissertation. Pharmacological PostC is a highly useful concept for clinical use. Signaling molecules known to be regulated by ischemic PostC are the main targets of therapeutic intervention using pharmacological PostC. While there have been clinical trials to investigate efficacy of interventions, there is not yet a conclusive method for decreasing damage incurred following MI. Two of the most promising exogenous pharmacological agents are the general KATP channel opener, nicorandil, and an inhibitor of MPTP formation, cyclosporine.

Adenosine release and accumulation during ischemia has also been shown to be important to ischemic PostC. There are four adenosine receptor subtypes; however, it is not yet clear which subtype is the main effector as data has shown involvement of each of them. The system of activation for protection is complex and may be confounded by changes in receptor binding affinity enacted by PKC (Przyklenk 2012). Adenosine has been shown to decrease cell death through PKC and RISK pathway activation (Philipp et al. 2006). A clinical trial, acute myocardial infarction study of adenosine (AMISTAD-II), enrolled 2,118 patients to study infusion of adenosine as adjunct therapy to percutaneous coronary intervention (PCI). While there was a significant decrease in infarct size in patients with anterior MI, there was not a corresponding decrease in clinical outcomes (Ross et al. 2005).

Bradykinin is increased during ischemia and has been shown to induce PostC in animal models. PostC of rat hearts with intermittent but not constant stimulation with bradykinin decreased infarct size (Penna et al. 2007). In mice, however, intermittent
exposure was not required as constant post-ischemic treatment with bradykinin 2 receptor agonist was shown to decrease infarct size. Protection was mediated by RISK signaling, specifically PI3K/Akt/GSK3β (Potier et al. 2013). Bradykinin has not yet been studied in post-ischemic treatment in humans.

Endogenous opioids that are released from CMs during ischemia seem to be involved in ischemic PostC since antagonizing opioid receptors decreases ischemic PostC. In particular, selective agonists for the δ and κ subtypes appear to reduce infarct size in rats (Gross et al. 2004). Pharmacological PostC with opioid receptor activation involves inactivation of GSK3β and decreased MPTP opening as a result (Gross et al. 2004). While there has not been a clinical trial involving post-ischemic opioid receptor stimulation, opioid agonists are often already administered to MI patients during percutaneous coronary intervention (PCI) for sedation and pain relief.

Nicorandil causes opening of the ATP-sensitive potassium (KATP) channels and is also a nitric oxide donor. A clinical trial with 613 patients found no difference in overall mortality with post-ischemic nicorandil treatment (Kitakaze et al. 2007). An additional clinical trial of 368 patients treated with a higher dose of nicorandil showed decreased cardiovascular death or hospital admission with perfusion of Nicorandil beginning five minutes prior to reperfusion (Ishii et al. 2005). Selective opening of mKATP with diazoxide has also been implicated in PostC human tissue. While there has not been a clinical trial, treatment with diazoxide post-ischemically increased the force of contraction in human right atrial trabeculae (Lemoine et al. 2010). Further studies will be
needed to assess involvement of either KATP or mKATP channel opening in post-ischemic protection.

Cyclosporine binds cyclophilin D which is a main component in the MPTP. By inhibiting MPTP formation cyclosporine has been shown to decrease cell death when added at reperfusion. There has been one clinical trial including 58 patients in which there was a significant decrease in infarct size (Piot et al. 2008). One limitation to cyclosporine is that it is also an immunosuppressant and an inhibitor of calcineurin. Data from a larger clinical trial will provide a more conclusive view of cyclosporine activation and its potential clinical use.

**Mechanisms of cardiac myocyte death in ischemia and reperfusion**

*Apoptosis*

Apoptosis is a type of programmed cell death that plays a role in normal tissue homeostasis as well as organism development (Wood et al. 2000). It can proceed by two main pathways known as the extrinsic and the intrinsic pathway of apoptosis. In the extrinsic pathway, an extracellular ligand binds to a death receptor, such as Fas, on the plasma membrane (Nagata and Golstein 1995). The death receptor activates intracellular death domain proteins which in turn cause cleavage and activation of caspases which proceed to cleave other caspases inducing cleavage of multiple proteins throughout the cell. The other major apoptotic pathway, the mitochondrial or intrinsic pathway, involves pro and anti-apoptotic Bcl-2 family members (Reed 1997) that accumulate at the mitochondrial membrane. Depending upon the ratio of these proteins, the mitochondrial
membrane becomes permeable and releases multiple effectors of apoptosis including cytochrome c (Li et al. 1997). Again multiple caspases are activated leading to cleavage of cellular proteins and DNA. Apoptotic cells undergo a decrease in cell size, characteristic chromosome condensation, and nuclear fragmentation (Wyllie et al. 1980). One definite advantage of apoptosis over necrosis is that apoptosis does not cause inflammation. The apoptotic cell regulates cleavage of intracellular proteins and packaging of cellular components into plasma membrane compartments. Cellular components enclosed in plasma membrane compartments break off from the cell keeping all membranes intact thus preventing the release of inflammatory cytokines. Finally, macrophages present within the tissue phagocytize the apoptotic membrane packets eliminating the need for an inflammatory response.

Necrosis

Necrosis has previously been thought to be largely unregulated, however, a growing amount of evidence suggests that at least some incidence of necrosis is dependent upon molecular events. The most prominent example of molecular regulation of necrosis is the opening of the MPTP (Kung et al. 2011). This pore, formed on the inner mitochondrial membrane, depletes mitochondrial membrane potential which causes a subsequent influx of water. Mitochondrial swelling eventually leads to membrane rupture and release of mitochondrial contents. This leads to cellular swelling and a loss of plasma membrane integrity which are both defining characteristics of necrosis. The shift in paradigm from necrosis as an unregulated process to programmed necrosis with specific
molecular effectors opens up opportunities for cardioprotection. One such example is the ligand cyclosporine, which blocks necrosis by inhibiting opening of the MPTP. Cyclosporine binds the molecule cyclophilin D effectively inhibiting its integration into MPTP structure (Elrod and Molkentin 2013). By decreasing MPTP formation and opening, cyclosporine has been shown to decrease incidence of necrosis. For specific implications in cardioprotection see section above on Cardiac PostC Ligands.

*Apoptosis vs. necrosis in ischemia and reperfusion*

While it is known that apoptosis and necrosis contribute to ischemic damage, there is controversy over which type of cell death prevails in the ischemic heart. In several studies in which there was a reperfusion period involved, levels of cardiac myocyte necrosis were higher than apoptosis. In a cell culture model of I/R, the level of necrosis was up to 4 times the level of apoptosis measured (Taimor et al. 1999). Consistent with the cell culture model, whole heart experiments with global ischemia and reperfusion showed twice the amount of cardiac myocyte necrosis as apoptosis (Takashi and Ashraf 2000). While these studies all provide convincing evidence that both necrosis and apoptosis are substantial during ischemia and reperfusion in the heart, there are limitations to their measurements. Some controversy exists around the specificity of TUNEL staining with the implication that exonucleases are released from necrotic cells and thus artificially increase values making apoptosis and necrosis difficult to distinguish (Dorn 2013). Despite this caveat, TUNEL assays alone are largely used to estimate the levels of apoptotic cell death. Also, Park et al. found that more than 80% of apoptotic
cells in the heart after MI were actually non-myocytes (2009). This demonstrates that additional cell types may be important in cardiac ischemic damage, as well as highlighting the importance of adequately separating the myocyte response from the rest of the cardiac cell population.

**Autophagy**

*Function of autophagy and molecular components*

Autophagy is a catabolic cellular process in which organelles and long lived cellular proteins are degraded. During states where nutrients are limiting, autophagy can be a source of amino acids and free fatty acids that can then be utilized for ATP production and protein synthesis. Autophagy has been shown to be increased following hypoxia and glucose starvation, as well as during infection (Hamacher-Brady et al. 2006, Yang and Klionsky 2010). It may also play a part in preventing the accumulation of protein aggregates within the cell (Marambio et al. 2010). Because the process of autophagy increases energy retention, decreases protein aggregation, and aids in protein and organelle turnover, increased autophagy is thought to be involved in mediating the cardioprotective response. However, some studies show evidence that excessive autophagy, can lead to cell death (Levine and Yuan 2005). Because autophagy can affect the cell differently depending upon environment and other complex factors, it is important to study autophagic regulation before, during, and after an ischemic episode to determine when this process might prove detrimental or beneficial. This information could lead to important new interventions to limit myocardial damage.
The molecular events regulating autophagy are only partially defined, but appear to be overall evolutionarily conserved between yeast and higher eukaryotes (Yang and Klionsky 2009). The process involves the participation of mTOR and various autophagy-related (Atg) proteins (Figure 4). Nutrient signals are normally mediated through mTOR (mammalian target of rapamycin), a serine-threonine kinase involved in regulating cell growth and survival. The lack of nutrient signals inhibits mTOR; lack of mTOR activity allows initiation of the autophagic process. Initiation, nucleation of autophagosomal membrane, and main signaling events are outlined in Figure 4. Importantly, microtubule-associated protein 1 light chain 3 I (LC3-I) is conjugated with phosphatidylethanolamine, to form LC3-II, a crucial component of autophagosomal membranes. This lipid bound LC3-II molecule is currently the best known marker of autophagosomes.

The initiation complexes involved in membrane assembly of autophagosomes gather at many sites in mammalian cells (Yang and Klionsky 2010). When autophagy is induced, a membrane structure elongates and encloses a portion of the cytoplasm to form the autophagosome, a double-membraned organelle. Autophagosomes then fuse with lysosomes for the final degradation of autophagosomal contents (Yang and Klionsky 2010).

**Autophagy and ischemia and reperfusion**

Studies have shown that autophagy appears to be differentially regulated between periods of ischemia and periods of reperfusion. In vivo autophagy studied in global GFP-LC3 transgenic mice showed inhibition of autophagy in the heart during both ischemia
Figure 4. Autophagic process. (A) When cells are subjected to nutrient starvation, mTOR is inactivated. When not phosphorylated by mTOR, ATG 13 is free to bind in a complex with ULK1/2 and FIP200 leading to ULK1/2 activation and autophagy progression. (B) Vesicle nucleation occurs with recruitment of proteins and lipids to initiate autophagosome construction; this requires the class III phosphatidylinositol 3-kinase vacuolar protein sorting 34 (Vps34) to form phosphatidylinositol-phosphate (PIP). Vps34 is activated by the formation of a complex with Beclin-1, the kinase Vps15 (p150 in humans), UV radiation resistance-associated gene (UVRAG), and Bcl2/Bcl-XL. (C) Vesicle elongation and completion involves a ubiquitin-like cascade to activate proteins LC3 and Atg12, both of which are substrates for ATP-requiring ubiquitin-like conjugation reactions that appear crucial for autophagosome formation. LC3 (Microtubule-associated protein 1 light chain 3), is cleaved by Atg4 to form LC3-I, and is then conjugated with phosphatidylethanolamine to form LC3-II. Modified LC3-II appears to be a critical component of the autophagosomal membrane. (D) Once the autophagosome is complete, it fuses to a lysosome in a still undefined manner that may involve numerous proteins, including Rab7 and SNAREs. Autophagosomal inner membrane and contents are then degraded upon fusion with a lysosome. Once an autophagosome and a lysosome fuse the resulting structure is called an autolysosome.
A. Autophagy Induction

ULK 1/2 → ATG13 → ATG16

RAPAMYCIN → mTOR

B. Vesicle Nucleation

Class III PI3K Complex

Vps34 → Vps15 → UVRAG → Beclin-1 → Bcl2/Bcl-XL

3-Methyladenine

C. Vesicle Elongation

ATG4 → ATG5 → ATG10 → ATG12

ATG5 → ATG16

ATG3 → ATG12

D. Engulfment, Fusion, and Degradation

Autophagosome → Autophagosome fuses with a Lysosome

Chloroquine

Autolysosome
and reperfusion (French et al. 2010). This data is supported with in vitro experiments that have shown that ischemia can impair fusion of the autophagosome with the lysosome, leading to accumulation of autophagosomes in the cytosol (Ma et al. 2012). The energy constraints of an ischemic environment make it likely that there would be a decrease in the formation of autophagic vesicles since ATP is required for many of the molecular interactions during autophagy.

On the other hand, experiments in GFP-LC3 transgenic mice have also shown increased LC3 during both ischemia and reperfusion (Matsui et al. 2007). This discrepancy may have to do with the type and severity of ischemia induced. Although its specific influence on cell death in ischemia-reperfusion injury remains to be fully defined, regulation of autophagy is a possible mechanism of action for pharmacological pre- or post-conditioning. Interestingly, long-term α1-AR stimulation has been shown to increase autophagy in CMs (Cao et al. 2011, Xu et al. 2014).

Because pharmacological activation of α1-AR stimulation can provide cardioprotection through pharmacological preconditioning, and also protect against apoptotic insults in the CMs, we hypothesized that α1-ARs can provide cardioprotection through pharmacological PostC. Specifically, we hypothesize that α1-ARs can mediate pharmacological PostC to protect CMs after an ischemic insult and that this protection is mediated through regulation of autophagy.
Hypotheses and Specific Aims

Reperfusion of the myocardium following MI leads to further injury and cell death leading to a larger infarct and decreased remaining cardiac function. Although the re-establishment of blood flow is essential to myocardial recovery, abrupt return of oxygen and nutrients stimulates production of ROS and mitochondrial Ca\(^{2+}\) overload, among other detrimental effects on the cell. Thus, one way to salvage myocardial tissue is to reduce the damaging aspects of reperfusion on the cardiac myocyte. Clinical trials have been conducted to determine an effective pharmacological treatment, but none have consistently prevented injury. The lack of cardioprotective interventions to be applied at the time of reperfusion in a clinical setting demonstrates a clear need for the definition and characterization of additional agents and therapeutic targets.

Multiple signaling pathways have been shown to decrease the amount of cell death; these pathways include those regulated by G protein-coupled receptors, or GPCRs. Previous studies have shown that stimulation of \(\alpha_1\)-adrenergic receptors (\(\alpha_1\)-AR) prior to an ischemic event (cardiac preconditioning) can induce protection in the heart, mainly through inhibition of cardiac myocyte apoptosis (Baghelai et al. 1999, Huang et al. 2007). Additionally, global inhibition of \(\alpha_1\)-ARs appears to be detrimental since the doxazosin (\(\alpha_1\)-AR antagonist) arm of ALLHAT (The Antihypertensive and Lipid-Lowering Treatment to Prevent Heart Attack Trial) was terminated early due to a doubled risk of congestive heart failure (ALLHAT Collaborative Research Group 2000). These studies indicate a putative role for \(\alpha_1\)-ARs in mediating protective responses in the heart. Interestingly, previous studies show that these receptors increase in number in the heart
during cardiac ischemia as well as in heart failure in humans (Froldi et al. 1994, Jensen et al. 2011), thus providing rationale for targeting these receptors for therapeutic intervention. The objective in this study is to define and characterize α1-AR impact on I/R injury in cardiac myocytes.

Accordingly, we hypothesize that stimulation of α1-ARs post-ischemically provides protection against cardiac myocyte cell death. Thus, this study is focused on the role of α1-ARs in cardioprotection and possible mechanisms that may be involved. We will examine the following specific aims:

1) Examine effects of post-ischemic stimulation of α1-ARs on cardiac myocyte cell death.

2) Determine whether post-ischemic α1-AR stimulation inhibits cardiac myocyte death through modulation of autophagy.

3) Examine the molecular pathway(s) stimulated by α1-ARs that lead to decreased cell death.

**Specific Aim 1. Examine effects of post-ischemic stimulation of α1-ARs on cardiac myocyte cell death.** We hypothesize that stimulation of α1-ARs post-ischemically provides protection against cardiac myocyte cell death. In order to investigate this aim it was necessary to develop an *in vitro* system of simulated ischemia and reperfusion in cultured cardiac myocytes. Experiments were conducted using cardiac myocytes, both established cell line (HL-1) and rat primary ventricular myocytes. Cells were subjected to
simulated ischemia and reperfusion and postconditioned pharmacologically at the onset of reperfusion by adding $\alpha_1$-AR agonists to the reperfusion buffer.

1a. Post-ischemic $\alpha_1$-AR stimulation inhibits cell death. LDH release and PI fluorescence, both measures of membrane integrity, were used as estimates of general cell death and damage. Following ischemia, cardiac myocytes were treated with $\alpha_1$-AR agonist and LDH and PI assays were conducted following various durations of reperfusion.

1b. Post-ischemic $\alpha_1$-AR stimulation reduces apoptosis. Previous studies have shown $\alpha_1$-AR stimulation prior to an ischemic event can inhibit cardiac myocyte apoptosis (Baghelai et al. 1999, Huang et al. 2007). Therefore, to investigate whether treatment after an ischemic episode will also decrease levels of apoptosis, TUNEL, Annexin V /Propidium Iodide (PI), and DNA Laddering assays were done.

**Specific Aim 2. Determine whether post-ischemic $\alpha_1$-AR stimulation inhibits cardiac myocyte death through modulation of autophagy.** Cardiac $\alpha_1$-AR stimulation has been shown to induce autophagy (Nakaoka et al. 2009); thus, autophagy in $\alpha_1$-AR-mediated protection was evaluated utilizing pharmacological inhibitors of autophagy following ischemia and reperfusion. PI uptake was measured to assess changes in cell death when autophagy was inhibited with and without $\alpha_1$-AR agonist.

2a. Post-ischemic $\alpha_1$-AR stimulation changes autophagic marker LC3-II. Immunoblots to detect LC3-II were done on cells after 2 and 6 hr of reperfusion to assess a general timeline of autophagy following I/R and pharmacological postconditioning.
2b. Post-ischemic $\alpha_1$-AR stimulation increases autophagic flux. To measure the dynamic process of autophagy, a tandem fluorescent-tagged LC3 plasmid was transfected into HL-1 cardiac myocytes. This allows quantitation of early and late autophagic vesicles because of differential stabilities of GFP and RFP. Additionally, LC3 was measured by immunoblot. Flux was calculated by comparison of LC3-II levels of cells with and without lysosomal fusion inhibitor.

**Specific Aim 3. Examine the molecular pathway(s) stimulated by $\alpha_1$-ARs that lead to decreased cell death.** We hypothesize that there is involvement of known G$\alpha_q$ stimulated $\alpha_1$-AR signaling and existing postconditioning pathways in $\alpha_1$-AR-mediated cardioprotection. To evaluate the importance of these pathways in decreasing cardiac myocyte cell death, PI assays were done on cells exposed to I/R and treated post-ischemically with pharmacological inhibitors of these pathways. Inhibitors for ERK1/2, PLC, Ca$^{2+}$, and PKC were used to determine whether $\alpha_1$-AR-mediated cardioprotection involved these signaling molecules. Immunoblotting experiments for specific proteins involved in these pathways were also conducted to more specifically evaluate the effects of post-ischemic $\alpha_1$-AR stimulation; ERK1/2 phosphorylation and Akt phosphorylation were investigated. These two molecules were chosen because of their known involvement in cardioprotective pathways (Hausenloy and Yellon 2007).
CHAPTER TWO

METHODS

**Animals and reagents**

The experimental design was approved by the Internal Animal Care and Use Committee (IACUC) of Northeast Ohio Medical University. All chemicals were purchased from Sigma-Aldrich, St. Louis, MO unless otherwise noted.

**Cell culture**

*HL-1 cardiac myocytes*

HL-1 cardiac myocytes (CMs) are a cell line developed by William Claycomb (Claycomb et al. 1998) and were a gift from him. They were originally derived from the AT-1 cell type which was isolated from an atrial tumor of a transgenic mouse expressing simian virus 40 (SV40) large T antigen controlled by the promoter for atrial natriuretic factor (ANF) (Claycomb et al. 1998). HL-1 CMs are commonly used as an *in vitro* cardiac myocyte model (Astrom-Olsson et al. 2009, Hamacher-Brady et al. 2006, Ikeda et al. 2005). They were chosen to use in this project because they are continuously dividing and passageable while maintaining a contractile cardiac phenotype indefinitely. They also provide a good system for genetic manipulation with lipophilic transfection (Hamacher-Brady et al. 2006).
Cell culture of HL-1 cardiac myocytes

HL-1 CMs were cultured in Claycomb media supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, and 0.1 mM norepinephrine in 30 mM L-ascorbic acid (Ikeda et al. 2005). HL-1 CMs were plated on dishes or coverslips coated for at least 1 hr with 5 μg/mL fibronectin in 0.02% (w/v) gelatin (BD Difco, Franklin Lakes, NJ). Cells were incubated at 37 °C in 5% CO₂ and 95% air. These conditions were used to maintain cells in a contractile cardiac phenotype.

Isolation of adult rat ventricular myocytes

For adult rat ventricular myocyte (ARVM) isolation from intact hearts all buffers were maintained at 37 °C using a water bath and jacketed chambers. ARVMs were isolated from ventricles of 280-350 gram male Sprague Dawley rats (Charles River Laboratories, Wilmington, MA). Rats were injected with 90 mg/kg ketamine and 10 mg/kg xylazine. The heart was promptly removed and retrogradely perfused with Isolation buffer (in mM: 118 NaCl, 4.7 KCl, 1.2 MgSO₄, 1.2 KH₂PO₄, 25 NaHCO₃, 11 glucose, 20 HEPES, 1 CaCl₂). Liberase Blendzyme (Roche Diagnostics, Indianapolis, IN) was added to digest hearts for 20-30 min. Ventricles were incubated in isolation buffer containing Liberase Blendzyme for 10 min at 37 °C with gentle agitation. Ventricles were then minced and undigested tissue was removed by filtration through a nylon mesh. Cells suspended in isolation buffer were centrifuged at 50 x g for 2-5 min and supernatant removed to remove the non-myocyte fraction. The myocyte fraction was
rinsed with isolation buffer before final resuspension of CMs in isolation buffer. Calcium was gradually reintroduced to the CMs over 15 min. The suspension was centrifuged at 50 x g for 2-5 min and the supernatant was removed and the cells were resuspended in cell culture media and plated.

Cell culture of ARVMs

ARVMs were plated immediately following isolation at 5-10 x 10^3 cells per coverslip, 5 x 10^4 cells per well in a 12-well dish, or at 2 x 10^5 cells per 60 mm dish. Cells were plated onto dishes or coverslips pre-coated with 10 µg/mL laminin (BD Biosciences, Franklin Lakes, NJ) and cultured at 37 °C in 5% CO₂ and 95% air. After 1 hr of incubation, non-adherent cells were removed and the remaining attached cells were cultured in M199 media supplemented with (in mM: 5.3 creatine, 5.6 taurine, 1.86 L-carnitine (Alfa Aesar, Ward Hill, MA)) 0.2% (w/v) BSA, 100 U/mL Penicillin, 100 µg/mL Streptomycin (Piper et al. 1982). ARVMs were plated for 16-24 hr before starting experiments.

Simulated ischemia and reperfusion

Cells undergoing simulated ischemia/reperfusion (I/R) were incubated in an ischemia mimetic buffer and subjected to low oxygen tension using a hypoxia chamber. Experimental cells undergoing the I/R protocol were incubated in modified Esumi ischemia mimetic buffer (in mM: 137 NaCl, 12 KCl, 0.49 MgCl₂, 0.9 CaCl₂ 2H₂O, 4 HEPES, 10 2-deoxy-D-glucose (Alfa Aesar, Ward Hill, MA), 20 sodium lactate, pH 6.2 (Esumi et al. 1991). Ischemia mimetic buffer simulates ischemia by decreasing the pH,
increasing potassium concentration to cause cellular swelling. Additionally, deoxyglucose was added in order to prevent anaerobic metabolism. The ischemia mimetic buffer was bubbled with N\textsubscript{2} for 30 min and then placed onto experimental cells which were immediately moved to a hypoxic environment of 1% O\textsubscript{2}, 5% CO\textsubscript{2}, 94% N\textsubscript{2} in 37 °C maintained by a ProOx Compact Oxygen controller (BioSpherix, Redfield, NY). Control cells not subjected to the I/R protocol were incubated in a non-ischemic (N-I) control buffer (in mM: 137 NaCl, 3.8 KCl, 0.49 MgCl\textsubscript{2}, 0.9 CaCl \textsubscript{2H\textsubscript{2}O, 4 HEPES, 10 glucose, pH 7.4}) in parallel with I/R cells and incubated in normoxic conditions (normal cell culture), 5% CO\textsubscript{2} and 95% air which contains 21% O\textsubscript{2} at 37 °C. At the end of the ischemic period the buffer on both the I/R and N-I control cells was removed and immediately replaced with fresh N-I control buffer and incubated in 5% CO\textsubscript{2} and 95% air at 37 °C to simulate reperfusion. For experiments, cells were subjected to varying durations of reperfusion. Pharmacological postconditioning (PostC) was achieved by adding drugs directly to N-I control buffer prior to adding buffer to cells at the onset of reperfusion. A schematic of the experimental protocol is shown in Figure 5.

**Measurements of cell death**

*Lactate dehydrogenase assay*

Lactate dehydrogenase (LDH) measurement assay was modified from a published protocol (Korzeniewski and Callewaert 1983). The cell culture media was collected from the cells following both the ischemia and the reperfusion periods and spun at 1500 x g for 5 min to remove any suspended cells. The supernatant was collected and stored at 4 °C
Ischemia/reperfusion with pharmacological postconditioning

Non-Ischemic Samples

N-I control buffer
137 mM NaCl
3.8 mM KCl
0.49 mM MgCl₂
0.9 mM CaCl₂ 2H₂O
4 mM HEPES
10 mM glucose
pH 7.4

Ischemia/Reperfusion Samples

Pharmacological postconditioning
N-I control buffer
Pharmacological agents

Figure 5. Schematic of ischemia and reperfusion protocol. Cells were treated with either non-ischemic (N-I) control buffer under normoxic conditions or Ischemic buffer under hypoxic conditions. Reperfusion was simulated by changing all media to fresh N-I control buffer after hypoxic stimulation. The effects of various pharmacological agents on ischemia/reperfusion (I/R) injury were assessed by adding these drugs to the N-I control buffer at the onset of ‘reperfusion’.
and was assayed within 24 hr to prevent degradation of LDH enzyme. Experimental sample (50 µL) was incubated with substrate solution (in mM, 100 Tris, pH 8.2, 2 sodium lactate, 1.3 β-nicotinamide adenine dinucleotide (NAD; Amresco, Solon, OH), 0.7 iodonitrotetrazolium (INT; Amresco, Solon, OH), and 0.3 phenazine methosulfate (PMS; Acros Organics, Pittsburgh, PA)). The reaction, in a 96-well plate, was mixed with a multi-well plate shaker and then incubated for 15 min. The reaction was read at 470 nm using a Beckman Coulter (Pasadena, CA), AD340 colorimetric plate reader. LDH standards (Cayman Chemical, Ann Arbor, MI) from 0-10 mU/mL were included in the assay to ensure readings were within the linear range. In parallel assays, the cells, including the pellets obtained from the ischemia and reperfusion media fractions were lysed with 0.1% triton X-100 in PBS and the lysate assayed to obtain total LDH for each sample, for which a percentage of total LDH was determined.

Propidium iodide assay

Following the experimental I/R protocol, propidium iodide (PI) in PBS was added to a 4 µg/mL final concentration onto cells in monolayer for 10 min at 37 °C. The buffer/PI solution was then removed and cells were rinsed twice with Hank’s balanced salt solution (HBSS; Corning Cellgro, Manassas, VA). PI fluorescence was read with a fluorescent plate reader (Synergy HT, BioTek, Wisnooski, VT) with a 528/20 nm excitation filter and a 620/40 nm emission filter. PI fluorescence is depicted by subtracting the value of background (cells not labeled with PI) followed by normalization to the appropriate I/R sample.
Detection of apoptosis

DNA laddering

Deoxyribonucleic acid (DNA) Laddering protocol was modified from Wildey et al. (Wildey et al. 2003). Cells were plated at 1 x 10^6 cells per 60 mm dish and grown in supplemented Claycomb media for 40 hr prior to experiments. Cells were scraped into the media and pelleted at 1,500 x g. Cells were lysed with 10 mM Tris hydrochloride, pH 8.0, 1 mM ethlenediaminetetraacetic acid (EDTA), and 0.2% triton-X 100 and centrifuged at 16,100 x g for 20 min at room temperature (RT). Supernatants were collected and a phenol/chloroform extraction of DNA was performed. Saturated phenol (Amresco, Solon, OH) was added to supernatants and mixed by inversion before centrifugation at 16,100 x g for 2 min at RT. The aqueous portion was then carefully removed to a new tube. Phenol: Chloroform: Isoamyl Alcohol (25:24:1, v/v) (Amresco, Solon, OH) was added and mixed before centrifugation at 16,100 x g for 2 min at RT. The aqueous portion was collected and DNA was precipitated with 700 µL isopropanol, 70 µL sodium acetate pH 5.2, and 0.04 mg/mL glycogen (MP Biomedicals, Santa Ana, CA) for pellet visibility. Tubes were placed at -80 °C overnight and then centrifuged at 16,100 x g for 20 min at 4 °C to precipitate DNA. Supernatant was removed and DNA pellet was washed with 1 mL 70% ethanol and centrifuged at 16,100 x g at 4 °C for 10 min. Ethanol was then removed and pellets were air dried. DNA was resuspended in 20 µL of Tris-EDTA (TE) buffer with RNAse (Amresco, Solon, OH) added to a concentration of 25 µg/mL. DNA was quantified using a NanoDrop 2000 UV-Vis Spectrophotometer (Thermo Scientific, Waltham, MA). Equal amounts of each sample
were loaded onto a 1.8% agarose gel (EMD Millipore, Billerica, MA) and electrophoresed at 80V in TBE buffer (89 mM Tris, 89 mM boric acid, 2 mM EDTA). DNA gels were imaged with a FluorChem M Simple Imager (Protein Simple, Santa Clara, CA).

Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling

The In Situ Cell Death Detection Kit (Roche, Indianapolis, IN) was used according to the manufacturer’s protocol for terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL). Cells were plated on coverslips, then following the I/R protocol the cells were washed once in PBS and then fixed in 4% paraformaldehyde (PFA). Two 5 min PBS washes were done, followed by 10 min of permeabilization in 0.1% triton X-100 in PBS. TUNEL reaction mixture was prepared as directed by adding 50 μL of Enzyme Solution containing TdT to 450 μL of Label Solution which contains fluorescein labeled nucleotides in a Reaction Buffer that includes cacodylate. Cells were incubated in TUNEL reaction mixture for 1 hr at 37 °C in the dark in a humidified chamber. Three 5 min washes with PBS were done and then coverslips were mounted with Vectashield with 4’,6-diamidino-2-phenylindole (DAPI; Vector Laboratories, Burlingame, CA). TUNEL staining was imaged using an epifluorescent microscope (Olympus, Center Valley, PA) at 20x magnification with standard band pass filters for DAPI and Fluorescein isothiocyanate (FITC) with excitation wavelengths of 340-380 nm and 465-495 nm, respectively and emission wavelengths of 435-485 nm and 515-555 nm, respectively. Images were acquired
digitally with a Retiga 2000R Fast cooled camera (QImaging, Surrey, BC, Canada) for each fluorophore separately with QCapture software (QImaging, Surrey, BC, Canada) and then merged and analyzed using ImageJ (NIH, Bethesda, MD). Quantitations of TUNEL and DAPI stained nuclei were done on at least 5 fields per sample.

**Annexin V/propidium iodide experiments**

HL-1 CMs were plated at 4 x 10^5 cells per well into 12-well dishes and subjected to the I/R protocol. Cells were incubated with Annexin V, Alexa Fluor 488 conjugate to visualize phosphatidylserine residues present on apoptotic cells and necrotic cells. PI (1 µg/mL) was added to assess cells with compromised membrane integrity. Total cell number was measured using nuclear stain, Hoechst 33342. Percent of cells stained with Annexin V and percent of nuclei stained with PI were calculated and normalized to I/R.

**Western blot**

Cells were lysed in Mammalian Protein Extraction Reagent (M-PER; Thermo Scientific, Waltham, MA), and lysates were incubated at 4 °C for 20 min and then centrifuged at 16,100 x g for 20 min. Supernatants were collected in a new tube and stored at -80 °C until protein quantitation. Protein concentrations were determined using the Bradford method (Bradford 1976) with a Coomassie Protein Assay Kit or with a BCA kit (Thermo Scientific, Waltham, MA). Aliquots were prepared using equal amounts of protein with Laemmli sample buffer (to final concentrations of: 65.5 mM Tris-Cl pH 6.8, 2% sodium dodecyl sulfate (SDS), 10% glycerol, 0.001% (w/v) bromophenol blue, 5% β-mercaptoethanol). Samples were then heated to 65 °C for 10 min to denature proteins.
then loaded onto 12 or 15% acrylamide gels for SDS-PAGE. Gels were run at 130V in Tris running buffer (25 mM Tris, 192 mM glycine, 0.1 % SDS). Proteins were transferred onto PVDF membrane (EMD Millipore, Billerica, MA) for 1 hr at 100V in cooled transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol). The membrane was blocked in 5% (w/v) milk in TBS with 0.05% tween-20 (TBS-Tw) for 1 hr then incubated with primary antibody overnight at 4 °C on an orbital shaker. The blot was then rinsed three times with TBS-Tw and incubated with the appropriate secondary antibody conjugated with horseradish peroxidase (Thermo Scientific, Waltham, MA). Signals were detected with the use of chemiluminescent substrate (Thermo Scientific, Waltham, MA) and a FluorChem M Simple Imager (Protein Simple, Santa Clara, CA) or a Medical Film Processor SRX-101A (Konica Minolta, Wayne, NJ) followed by positive film scanning with an EPSON scanner (Long Beach, CA). Densitometry was performed using ImageJ software (NIH, Bethesda, MD). Primary antibodies used for western blotting were MAP LC3β (Santa Cruz Biotechnology, Santa Cruz, CA) MAP LC3β, p44/42 MAP Kinase, Phospho-p44/42 MAP Kinase (Thr202/Tyr204), Akt, Phospho-Akt (Ser 473), Pan-Actin, GAPDH. Primary antibodies were from Cell Signaling Technology (Danvers, MA) unless otherwise stated.

**DNA transfection**

Plasmid ptf-LC3 was obtained from Addgene (Kimura et al. 2007). Plasmids were propagated in competent *E. coli* (Top10, Invitrogen, Carlsbad, CA) and isolated with Qiagen midi prep kit (Valencia, CA).
Cells were plated on fibronectin coated coverslips at 1.5 x 10^5 cells per coverslip and grown in supplemented Claycomb media for 24 hr before transfection, and 40 hr before experiment. Transfections were carried out using Lipofectamine reagent (Invitrogen, Carlsbad, CA) added to 1 μg of DNA per well. Transfection mixture was left on cells for 24 hr before subjecting cells to experimental I/R protocols.

**Quantifying LC3 puncta**

Following I/R protocol, cells were fixed with 4% PFA (Thermo Fisher Scientific, Waltham, MA) for 10 min. After 3 washes in PBS, coverslips were mounted with Vectashield containing DAPI (Vector Laboratories, Burlingame, CA). Images were taken at 60x magnification with an FV1000 Confocal Microscope (Olympus, Center Valley, PA) using 405 nm, 488 nm, and 559 nm lasers and FluoView software (Olympus, Center Valley, PA).

LC3-I is typically distributed diffusely throughout the cell while the lipid bound LC3-II protein is incorporated into the membranes of autophagosomes. Tandem fluorescent-tagged LC3 is tagged with both GFP and mRFP allowing visualization of autophagosomes and autolysosomes as small dots, or puncta, within cells. This method can be used for evaluation of autophagic flux because of the acid sensitivity of the GFP tag which fluoresces when LC3 is incorporated into the membrane of an autophagosome, but is degraded (no longer fluorescent) in the acidic environment present once an autophagosome and lysosome fuse forming an autolysosome. Using fluorescence microscopy, autophagosomes appear in both the green and red fields, while the more
acidic autolysosomes appear only in the red field. The tagged LC3 protein allows measurement of autophagosomes which are present during the early phase of autophagy and measurement of autolysosomes which are only present during the late degradation phase of autophagy. By comparing changes in these numbers between samples we can establish whether there is an increase or decrease in autophagic flux including whether there is a change in induction or degradation.

Puncta were evaluated per cell with between 80-200 cells per treatment. LC3 puncta counts of both GFP- and RFP-expressing cells were done using a custom script written with MatLab software (MathWorks, Natick, MA). The MatLab script was written and applied to images by Douglas C. Crowder. Maximum z-projections of confocal image stacks were edited to include one cell per image. The MatLab script processed the red (RFP) and green (GFP) channels separately. For each channel, images were converted to a binary pixel map using intensity thresholds. The threshold for red pixels was set at 100, and the threshold for green pixels was set at 175. These numbers were chosen from the possible intensity values between 0 and 255, where 255 represents the most saturated (intense) colors. The threshold values were visually validated by comparing the binary pixel maps to the original images. Threshold values remained consistent for each channel (red or green) across all treatment groups.

Pixels contained in the binary map were then segmented into individual puncta based on immediate proximity to other pixels. Pixel clusters containing more than 20 pixels were excluded from the analysis since such clusters tended to include background that could bias results. Additionally, pixel clusters containing less than 2 pixels were
excluded from analysis, since puncta containing only one pixel were difficult to identify accurately. The size thresholds were visually validated by comparing the binary pixel maps to the original images and were kept consistent across all treatment groups, for all channels.

The binary pixel maps were then used to quantify: 1.) the total number of puncta, 2.) the average size of the puncta (in pixels), and 3.) the average intensity of the pixels contained within the puncta, expressed as a percent of the maximum intensity (100% = 255).

The script makes the following assumptions: 1.) that staining intensity is the same for all treatment groups, given that the image acquisition parameters were held constant for all treatments, 2.) that puncta greater than 20 pixels in size generally contained "noise" and should be excluded from analysis, and 3.) that puncta smaller than 2 pixels in size could not be reliably identified as puncta, rather than noise and should be excluded from analysis. These assumptions were supported by visual analysis of the binary pixel maps.

**Reverse transcriptase polymerase chain reaction**

RNA was isolated using Triazol (Life Technologies Corporation, Carlsbad, CA). Media was removed and Triazol was added directly onto cells in plate. Triazol and cells were transferred to new RNAse-free microcentrifuge tubes and allowed to sit for 5 min at RT. Chloroform was then added at 0.2 mL per mL of Triazol initially used. Tubes were then shaken and incubated for 3 min at RT. Tubes were centrifuged at 12,000 x g for 15
min at 4 °C. Aqueous phase was removed to a new RNase-free microcentrifuge tube and isopropanol was added at 500 µL per mL of Triazol initial used. Tubes were incubated at RT for 10 min and then centrifuged at 12,000 x g for 10 min at 4 °C. The supernatant was removed and RNA was washed with 75% ethanol followed by centrifugation at 7,500 x g for 7 min at 4 °C. Ethanol was removed and the pellets were allowed to dry. Pellets were dissolved in RNase-free water and heated to 55 °C for 10 min. RNA was then DNase treated to remove any possible DNA contamination. RNA was quantified using a NanoDrop 2000 UV-Vis Spectrophotometer (Thermo Scientific, Waltham, MA). Complementary DNA (cDNA) was then synthesized with reverse transcriptase enzyme using RNA as the template. Reverse transcriptase polymerase chain reaction (RT-PCR) was performed using PCR Master Mix (Promega, Madison, WI) in a Thermo Hybaid PCR Sprint thermal cycler (Thermo Scientific, Waltham, MA). The PCR consisted of an initial denaturation step at 95 °C for 3 min, followed by 30 cycles of 30s 95 °C denaturation, 45s 60 °C annealing temperature, and 30s 72 °C extension with a final step of 72 °C for 5 min. PCR products were then loaded and ran on 3% agarose gels with ethidium bromide added before hardening in 1x Tris-Acetate-EDTA (TAE) buffer using electrophoresis.

Three separate sets of primers were used to detect the α1A and α1B subtypes. Primer set 1 was designed to cross an exon-exon junction eliminating the possibility of amplification of genomic DNA. Primer sets 2 and 3 do not cross an exon-exon junction and thus were used to amplify HL-1 genomic DNA to ensure that all primers were able to detect their prospective target. Primer set 2 was designed to detect all four current
transcript variants of the $\alpha_{1A}$-AR subtype found on the NCBI website. Additionally, primer set 3 was verified against genomic DNA from $\alpha_{1A}$ and $\alpha_{1B}$-AR subtype specific knock out mice. Sets 1 and 3 were designed within our lab with the aid of Primer-BLAST which was developed by NCBI (Ye et al. 2012). Primer set 2 was previously described in a publication (Gupta et al. 2009). A control sample was run with water instead of cDNA to ensure the absence of contaminants or primer-primer interaction. Primers used are listed in Table 1.

**Statistical analysis**

Statistical analyses were carried out using GraphPad Prism SoftwareV6. Analyses were completed using one-way ANOVA followed by Tukey’s post test to correct for multiple comparisons or an unpaired t test as indicated. A value of P<0.05 was considered significant.
<table>
<thead>
<tr>
<th>Primer Set</th>
<th>Target</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Set 1</td>
<td>α1A</td>
<td>sense 5'-CCT CGT GAT GCC CAT TGG GTC C-3',&lt;br&gt;antisense 5'-CTC CTG GCT GGA GCA TGG GT-3'</td>
</tr>
<tr>
<td>Set 1</td>
<td>α1B</td>
<td>sense 5'-CGC TCT CCC ACT TGG CTC CC-3',&lt;br&gt;antisense 5'-ACG CAT GAA GGC GCG TTT GA-3'</td>
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<tr>
<td>Set 2</td>
<td>α1A</td>
<td>sense 5'-GGT TCC CAA AGG AAA CCT GT-3',&lt;br&gt;antisense 5'-GGT TTC ATA CCA GGG TGG TG-3'</td>
</tr>
<tr>
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<td>sense 5'-TCT CAG CCA AGT CCT GGT TT-3',&lt;br&gt;antisense 5'-GCG AAC ACC TTT ACC TGC TC-3'</td>
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<tr>
<td>Set 3</td>
<td>α1A</td>
<td>sense 5'-ACA TTG GTG TGA GCT ACC CG-3',&lt;br&gt;antisense 5'-AAC AGG GGT CCG ATG GAG AG-3'</td>
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<tr>
<td>Set 3</td>
<td>α1B</td>
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</tr>
<tr>
<td></td>
<td>α1D</td>
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<tr>
<td></td>
<td>GAPDH</td>
<td>sense 5'-CTT CAT TGA CCT CAA CTA CAT G-3',&lt;br&gt;antisense 5'-TGT CAT GGA ATG ACC TTG GGC CAG-3'.</td>
</tr>
</tbody>
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Table 1. RT-PCR Primers used to investigate α1-adrenergic receptor subtypes expressed in HL-1 CMs.
CHAPTER THREE

RESULTS

1. Examine effects of post-ischemic stimulation of α₁-adrenergic receptors on cardiac myocyte cell death

*Determinination of optimal duration of initial ischemic insult*

To test the hypothesis that post-ischemic alpha₁-adrenergic receptor (α₁-AR) stimulation will decrease cell death, initial experiments focused on developing a protocol for a cell culture model to simulate ischemia and reperfusion of HL-1 cardiac myocytes (CMs) and primary adult rat ventricular myocytes (ARVMs.) The experiments were used to determine the appropriate durations of ischemic periods for subsequent experiments. The endpoint of cell death, as assessed by determining cell membrane integrity, was measured using either propidium iodide (PI) uptake or lactate dehydrogenase (LDH) activity in the media, denoting LDH released by cells.

HL-1 CMs were exposed to constant 1% O₂ and ischemia mimetic buffer for increasing durations of ischemia (2, 4, and 8 hr) which resulted in increased PI uptake (increased fluorescence) as shown in Figure 6A. A similar effect was observed in ARVMs exposed to 1, 2, and 3 hr ischemia and measured with LDH release (Figure 6B). As expected, longer exposure of cells to ischemia produced the most dramatic effects in cell death. However, in order to assess the effects of postconditioning (PostC) during the reperfusion phase, the length of ischemic insult was chosen such that most cells were not
Figure 6. Effects on cardiac myocyte death with increasing durations of ischemia. (A) Propidium Iodide (PI) measurements from HL-1 cardiac myocytes exposed to simulated ischemia from 2-8 hrs. PI fluorescence is expressed as fold-change over non-ischemic control (N-I) ± SEM. N≥1. (B) LDH measurements from ARVMs exposed to simulated ischemia for 1-3 hrs. Percent LDH released from cells is expressed as fold-change over N-I ± SEM. N≥3.
irreversibly damaged by the initial ischemic insult. Thus, conditions were chosen in which there was not a significant level of cell death over the non-ischemic (N-I) sample. Based on conditions previously described in the literature (Hamacher-Brady et al. 2006, Astrom-Olsson et al. 2009), data shown in Figure 6, and morphological analysis, the experimental conditions chosen were 2 hr of ischemia for HL-1 CMs and 30 min of ischemia for the more sensitive primary ARVMs. These durations of ischemia were used for the remainder of experiments in this dissertation, while durations of reperfusion varied depending on the experiment.

Determination of optimal duration of reperfusion

Reperfusion injury was evaluated by comparing levels of cell death occurring after ischemia during the reperfusion period. This comparison was essential to the project as cell death occurring during reperfusion is the target of both ischemic and pharmacological PostC. After an initial ischemic insult for the times described above, HL-1 CMs and ARVMs were subjected to various periods of reperfusion between 0 and 24 hr. Cell death was evaluated using LDH release. Differences between reperfusion times were subtle but displayed a clear trend of increased LDH release with increased duration of reperfusion (Figure 7). Because differences in levels of cell death during reperfusion were relatively small between early reperfusion time points such as 2 and 4 hr, it was necessary to evaluate cell death at longer time points such as 6 and 24 hr of reperfusion. This was done to adequately assess differences in cell death, with PI fluorescence and LDH release, and differences in apoptosis with TUNEL staining.
Figure 7. Effects on cardiac myocyte death with increasing durations of reperfusion. LDH released from (A) HL-1 cardiac myocytes (N≥3) and (B) Primary ARVMs (N≥4). LDH released is expressed as relative change in % LDH as compared to time matched non-ischemic control (N-I) for the entire time encompassing ischemia (I) and reperfusion (R), ± SEM.
Laddering, and Annexin V staining. Other end points such as autophagic flux were evaluated at an earlier reperfusion time (2 hr) to investigate the contribution of early molecular events that lead to differences in cell death seen after longer durations (e.g., 6 and 24 hr) of reperfusion.

1a. *Post-ischemic treatment of cardiac myocytes with α₁-adrenergic receptor agonist (pharmacological postconditioning) decreases cell death*

Ischemia and reperfusion (I/R) experiments were carried out using the previously optimized times and pharmacological PostC was assessed by addition of the α₁-AR agonist phenylephrine (Phe) at the onset of reperfusion. Drugs remained in the media for the duration of the reperfusion. ARVMs treated with increasing concentrations of Phe showed a decrease in LDH release only with the 10 µM concentration of Phe (Figure 8A). Phe was not effective at decreasing cell death at nanomolar concentrations or at millimolar concentrations. This is expected since Phe at higher concentrations has been shown to lose its selectivity and may stimulate β-adrenergic receptors (β-ARs). Increasing concentrations of phentolamine (Pnt), an α-adrenergic receptor antagonist effectively inhibited Phe at every concentration tested ranging from subnanomolar to micromolar (Figure 8B). Based on these experiments, concentrations of 10 µM Phe (agonist) and 1 µM Pnt (antagonist) were used for all subsequent experiments.

To evaluate effects of post-ischemic treatment with the chosen concentrations of Phe (10 µM) and Pnt (1 µM) on HL-1 CMs, morphological studies were done using phase contrast microscopy. Figure 9 (B-E) shows images of HL-1 CMs subjected to the I/R protocol and treated post-ischemically with pharmacological agents. Exposure to 2hr
Figure 8. Evaluation of phenylephrine and phentolamine concentration response in ARVMs. (A) Shows ARVMs treated with increasing concentrations of phenylephrine (Phe) following 30 minutes of ischemia, measured after 24 hr of reperfusion (30I/24R). The graph in (B) shows ARVMs exposed to 30 minutes of ischemia, measured after 24 hr of reperfusion (30I/24R) and treated with 10 µM Phe along with increasing concentrations of phentolamine (Pnt) during reperfusion. P≤0.05:* vs 30I/24R, + vs Phe. Values represent fold-change from 30I/24R, mean ± SEM. N≥3. Significant differences between conditions were determined with one-way ANOVA followed by Tukey’s post test.
Figure 9. HL-1 cardiac myocytes exposed to ischemia and reperfusion protocol. Phase contrast images taken at 10x magnification. In (A) cells were non-ischemic (N-I) while (B-D) were exposed to ischemia and reperfusion (I/R) protocol with or without pharmacological postconditioning. Specifically, HL-1 cardiac myocytes (B) were exposed to 2 hr ischemia and 24 hr reperfusion (2I/24R), (C) were exposed to I/R protocol with phenylephrine (Phe) added post-ischemically, (D) were exposed to I/R protocol with Phe and prazosin (Pz), (E) were exposed to the I/R protocol with Phe and propranolol (Prop).
ischemia and 24 hr reperfusion (Figure 9B) caused more rounded cells and fewer adherent cells as compared to N-I (Figure 9A). Addition of Phe appears to reverse the change in rounded vs. adherent cells (Figure 9C) which is inhibited by the inclusion of α₁-AR antagonist, prazosin (Pz) (Figure 9D). The addition of the β-AR antagonist propranolol (Prop) had no effect on the changes seen with Phe (Figure 9E), indicating that β-AR signaling was not involved in this process.

The results of LDH release assays performed with 2 hr ischemia and 24 hr reperfusion (2I/24R) supported the results of the morphological studies (Figure 10). Exposure of HL-1 CMs to 2I/24R significantly increased LDH release as expected. Phe added at the time of reperfusion significantly decreased the amount of LDH released during a 24 hr reperfusion translating to an average of 10% decrease in cell death. This protection was successfully reversed with addition of Pz indicating a necessity of α₁-AR activation for the cardioprotective effect (Figure 10A). This effect was also observed in primary ARVMs, which is the more physiologically relevant cell type (Figure 10B).

Because of the large amount of cell death found at 24 hr of reperfusion, cell death was further assessed at a shorter duration of 6 hr reperfusion as well. Using PI uptake as an estimate of cell death, HL-1 CMs showed a similar result to previous LDH release assays at both 6 and 24 hr durations of reperfusion (Figure 11). There was a significant decrease of PI fluorescence with post-ischemic Phe treatment compared to I/R. This decrease in cell death with Phe was inhibited by Pnt. A control sample with Pnt alone did not show a significant difference from I/R without pharmacological treatment.
Figure 10. Cell death is decreased with post-ischemic $\alpha_1$-adrenergic receptor stimulation following simulated ischemia and reperfusion: 24 hr reperfusion. (A) LDH released from HL-1 cardiac myocytes after 2 hr ischemia and 24 hr reperfusion (2I/24R). N≥4. Values represent fold-change from 2I/24R. (B) LDH release from ARVMs following 30 min ischemia and 24 hr reperfusion (30I/24R). N≥11. P≤0.05: * vs I/R, + vs Phe. Values represent fold-change from 30I/24R, mean ± SEM. Significant differences between conditions were determined with one-way ANOVA followed by Tukey’s post test. Phenylephrine (Phe), Prazosin (Pz), Phentolamine (Pnt).
Figure 11. Cell death is decreased with $\alpha_1$-adrenergic receptor stimulation following simulated ischemia and reperfusion: propidium iodide fluorescence of HL-1 cardiac myocytes. (A) PI fluorescence of HL-1 cells following 2 hr ischemia and 6 hr reperfusion (2I/6R). N\geq3. Values represent fold-change from 2I/6R. P\leq0.05: * vs 2I/6R, + vs Phe. (B) PI fluorescence of HL-1 cells following 2 hr ischemia and 24 hr reperfusion (2I/24R). N\geq3. Values represent fold-change from 2I/24R, mean ± SEM. P\leq0.05: * vs 2I/24R, + vs Phe. Significant differences between conditions were determined with one-way ANOVA followed by Tukey’s post test. Phenyplephrine (Phe), Prazosin (Pz), Phentolamine (Pnt). Non-ischemic (N-I), Phenyplephrine (Phe), Phentolamine (Pnt).
Additional treatments were done to confirm previous results indicating that selective stimulation of $\alpha_1$-ARs is involved in Phe-mediated cardioprotection and to investigate effects of concurrent activation of $\beta$-ARs. HL-1 CMs subjected to 2 hr ischemia and 6 hr reperfusion (2I/6R) and treated with the non-selective adrenergic agonist norepinephrine (NE) post-ischemically showed no significant change in PI fluorescence. This indicated that concurrent stimulation of $\alpha$ and $\beta$-ARs does not produce cardioprotective effects (Figure 12A). When Prop was added with NE to inhibit $\beta$-AR stimulation, there was decreased PI fluorescence compared to NE alone, but compared to 2I/6R the difference did not reach significance. Selective stimulation of $\beta$-ARs with Isoproterenol (Iso) showed no significant difference from 2I/6R. Addition of $\beta$-AR antagonist Prop in addition to Iso or alone also showed no change from 2I/6R. These experiments were repeated in primary ARVMs (Figure 12B). Treatment of ARVMs with NE did not show significant cardioprotective effects indicating that only $\alpha_1$-AR stimulation and not $\beta$-AR stimulation post-ischemically results in cardioprotection. Further, NE and Prop together significantly decreased LDH release in ARVMs. Inhibition of $\beta$-ARs with Prop decreased levels of cell death almost to those seen with Phe treatment alone in ARVMs.

Overall, data obtained with HL-1 CMs and primary ARVMs supported the hypothesis that post-ischemic $\alpha_1$-AR stimulation decreases general cell death as measured with LDH release and PI fluorescence at multiple reperfusion periods. Additionally, the effects of Phe appear to be mediated by $\alpha_1$-ARs and there is no indication of protection.
Figure 12. Decrease in cell death with post-ischemic treatment is dependent upon $\alpha_1$-adrenergic receptor stimulation and is independent of $\beta$-adrenergic receptor stimulation. (A) PI fluorescence of HL-1 cells following 2 hr ischemia and 6 hr reperfusion (2I/6R). Values represent fold-change from 2I/6R, mean ± SEM. P≤0.05: * vs 2I/6R. N≥3. (B) LDH released from ARVMs exposed to 30 min of ischemia and 24 hr reperfusion (30I/24R). Values represent fold-change from 30I/24R, mean ± SEM. P≤0.05: * vs 30I/24R. N≥4. Significant differences between conditions were determined with one-way ANOVA followed by Tukey’s post test. Non-ischemic (N-I), Phenylephrine (Phe), Norepinephrine (NE), Propranolol (Prop), Isoproterenol (Iso).
conferred by β-AR stimulation. Further, α₁-AR-mediated protection was actually blunted by concurrent β-AR stimulation.

1b. Post-ischemic α₁-adrenergic receptor stimulation decreases levels of apoptosis

Further characterization was done to more specifically assess Phe-mediated protection; apoptosis was measured using several techniques. Pre-ischemic α₁-AR stimulation and α₁-AR stimulation concurrent with ischemia has been shown to decrease apoptosis in CMs (Baghelai et al. 1999, Rorabaugh et al. 2005, Huang et al. 2007). Therefore it was imperative to examine the impact of α₁-AR stimulation on apoptosis in this system as this has not been previously investigated in cells treated post-ischemically. To test the hypothesis that post-ischemic α₁-AR stimulation will decrease apoptosis, in-plate staining was done on HL-1 CMs using Annexin V to label outer membrane phosphatidylserine. PI was used to label cells with compromised membrane integrity, and Hoechst nuclear stain was used to quantitate the total cells present. Representative images and graphs of their quantitation in Figure 13 show an increase in Annexin V and PI staining with 2 hr ischemia and 6 hr reperfusion. Phe added at reperfusion significantly inhibited both Annexin V and PI staining. Pnt did not significantly reverse Phe-mediated cardioprotection, although there was a trend towards inhibition. This result was consistent with evaluation of apoptotic DNA cleavage using terminal deoxynucleotidyltransferase-mediated nick end labeling (TUNEL) staining (Figure 14). There was a significant decrease in TUNEL stained cells with Phe treatment and while Pnt partially inhibited the effect seen with Phe treatment, it did not reach statistical significance. To confirm these
Figure 13. Levels of apoptosis evaluated with Annexin V and PI staining in HL-1 cardiac myocytes. (A) Representative images of adherent HL-1 CMs exposed to 2hr ischemia and 6 hr reperfusion (2I/6R) and stained with Annexin V-488, PI, and Hoechst. (B) Counts of PI positive cells normalized to 2I/6R sample. (C) Counts of Annexin V labeled cells normalized to 2I/6R sample. P≤0.05: * vs 2I/6R, + vs Phe. N=4. Significant differences between conditions were determined with one-way ANOVA followed by unpaired t-tests. Phenylephrine (Phe), Phentolamine (Pnt).
Figure 14. Levels of apoptosis evaluated using TUNEL staining. (A) Representative images of HL-1 CMs exposed to 2 hr ischemia and 24 hr reperfusion (2I/24R) stained with TUNEL (Green) and DAPI (Blue). (B) Graph shows percent of positively stained nuclei normalized to 2I/24R. P≤0.05: * vs 2I/24R. N=4 at least 5 fields per experiment. Significant differences between conditions were determined with one-way ANOVA followed by unpaired t-tests. Phenylephrine (Phe), Phentolamine (Pnt).
results, DNA Laddering assays were performed using HL-1 CMs exposed to 2 hr ischemia and 24 hr reperfusion (Figure 15). There was a decrease in laddering when Phe was added post-ischemically supporting results obtained with Annexin V and TUNEL staining. Pnt appeared to reverse this effect. These results support the hypothesis that post-ischemic α₁-AR stimulation contributes to reduced CM apoptosis in I/R injury.

2. Alpha₁-adrenergic receptor-mediated cardioprotection may involve autophagy

*Pharmacological modulation of autophagy alters cell death in ischemia and reperfusion exposed cardiac myocytes*

Because autophagy has been implicated in I/R injury in CMs (Hamacher-Brady et al. 2006), and long-term α₁-AR stimulation has been shown to induce autophagy (Cao et al. 2011, Xu et al. 2014), we assessed autophagy in our simulated I/R system to determine its involvement in cardiac myocyte death and Phe-mediated protection. Additionally, autophagy is also implicated in regulating apoptosis (Maiuri et al. 2010). The autophagy inhibitor 3-methyladenine (3-MA) is a class three PI3Kinase inhibitor and blocks the induction of autophagy. Rapamycin (Rp) is an autophagy activator that blocks the activity of mTOR allowing autophagy to proceed as depicted in Figure 4. As shown in Figure 16A, 3-MA significantly inhibited Phe-mediated cardioprotection in HL-1 CMs exposed to 2 hr ischemia and 6 hr reperfusion. Treatment with 3-MA alone was not different from 2I/6R. These results were similar in primary ARVMs measured with LDH release (Figure 16B). While post-ischemic Rp treatment showed a trend toward protection, values did not reach significance. The observed changes in PI fluorescence
Figure 15. Levels of apoptosis estimated using DNA laddering. Representative image of DNA laddering gel done with HL-1 cardiac myocytes exposed to 2 hr ischemia and 24 hr reperfusion (2I/24R). N=3. Non-ischemic (N-I), Phenylephrine (Phe), Phentolamine (Pnt).
Figure 16. Modulation of autophagy post-ischemically changes the cell death response in cardiac myocytes. (A) PI fluorescence of HL-1 cells following 2 hr ischemia and 6 hr of reperfusion (2I/6R). Values represent fold-change from 2I/6R, mean ± SEM. P≤0.05: * vs 2I/6R, + vs Phe. N≥5. (B) LDH released from ARVMs exposed to 30 min ischemia and 24 hr of reperfusion (30I/24R). Values represent fold-change from 30I/24R, mean ± SEM. P≤0.05: * vs 30I/24R, + vs Phe. N≥3. Significant differences between conditions were determined with one-way ANOVA followed by Tukey’s post test. Non-ischemic (N-I), Phenylephrine (Phe), Phentolamine (Pnt), 3-methyladenine (3-MA), Rapamycin (Rp).
with pharmacological modulation of autophagy suggest that autophagy is necessary for Phe-mediated cell survival during reperfusion.

2a. *Post-ischemic \( \alpha_1 \)-adrenergic receptor stimulation changes autophagy marker LC3-II*

To measure autophagy, immunoblots were done of autophagosomal marker protein, LC3-II. HL-1 CMs were exposed to 2 hr of ischemia and various durations of reperfusion (2 and 6 hr). Change in LC3-II was measured with and without Phe. This experiment was done with chloroquine added to all samples to allow autophagosome accumulation. As shown in Figure 17, increased LC3-II was detected at 2 hr reperfusion with Phe treatment compared to 2I/2R. On the other hand, there was no change in LC3-II between 2I/6R and with Phe treatment. These results suggested that \( \alpha_1 \)-AR activation post-ischemically induces an increase in LC3-II levels early in reperfusion that then tapers off between 2 and 6 hr of reperfusion.

2b. *Post-ischemic \( \alpha_1 \)-adrenergic receptor stimulation increases autophagic flux*

Autophagy is dynamic process and requires measurement of autophagic flux, or the rate of clearance of autophagosomal contents. Changes in LC3-II levels can be misleading as increased induction and decreased degradation both appear as an increase in LC3-II (Mizushima et al. 2010). For this reason, several methods to measure autophagic flux were used to determine whether the Phe-mediated changes in LC3-II were caused by increased autophagic induction (increased flux) or an error in degradation (decreased flux). Autophagic flux was evaluated using western blotting of LC3-II in the presence and absence of chloroquine (CQ). CQ blocks autophagosome-lysosome fusion
Figure 17. Autophagosomal marker LC3-II measured in HL-1 cardiac myocytes during increasing durations of reperfusion. (A) Representative western blot of LC3-II (lower band) in HL-1 cardiac myocytes exposed to 2 hr ischemia and 2 and 6 hr reperfusion (2I/2R, 2I/6R). (B) Graph shows levels of LC3-II compared to GAPDH and measured using densitometry. Values represent fold-change from 2I/2R or 2I/6R, mean ± SEM. N=3. Significant differences between conditions were determined with one-way ANOVA followed by Tukey’s post test. Non-ischemic (N-I), Phenylephrine (Phe).
which inhibits autophagosome degradation allowing measurement of accumulated autophagosomes. Referred to as an LC3 turnover assay, ratios comparing LC3-II levels were determined with and without CQ to assess the change in autophagosomes that occurred during the time period of treatment. LC3 turnover assays in HL-1 CMs were conducted after 2hr of ischemia and 2 hr of reperfusion, conditions in which we previously saw modulation of autophagy by α1-ARs (Figure 17). Turnover was significantly increased by post-ischemic addition of Phe as compared to 2I/2R and this effect was significantly inhibited by the addition of Pnt (Figure 18). This increase in turnover is indicative of an increase in induction. Taken together with the cell death data there is a correlation between increased induction of autophagy and decreased CM death with α1-AR activation following ischemia.

As additional confirmation of a change in autophagic flux, a plasmid encoding a tandem fluorescent-tagged LC3 protein (Kimura et al. 2007) was transfected into HL-1 CMs. HL-1 CMs were used for this experiment because lipophilic agent-induced transfection is possible; ARVMs, on the other hand, are more sensitive and are unable to be transfected in this way. The LC3 is fused with both GFP and mRFP. LC3 incorporated into autophagosomes appears as GFP and mRFP labeled puncta and represents the early phase of autophagy. Once the autophagosome fuses to a lysosome, the acidic environment degrades GFP but not RFP. The remaining RFP labels autolysosomes only red with no corresponding signal in the green field. This allows quantitation and comparison of the early phase of autophagy with autophagosomes (GFP and mRFP) with
**Figure 18. Autophagic flux measured by LC3 turnover assay in HL-1 cardiac myocytes.** (A) HL-1 CMs were exposed to 2 hr ischemia and 2 hr reperfusion (2I/2R). Representative western blot of LC3-II without (top) and with (bottom) chloroquine (CQ) to show accumulation of autophagosomes during a 2 hr reperfusion. (B) Densitometry measurements of graph shown indicate the change in LC3-II amounts with addition of CQ. Values represent fold-change from 2I/2R, mean ± SEM. P ≤ 0.05, # vs 2I/2R, + vs Phe. N=5. Significant differences between conditions were determined with one-way ANOVA followed by Tukey’s post test. Non-ischemic (N-I), Phenylephrine (Phe), Phentolamine (Pnt).
the late phase of autophagy after fusion with lysosomes has been completed (autolysosomes, only mRFP). Autophagic flux can be determined using relative levels of early and late autophagy present. If there is an increase in autophagic flux there will be an increase in numbers of both autophagosomes and autolysosomes. If there is a decrease in flux, it is possible to determine whether there is a defect in autophagic induction or a defect in autophagosome-lysosome fusion. With a defect in autophagic induction, there will be a decrease in both autophagosomes and autolysosomes. A defect in fusion would show an accumulation of autophagosomes due to a lack of autophagosome-lysosome fusion, and a decrease in autolysosomes (Klionsky et al. 2008). Using these guidelines, we were able to use transfected cells to determine changes in autophagic flux in HL-1 CMs. Figure 19A shows representative confocal images of tandem-LC3 in HL-1 CMs exposed to 2 hr ischemia and 2 hr reperfusion (2I/2R). The average number of red and green colocalized puncta, or autophagosomes, per cell was similar between N-I and 2I/2R samples, but there was a significant increase when cells were treated with Phe indicating an increase in autophagosome number (Figure 19B). Puncta that fluoresce only in the red field, or autolysosomes, decreased with I/R as compared to N-I but this change was not statistically significant. Post-ischemic addition of Phe caused a significant increase in puncta fluorescing only red indicating an increase in autolysosomes. These data indicate that post-ischemic Phe addition increased the average number of autophagosomes and autolysosomes per cell indicating an increase in autophagic flux through increased autophagic induction.
Figure 19. Autophagic flux examined in HL-1 cardiac myocytes expressing a tandem fluorescent-tagged LC3 molecule. (A) Representative confocal images of tandem fluorescent-tagged LC3 with DAPI (Blue) nuclear stain. (B) Puncta counts in HL-1 cardiac myocytes exposed to 2 hr ischemia and 2 hr reperfusion (2I/2R) of both autophagosomes (red and green colocalized puncta) and autolysosomes (only red puncta). P≤0.05: * vs 2I/2R. N=3. Significant differences between conditions were determined with an unpaired t test. Non-ischemic (N-I), Phenylephrine (Phe).
There was an increase in autophagic flux with post-ischemic Phe treatment as measured with two techniques. Increased LC3-II levels seen on western blots represent an increase in autophagy induction and not an error in autophagosome fusion and degradation. These results support the hypothesis that post-ischemic α₁-AR stimulation increases autophagy, an event that occurs early in reperfusion, ie, 2 hr of reperfusion. This increase in autophagic flux may contribute to the protective effect mediated by addition of Phe at reperfusion.

3. Possible signaling components involved in α₁-adrenergic receptor-mediated cardioprotection

In order to examine the signaling mechanism of α₁-AR-mediated cardioprotection, pharmacological inhibitors were used in PI assays of cell death. Stimulation of α₁-ARs in CMs has been shown to induce increases in ERK1/2 phosphorylation (Huang et al. 2007). Therefore we used ERK1/2 pathway inhibitor PD98059 (PD) to evaluate ERK1/2 involvement in Phe-mediated cardioprotection (Figure 20). Post-ischemic addition of PD was able to inhibit the protective effect of Phe on HL-1 CMs in a dose dependent manner at both 1 and 10 µM. PD alone caused no significant changes from I/R alone indicating that ERK1/2 pathway activation does not cause cell death. To further investigate ERK1/2 involvement in α₁-AR-mediated cardioprotection, immunoblotting was completed to detect changes in ERK1/2 activation by phosphorylation at various time points (5 min, 15 min, and 2 hr reperfusion). There was not a difference in levels of phospho-ERK1/2 with Phe treatment at the time points tested (Figure 21). ERK1/2 is one of the members of the RISK pathway that participates
Figure 20. Effect of ERK1/2 inhibition. PI fluorescence measured in HL-1 cardiac myocytes after 2 hr ischemia and 6 hr reperfusion with pharmacological treatments applied post-ischemically. PD98059 (PD) was used to evaluate ERK1/2 effects in Phe-mediated protection. Values represent fold-change from 2I/6R (data not shown), mean ± SEM. P≤0.05# vs Phe. N≥7. Significant differences between conditions were determined with one-way ANOVA followed by Tukey’s post test. Phenylephrine (Phe).
Figure 21. Post-ischemic phenylephrine does not change ERK1/2 phosphorylation. (A) Shows a representative blot for each time point measured. Detected are phosphorylated ERK1/2, Total ERK1/2, and Pan-Actin in HL-1 cardiac myocytes at 2 hr ischemia and various times of reperfusion. (B) Densitometric measurements of N=3 immunoblots. Values represent fold-change from I/R, mean ± SEM. Significant differences between conditions were determined with an unpaired t test. Phenylephrine (Phe).
in decreasing cell death in ischemic PostC. One additional effector of this cardioprotective pathway is Akt (Hausenloy and Yellon 2007). Immunoblotting to detect changes in phospho-Akt was done as well (Figure 22). Treatment with Phe post-ischemically did not change levels of phospho-Akt in HL-1 CMs exposed to 2 hr ischemia and 2 hr reperfusion.

Next, we inhibited Protein kinase C (PKC), which has been shown to contribute to preconditioning and PostC of cardiac tissue (Lemoine et al. 2010), and has been specifically implicated in α₁-AR-mediated preconditioning (Tsuchida et al. 1994). Inhibition of PKC with a general PKC inhibitor, GF 109203X (GFX) inhibited cardioprotection at 1 µM but not 0.1 µM (Figure 23). Finally, U73122 was used to inhibit the action of Phospholipase C (PLC) which is known to be activated by α₁-AR signaling through the Gαq pathways. Post-ischemic addition of U73122 reversed the cardioprotective effects of Phe at 0.2 µM (Figure 24). Although not showing actual function, these assays reveal that there is possible involvement of PKC and PLC in α₁-AR-mediated post-ischemic protection in CMs.

To further investigate mechanism of protection, we also evaluated α₁-AR subtype expression in HL-1 CMs. Alpha₁-AR subtype specific pathways have been studied in HL-1 CMs using heterologous receptor expression (McWhinney et al. 2000) but have yet to be fully defined. Knowing subtype contributions to α₁-AR-mediated cardioprotection could aid in distinguishing differential effects of the α₁A and α₁B-AR subtypes. CMs of both rodents (O'Connell et al. 2003), and humans (Jensen et al. 2009) are known to express α₁A and α₁B-adrenergic receptor subtypes. We performed RT-PCR to detect
Figure 22. Post-ischemic phenylephrine does not change Akt phosphorylation. HL-1 cardiac myocytes exposed to 2 hr ischemia and 2 hr reperfusion (2I/2R). (A) Shows representative blots of phosphorylated and total Akt. (B) Densitometric values of immunoblots. Values represent fold-change from I/R, mean ± SEM. N≥3. Phenylephrine (Phe), Phentolamine (Pnt).
Figure 23. Effect of PKC inhibition. PI fluorescence measured in HL-1 cardiac myocytes after 2 hr ischemia and 6 hr reperfusion with pharmacological treatments applied post-ischemically. GF 109203X (GFX) was used to inhibit activity of PKC with Phe treatment. Values represent fold-change from 2I/6R (data not shown), mean ± SEM. P≤0.05# vs Phe. N≥4. Significant differences between conditions were determined with one-way ANOVA followed by Tukey’s post test. Phenylephrine (Phe).
Figure 24. **Effect of PLC inhibition.** PI fluorescence measured in HL-1 cardiac myocytes after 2 hr ischemia and 6 hr reperfusion with pharmacological treatments applied post-ischemically. U73122, a PLC inhibitor, was used to evaluate the effects of Phospholipase C (PLC) with and without Phe. Values represent fold-change from 2I/6R (data not shown), mean ± SEM. P≤0.05# vs Phe. N≥4. Significant differences between conditions were determined with one-way ANOVA followed by Tukey’s post test. Phenylephrine (Phe).
expression of α₁-AR subtypes in HL-1 CMs. Surprisingly, we were only able to detect the α₁B subtype in HL-1 CMs (Figure 25). This result was confirmed with three separate sets of primer pairs to detect α₁A- and α₁B-ARs and one primer pair for the α₁D subtype. Primer sets 2 and 3 were both shown to amply a product of the correct size when using HL-1 genomic DNA as the template. Primer set 2 was designed to amplify all four current transcript variants of the α₁A-AR subtype available on NCBI. Also, primer set 3 was verified for specificity against α₁A and α₁B-AR subtype specific knock out mice. According to these results, a lack of expression found in HL-1 CMs of either the α1A or α1D-AR subtypes suggests that α1B-AR activation is sufficient to confer protection in the HL-1 CMs exposed to ischemia and reperfusion.
Figure 25. Alpha_1-adrenergic receptor subtypes in HL-1 cells. Representative gel of PCR using primers to detect subtypes of α1-ARs in HL-1 CM cDNA. GAPDH was used as a housekeeping gene. 100 base pair marker (M) shown in first lane of each section of gel. α_1A product is 135 nt, α_1B product is 150 nt, α_1D product is 144 nt, and GAPDH product is 432 nt.
CHAPTER FOUR

DISCUSSION

The data presented in this dissertation suggest that $\alpha_1$-ARs play a protective role in ischemia and reperfusion (I/R) injury. Furthermore, this protective effect on cardiac myocytes can be conferred post-ischemically, and through modulation of autophagy in the cardiac myocytes (CMs), both of which are novel findings. Pharmacological modulation of autophagy during our simulated I/R protocol reveals autophagy induction as a process involved in protection in CMs. Our data indicate that post-ischemic $\alpha_1$-AR stimulation increases autophagic flux and decreases necrosis and apoptosis.

Drugs targeting $\alpha_1$-ARs are used clinically; $\alpha_1$-AR antagonists are used in treatment of benign prostatic hyperplasia and agonists for nasal congestion. In addition, $\alpha_1$-AR antagonists have been tested in several clinical trials aimed at hypertension or heart failure. Interestingly, inhibition of $\alpha_1$-ARs with doxazosin increased adverse cardiac events (ALLHAT Collaborative Research Group 2000). This implies a protective role for $\alpha_1$-AR activation. At a molecular level, $\alpha_1$-ARs have been implicated in the stimulation of antiapoptotic mechanisms mediated through ERK1/2 activation (Zhu et al. 2000). There is currently limited data available examining post-ischemic stimulation of $\alpha_1$-ARs in CMs. Most information available about $\alpha_1$-ARs in I/R injury comes from multiple studies into mechanistic pathways involving pre-ischemic stimulation of $\alpha_1$-ARs resulting in cardioprotection. Alpha$_1$-AR stimulation in the heart has been shown to be
cardioprotective when applied prior to an ischemic episode (Tsuchida et al. 1994, Hu and Nattel 1995, Baghelai et al. 1999, Rorabaugh et al. 2005, Gao et al. 2007). The mechanisms involved in α₁-AR-mediated preconditioning include stimulation of PKC isoforms (Zhao et al. 2003), ERK1/2, and mKATP opening (Gao et al. 2007). Additionally, α₁-ARs have been shown to be involved in modulation of antiapoptotic molecules (Baghelai et al. 1999, Zhu et al. 2000). Our research, exploring cardioprotective effects of post-ischemic α₁-AR stimulation, presents consistent and meaningful data towards activation of these receptors at a different and more practical time of therapeutic intervention for I/R injury.

*Alpha₁-adrenergic receptor postconditioning decreases cardiac myocyte cell death*

In our experiments we saw a consistent decrease in cell death with the addition of Phe immediately following an ischemic episode at the onset of reperfusion. These experiments were conducted with two cell types, HL-1 CMs which are an adult atrial cell line, and primary ARVMs. When both HL-1 CMs and ARVMs were exposed to simulated I/R, post-ischemic stimulation of α₁-ARs with phenylephrine (Phe) decreased cell death measured with LDH release and PI fluorescence (Figures 10,11). This result was inhibited when α antagonist phentolamine (Pnt) or α₁ antagonist prazosin (Pz) was added with Phe implicating α₁-receptor involvement in this response.

We used multiple methods to investigate levels of apoptosis as both necrosis and apoptosis are triggered by I/R. These experiments were conducted because Phe has been shown to decrease apoptosis in CMs when added prior to ischemia but has not been well
studied when added after. Apoptosis was measured using TUNEL, Annexin V/PI staining, and DNA laddering. Park et al. (2009) reported that most apoptosis in MI is occurring in non-myocytes thus making it exceedingly important to study cell type specific events as opposed to whole tissue assessments. Our cell culture model allowed us to isolate the cardiac myocyte-specific effects of $\alpha_1$-AR post-ischemic activation. Apoptotic cell death in CMs was decreased when cells were treated post-ischemically with Phe (Figures 13, 14, and 15). This effect was shown in all three assays used and was quantified in the TUNEL and Annexin V/PI experiments. Thus, our data indicate that post-ischemic stimulation of HL-1 CMs decreases levels of apoptosis.

**Beta-adrenergic receptor involvement**

CMs express other members of the adrenergic receptor family; $\beta$-ARs are present at a higher density than $\alpha_1$-ARs and can be stimulated by $\alpha_1$-AR agonists at high concentrations (100 $\mu$M Phe) (Valks et al. 2002). There is some discrepancy over regulation of $\beta$-ARs in a hypoxic environment. Some reports have shown that hypoxia induces a decrease in density of $\beta$-ARs (Iwatsubo et al. 2003), while others did not see any change (Froldi et al. 1994). Alpha$_1$-AR density has been shown to increase on the plasma membrane with exposure to hypoxia (Froldi et al. 1994), in which case, $\alpha_1$-AR stimulation at reperfusion may be of importance.

In our system, treatment of HL-1 CMs with $\beta$-AR agonists for 6 hr did not produce any significant changes in cell death. (Figure 12A). Norepinephrine (NE), which stimulates both $\alpha$ and $\beta$-ARs, also did not cause a significant change compared to I/R in
HL-1 CMs and ARVMs. The lack of protection with NE suggests that stimulating both β-ARs and α₁-ARs blunts the protective response seen with stimulation of α₁-ARs alone. It has been shown that β-AR activation can inhibit α₁-AR activated PKC (Schafer et al. 2001), indicating that β-AR stimulation is capable of inhibiting the α₁-AR response. Alternatively, β-AR (β₁) stimulation can induce apoptosis which may override the α₁-AR-mediated protection of ARVMs at 24 hr of treatment. There is a chance that activation of both signaling systems simultaneously negated the effects of either one in adult CMs as similar results have been shown in whole rat hearts (Lochner et al. 1996).

**Alpha₁-adrenergic receptor-mediated autophagy**

Autophagy has been shown to be involved in the beneficial effects of ischemic postconditioning (PostC) (Wei et al. 2013). While it has not been tested in the short term, α₁-AR stimulation has been shown to increase autophagy with long-term treatment (Cao et al. 2011, Xu et al. 2014). With chronic Phe treatment increases in autophagy are present but have not been linked to changes in cell death. We hypothesized that α₁-AR stimulation post-ischemically would regulate autophagy. Utilizing techniques to measure autophagy, we found that post-ischemic Phe treatment caused increased LC3-II (Figure 17), and an increase in autophagic flux, specifically at an earlier time point of 2 hr of reperfusion (Figures 18 and 19). It is important to note that the beneficial effects in our experiments are achieved with acute low concentration Phe addition at the time of reperfusion and will not induce cardiac hypertrophy.
Whether autophagy creates a positive or negative outcome in ischemia and reperfusion is controversial. In our experiments, an increase in autophagic flux during the reperfusion period was associated with a decrease in CMs cell death. This is in agreement with publications from the Gottlieb lab in which they found that increased autophagic flux was protective during reperfusion (Hamacher-Brady et al. 2006), as well as the result that autophagy is indispensable to cardioprotection incurred by ischemic preconditioning (Huang et al. 2010) and ischemic postconditioning (Wei et al. 2013). Others have concluded that autophagy contributes to cell death based on experiments on neonatal rat ventricular myocytes (NRVMs) treated with autophagy inhibitor, 3-MA (Valentim et al. 2006). Many have speculated that this discrepancy exists because of cell sensitivity to excessive levels of autophagy. In this theory, autophagy is cardioprotective by recycling cellular material into metabolically useful substrates and through the removal of damaged organelles, however, autophagy becomes detrimental if autophagic degradation reaches excessive levels leading to cellular damage (Gustafsson and Gottlieb 2008, Sciarretta et al. 2011).

Signaling molecules in α₁-adrenergic receptor postconditioning

Cardioprotective molecules and pathways have been shown to be largely similar whether induced pre- or post-ischemia (Hausenloy and Yellon 2007). This led us to investigate signaling molecules already shown to be important to α₁-AR-mediated preconditioning including PKC and ERK1/2. We also confirmed signaling through Gαq by evaluation of PLC (Figure 24). We were able to reverse α₁-AR PostC protection in CMs by the inhibiting ERK1/2, PKC, and PLC (Figures 20,23, and 24). While this tells
us that there is a strong possibility that these molecules are involved in this response, additional data are needed for this to be conclusive. Immunoblots were done to evaluate ERK1/2 activation. Post-ischemic treatment with Phe did not result in an increase in ERK1/2 activation (Figure 21). Additionally, we measured activation of Akt using immunoblots detecting levels of phosphorylated or active Akt and total Akt (Figure 22). At 2 hr of reperfusion there was no difference in Akt activation between I/R and post-ischemic Phe treated cells. Additional experiments are needed to confirm the complete mechanism of post-ischemic Phe-mediated cardioprotection. While we did not detect changes in ERK1/2 or Akt activity in our system, phosphorylation events of these molecules has been shown to be transient. It is possible that the times surveyed for phosphorylation were not sufficient to detect ERK1/2 or Akt activation (Wang et al. 2001).

Alpha₁-adrenergic receptor subtype expression in HL-1 cardiac myocytes

We also evaluated HL-1 CMs for expression of α₁-AR subtypes in order to investigate subtype selective effects. Interestingly, RT-PCR experiments showed expression of α₁B-AR but not the α₁A-AR subtype which suggests that the observed cardioprotection was mediated by the α₁B-AR subtype. Indeed, heterologous expression studies of constitutively active α₁A- and α₁B-ARs in HL-1 CMs show that different signaling pathways are activated by each receptor subtype (McWhinney et al. 2000). This is interesting because, unlike in HL-1 CMs, both α₁A-AR and the α₁B-AR subtypes are expressed at an mRNA level in cardiac tissue of multiple species including human.
(Jensen et al. 2009), rat (Luther et al. 2001), and mouse (Cavalli et al. 1997). Additionally, both $\alpha_{1A}$- and the $\alpha_{1B}$-ARs are expressed in cultured neonatal and ARVMs, while rat cardiac fibroblasts do not express $\alpha_1$-ARs at all (Stewart et al. 1994). Although $\alpha_1$-ARs do not appear to contribute to PostC in HL-1 CMs, it remains to be studied whether they contribute to the $\alpha_1$-AR-mediated PostC in ARVMs.

Alpha$\alpha_1$-adrenergic receptor postconditioning: an emerging paradigm

During the writing of this dissertation Buchholz et al. published a study that investigated $\alpha_1$-AR PostC in whole rat hearts (Buchholz et al. 2014). This recently published data using Langendorff perfusion of rat hearts corroborates our data and showed that post-ischemic treatment with $\alpha_1$-AR agonists results in a decrease in infarct size. Additionally, the benefit relayed by ischemic PostC was blocked by $\alpha_1$-AR antagonist. Several signaling pathways were investigated in the study including Akt and GSK3$\beta$. With treatment of Langendorff perfused rat hearts with Phe at the onset of reperfusion they observed an increase in both Akt and GSK3$\beta$ phosphorylation implicating these molecules in Phe-mediated post-ischemic cardioprotection (Buchholz et al. 2014). Differences between their signaling results and ours may stem from a difference in experimental models. Their results were obtained using cytosolic and mitochondrial protein samples isolated from whole hearts containing multiple cell types in which Akt could be regulated. For example, $\alpha_1$-ARs are also expressed on vascular smooth muscle which may contribute to these findings. Our results represent Phe effects on CMs in which we did not find Akt phosphorylation. It thus remains to be seen whether these
effects are due to other cell types in the heart, rather than the cardiac myocyte. Indeed, evidence for other cellular involvement is supported by studies where Phe treatment does not lead to Akt activation in ARVMs (Wang et al. 2001, Wang et al. 2008), and adult mouse CMs (O'Connell et al. 2003).

Buchholz et al. (2014) conducted measurement of coronary perfusion pressure to find an optimal concentration of Phe without stimulating vasoconstriction of coronary arteries. It was found that a 50 nM concentration of Phe is capable of both decreasing infarct size in rat hearts while maintaining consistent coronary perfusion pressure (Buchholz et al. 2014). It is interesting that 10 nM concentration of Phe in our cell culture model showed no protective effect. Further, the concentration of Phe that we found to be cardioprotective was 10 µM (Figures 8, 10, and 11), while Buchholz et al. found that addition of 12.5 µM Phe following global ischemia was detrimental and actually increased infarct size (Buchholz et al. 2014). It is possible that α1-ARs present in smooth muscle of the coronary arteries induce vasoconstriction at the 12.5 µM concentration of Phe thus limiting myocardial blood flow. These studies support the importance of identifying subtype-selective effects in different cardiac cell types in order to maximize cardioprotection.

**HL-1 cardiac myocytes used for in vitro ischemia and reperfusion simulation**

HL-1 CMs developed by William Claycomb (Claycomb et al. 1998), are a mouse atrial cardiac myocyte cell line and are an ideal model to study I/R injury. In contrast to ARVMs, HL-1 CMs are able to be transfected using liposomal methods allowing
transfection of plasmid DNA for expression of fluorescent labeled protein. HL-1 CMs were appropriate for plate-based and cover slip based fluorescence assays because they grow in a confluent monolayer allowing for accurate measurement. Like primary ARVMs, HL-1 CMs express all ion channels necessary for contraction (Xia et al. 2004), they have myofibrillar structures, and express protein isoforms consistent with adult myocardium (Claycomb et al. 1998). Especially important to the experiments presented in this dissertation, HL-1 CMs have been shown to have functional hypoxia-inducible factor 1α (HIF1α) (Nguyen and Claycomb 2000) indicating molecular responses to hypoxia parallel to those seen in adult myocardium. Molecular components have also been confirmed that are important to this study including ERK1/2 signaling (McWhinney et al. 2000), PKC isoforms (Chaudary et al. 2002), and multiple GPCRs (White et al. 2003), including α1-ARs (McWhinney et al. 2000).

HL-1 CMs and ARVMs display metabolic differences that should be taken into account for ischemia experiments. While it is known that ATP production in ARVMs is accomplished by oxidative phosphorylation, HL-1 CMs have been shown to rely more heavily on glycolytic pathways instead (Monge et al. 2009). This lower dependence on oxidative phosphorylation may impart additional resistance against ischemia to HL-1 CMs. ARVMs. While optimizing durations of ischemia, we noticed that HL-1 CMs could withstand a longer ischemic insult than ARVMs (2 hr I vs. 30 min I). Therefore we found it appropriate to choose times that resulted in similar damage to allow relative comparison of I/R injury and PostC protection in both cell types.
Possible effects of post-ischemic $\alpha_1$-adrenergic receptor stimulation

Autophagic degradation of mitochondria: mitophagy

In such a complex system as a cardiac myocyte there could be multiple ways in which $\alpha_1$-AR stimulation could positively influence cellular dynamics to result in increased survival. It has been suggested that post-ischemic autophagy benefits the cell by decreasing the presence of damaged mitochondria. Cells exposed to I/R may accrue ongoing damage during reperfusion because of increased mitochondrial ROS production. By containing and degrading damaged mitochondria, an increase in autophagy could lessen the negative impact of ROS on cellular components. In this study we found that $\alpha_1$-AR activation led to increased autophagic induction. The cellular components being degraded have yet to be defined in our system. General autophagy in a cardiac myocyte could increase mitochondrial degradation; however, additional studies could be done to assess specific autophagic activity such as mitophagy. Mitophagy is a form of autophagy in which several molecules collaborate to allow specific degradation of mitochondria.

Mitochondrial dynamics

One way in which $\alpha_1$-AR stimulation could be beneficial is by changing mitochondrial network dynamics and in turn levels of mitophagy. Mitochondria have been shown to undergo fusion and fission in order to modulate differences in mitochondrial membrane potential or redox state (Beraud et al. 2009). This mitochondrial fusion and fission is needed to allow for a balance between mitochondrial degradation
and biogenesis, which has been shown to be vital in times of increased stress. Mitochondrial fission has been shown to be a prerequisite of mitochondrial degradation by autophagy or mitophagy in CMs (Twig et al. 2008). Mitochondrial division would result in the segregation of damaged components to damaged mitochondria, which can then be cleared by autophagy. Because we see an increase in autophagy it would be interesting to see if there is any regulation of mitochondrial fusion and fission dynamics by α1-ARs in our system. Cell experiments with long term phenylephrine treatment provide some information on this subject. Javadov et al. (2011) performed experiments in neonatal rat ventricular myocytes (NRVMs) treated with Phe (10 µM) for 24 hr and saw increased levels of mitochondrial fission proteins Fis1 and DRP1. In addition, measurements of a fusion protein, mitofusin 2, were significantly decreased changing the ratio of fission to fusion proteins to promote mitochondrial fission (Javadov et al. 2011). While these changes were the results of long term Phe treatment in which they may or may not have been beneficial, an increase in mitochondrial fission at the onset of reperfusion would be beneficial and could interplay with the increase in autophagy seen in our experiments. Therefore, cardioprotection by α1-AR stimulation may be induced in part by increased cardiac myocyte mitophagy that is secondary to the modulation of fusion and fission proteins in a way that favors mitochondrial fission.

**Clinical relevance of α1-adrenergic receptor stimulation**

Clinically, post-ischemic as opposed to pre-ischemic interventions are more practical and achievable in patients who experience an MI. Treatment options for MI are
the induction of reperfusion of the ischemic tissue achieved with either thrombolysis or percutaneous coronary intervention (PCI). Interventions, such as ischemic pre- or post-conditioning, done by using several inflations and deflations of an angioplasty balloon have raised concerns among clinicians. For example, multiple balloon inflations could increase damage during intervention, especially in regard to the endothelium that is in contact with the angioplasty balloon or the possibility of further damage by embolism. There has also been the argument of ischemic pre- or post-conditioning increasing time, whether during post-ischemic treatment, or interventions applied in conjunction with cardiac surgery (Kloner and Rezkalla 2006). Ischemic PostC has shown promising results in the human heart in several small scale studies (Laskey 2005, Staat et al. 2005), but results have not been proven significant in a larger trial (Limalanathan et al. 2014). Regardless, only the subset of patients undergoing PCI can benefit from ischemic PostC. Pharmacological PostC, on the other hand, can be used for all patients presenting with MI along with either method of reperfusion. Several clinical trials have been conducted to investigate pharmacological PostC, for example, using adenosine, but have failed to establish a consistent decrease in infarct size (Quintana et al. 2003, Ross et al. 2005). It is therefore important to continue to pursue viable agents and to define mechanisms underlying pharmacological PostC.

PostC with $\alpha_1$-AR targeted agonists is the long-term goal of the study presented here. While it might be feasible to postcondition the human heart with Phe, postconditioning with $\alpha_1$-AR stimulation might be improved with the development and use of subtype selective agonists. Subtype selective agonists would be useful because of
receptor subtype distribution differences between cardiac myocytes and coronary vasculature. The $\alpha_{1D}$ subtype is expressed in the smooth muscle of coronary vasculature (Jensen et al. 2009), while the myocardium expresses functional $\alpha_{1A}$ and $\alpha_{1B}$ only (Jensen et al. 2009). Activation of $\alpha_1$-ARs in smooth muscle cells induces vasoconstriction which in turn decreases blood flow to the myocardium. This necessitates receptor activation in the myocardium that is independent of coronary vessel constriction for cardioprotection to be possible. This obstacle might also be overcome by using a lower concentration of $\alpha_1$-AR agonist although this may change the level of cardioprotection. With further study of the subtype involved and selective stimulation of that subtype, the myocardium could be protected without eliciting unwanted vasoconstriction.

Importantly, Baumgart et al. (1999) measured coronary vasoconstriction after intracoronary infusion of $\alpha_1$-AR agonist (methoxamine) in both healthy patients and those with atherosclerosis. With $\alpha_1$-AR stimulation there was no change in vessel tone in healthy patients, however, there was significant vasoconstriction in those with atherosclerosis (Baumgart et al. 1999). This result presents a substantial caveat for general $\alpha_1$-AR stimulation in patients undergoing PCI and presents further questions about in vivo subtype specific effects. In addition, there have been multiple accounts of single receptor subtype cardioprotection involving either the $\alpha_{1A}$ (Rorabaugh et al. 2005, Huang et al. 2007) or $\alpha_{1B}$ receptors (Anyukhovsky and Rosen 1991, Tsuchida et al. 1994, Gao et al. 2007, Gao et al. 2014) in animal models. Data presented herein suggest that $\alpha_1$-AR stimulation could protect the reperfusion-exposed cardiac myocyte from cell death. Limited clinical studies in humans discussed above indicate that non-specific $\alpha_1$-AR
stimulation in myocardium could be detrimental, which precipitates the need for further development of subtype selective ligands to possibly expand upon this cardioprotection. Currently there is only one subtype selective agonist which targets the $\alpha_{1A}$ subtype (A61603) which displays at least 35 times the potency at the $\alpha_{1A}$ subtype over the $\alpha_{1B}$ or $\alpha_{1D}$. The development of an $\alpha_{1B}$ selective agonist would be helpful in advancing the study and viability of post-ischemic stimulation of $\alpha_1$-ARs to induce cardioprotection.

**Conclusion**

Described in this dissertation was the examination of the hypothesis that stimulation of $\alpha_1$-ARs post-ischemically provides protection against CM cell death. The aims approached were 1.) to examine effects of post-ischemic stimulation of $\alpha_1$-ARs on cardiac myocyte cell death, 2.) to determine whether post-ischemic $\alpha_1$-AR stimulation inhibits cardiac myocyte death through modulation of autophagy, and, (3) to examine the molecular pathway(s) stimulated by $\alpha_1$-ARs that lead to decreased cell death.

Overall, stimulation of $\alpha_1$-ARs at the time of reperfusion was found to be cardioprotective against general cell death and apoptotic cell death in both HL-1 CMs and ARVMs. This protection was found to be associated with an increase in autophagic flux early in the reperfusion process (2 hr) measured using LC3-II turnover and tandem fluorescent-tagged LC3. Pharmacological agents used to inhibit signaling of ERK1/2, PKC, and PLC were able to reverse the beneficial effect of Phe-mediated protection from cell death. Verification of involved molecules using immunoblotting failed to reveal activation of either ERK1/2 or Akt in Phe treated HL-1 CMs. This result is possibly due
to the transient nature of ERK1/2 and Akt phosphorylation usually seen in response to $\alpha_1$-AR stimulation. Finally, RT-PCR data indicate that HL-1 CMs express $\alpha_{1B}$ but not $\alpha_{1A}$ mRNA indicating that $\alpha_{1B}$-AR activation alone is sufficient for protection in this cell type. Additional data are needed to completely elucidate mechanistic effects of $\alpha_1$-AR stimulation and its means of cardioprotection.

**Future Directions**

Additional experiments are required to fully elucidate the mechanism of Phe-mediated cardioprotection including functional data evaluating involvement of the studied signaling molecules (ERK1/2, PKC, and PLC). It is important to know whether or not there are connections between protective signaling and increased autophagic induction. There could be several different pathways stimulated by $\alpha_1$-ARs that may or may not be related to increased levels of autophagy or decreased levels of apoptosis. Because of cardiac myocyte signaling complexity, it is likely that other factors are involved in addition to modulation of autophagy.

Further, investigations into whether $\alpha_1$-ARs increase levels of mitophagy through regulation of mitochondrial fusion and fission proteins. Autophagic signaling pathways are still being defined, however, there is a possibility that $\alpha_1$-AR stimulation indirectly induces an increase in mitophagy by increasing mitochondrial fission.

The use of pharmacological agents and $\alpha_{1A}$-AR and $\alpha_{1B}$-AR subtype specific knock out mice would be useful in discriminating subtype specific effects. Isolation of cardiac myocytes from $\alpha_{1A}$-AR and $\alpha_{1B}$-AR knock out mice would allow targeted
investigation into signaling and function of α₁-ARs in cardiac myocytes as opposed to whole tissue. In addition, using a protocol for global ischemia, there could be assessment of the effectiveness of α₁-AR-mediated postconditioning in a whole heart. In these experiments there would be discrimination between the effects of the α₁A and α₁B-AR subtypes not only in pharmacological postconditioning, but also their involvement in ischemic postconditioning (as was implicated by Buchholz et. al, 2014). Additionally, utilization of an in vivo model of myocardial infarction would allow continued measurements of cardiac function using echocardiography. By assessing the initial infarct, as well as the subsequent damage and remodeling, a better prediction of clinical outcomes of α₁-AR postconditioning would be possible.
REFERENCES


