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New roles for alpha B-crystallin in protecting the myocardium from ischemia/reperfusion injury

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SAN DIEGO STATE UNIVERSITY

New Roles for Alpha B-Crystallin in Protecting the Myocardium

from Ischemia/Reperfusion Injury

A dissertation submitted in partial satisfaction of the

requirements for the degree Doctor of Philosophy

in

Biology

by

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2008

DEDICATION

This dissertation is dedicated to my family and all of my friends; their love and

support have made this work possible.

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LIST OF ABBREVIATIONS

ADH	Alcohol dehydrogenase
ANT	Adenine nucleotide transporter
ASK 1	Apoptosis regulating kinase 1
ATF6	Activating transcription factor 6
BAD	Bcl-2 associated death promoter
BAX	Bcl-2 associated protein X
Bid	BH3 interacting domain death agonist
CPP	Cell penetrating peptide
CVD	Cardiovascular disease
EK	Enterokinase
ER	Endoplasmic reticulum
ERK	Extracellular signal regulated kinase
G6PD	Glucose-6-phosphate dehydrogenase
GPX	Glutathione Peroxidase
GR	Glutathione reductase
GRP	Glucose response protein
GS	Glutathione synthase
GSH	Reduced form of glutathione
GSSG	Glutathione disulfide, oxidized glutathione
HSP	Heat shock protein
I/R	Ischemia-reperfusion

JNK	c-Jun n-termial kinase
LAD	Left anterior descending coronary artery
LVDP	Left ventricle developed pressure
MAPK	Mitogen activated protein kinase
MAPKAPK2	MAPK activated protein kinase 2
Mcl-1	myeloid cell leukemia sequence 1
MI	Myocardial Infarction
MKK	Mitogen activated protein kinase kinase
MKKK	Mitogen activated protein kinase kinase kinase
MLK	mixed lineage kinase
MOM	Mitochondria outer membrane
MPTP	Mitochondrial permeability transiiton pore
MTK1	AKA: MKK4
MudPIT	Multi-dimensional protien identification techonology
NADP	Nicotinamide adenine dinucleotide phosphate
NADPH	Nicotinamide adenine dinucleotide phosphate, reduced
NRVCM's	Neonatal rat ventricular cardiomyocytes
OxPhos	Oxidative phosphorylation
PDK1	Phosphoinositide dependent kinase
PI3K	Phosphoinositol-3 kinase
PKC	Protein kinase C
PRX	Peroxiredoxin

PUMA	p53 upregulated modulator of apoptosis
RIPA	Radio immuno precipitation assay
ROS	Reactive oxygen species
SERCA	Sarco/Endo plasmic reticulum ATPase
SOD	Superoxide dismutase
SR	sarcoplasmic reticulum
TAK1	Transforming growth factor β activated kinase 1
TNFα	Tumor necrosis factor alpha
TR	Thioredoxin reductase
VDAC	Voltage dependent anion channel
αBC	Alpha B-Crystallin
γGCS	Gamma glutamyl -cysteine synthase

LIST OF FIGURES

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Jin JK, **Whittaker R**, Glassy M, Gottleib RA, Barlow SB, and Glembotski C.C., *Localization of phosphorylated alphaB-crystallin to heart mitochondria during ischemia-reperfusion.* AJP-Heart and Circulatory Physiology. 2008 Jan:294(1):H337-44

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ABSTRACT OF THE DISSERTATION

New Roles for Alpha B-Crystallin in Protecting the Myocardium

from Ischemia/Reperfusion Injury

by

Ross J. Whittaker

Doctor of Philosophy in Biology

University of California, San Diego 2008

San Diego State University 2008

Professor Chris Glembotski, Chair

Expression of αBC in the heart has been implicated in providing

protection from acute stresses, such as ischemia and reperfusion, by binding

to specific target proteins.   This dissertation explores two new mechanisms by

which αBC can protect the heart from I/R. First, the role of αBC in maintaining

mitochondrial integrity and function during I/R is examined. Previous work has

demonstrated that αBC associates with and protects mitochondria during I/R. This work expands upon these studies by examining the kinetics of αBC translocation and phosphorylation of αBC, an event that is required for αBC to provide maximal protection against stress. This study shows that, in response to ischemia, αBC reaches maximal accumulation at the mitochondria following 20 minutes of ischemia. Furthermore, there is an accumulation of phospho-αBC-S59, the most protective form of αBC, at the mitochondria. This study goes further to demonstrate that the presence of a phospho-αBC-S59 mimic, at the mitochondria, provides protection against H_2O_2-induced cytochrome c release. Moreover, this study demonstrates that αBC physically interacts with VDAC, a possible mechanism by which αBC prevents cytochrome c release.

The second study explores a role for αBC in regulating the ceullar redox environment. Hearts from mice harboring deletions of both αBC and HSPB2 were found to have significantly lower levels of glutathione compared to controls. Consistent with this finding, glutathione reductase (GR) activity was significantly impaired in the knockout mice. HeLa cells were used as a model to further examine a role for αBC in glutathione recycling since, like the knockout mice, they lack both αBC and HSPB2. Expression of αBC in HeLa cells was found to enhance glutathione levels during H_2O_2 treatment, enhance GR activity, and prevent apoptosis. Moreover, αBC was shown to directly interact with GR and enhance its activity.

The third study of this dissertation examines the use of protein transduction domains to facilitate the delivery of αBC proteins across cell membranes. This study introduces a novel system that could be used as a research or a therapeutic platform for delivering proteins, nucleic acids or small molecules.

Chapter One

1. Introduction

Cardiovascular disease (CVD) continues to be the leading cause of death among Americans. Of the various conditions which fall under the umbrella of cardiovascular disease, myocardial infarction (MI) is directly responsible for a significant number of the deaths attributed to CVD and is a contributing factor in mortality attributed to other forms of CVD[1]. With 1.2 million new or recurrent cases of MI reported every year, more than a third of which result in death, improving on current treatments and developing new, more effective treatments for MI are top priorities for cardiovascular researchers. One of the keys to creating new and better treatments is to understand the mechanisms employed by the heart to protect itself during stress, such as MI.

A. Ischemia/Reperfusion Injury of the Myocardium

MI occurs when there is a blockage of the coronary arteries. During an MI, tissue immediately downstream of the blockage is deprived of oxygen and nutrients and there is a decrease in the clearance of CO_2. In this state the tissue is described as being ischemic. The myocytes within this ischemic region of the heart face an intertwined series of events that challenge their abilities to survive and contribute to the contractile action of the heart **(Figure 1)**.
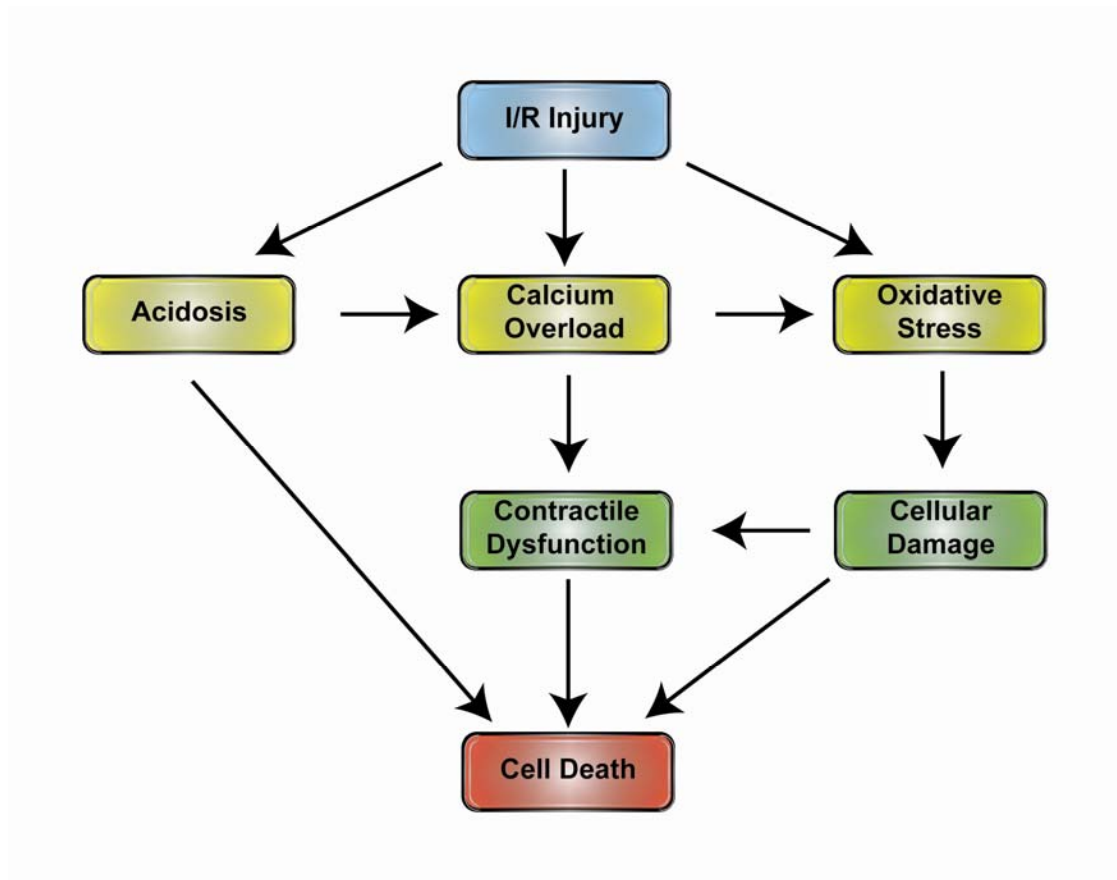
Figure 1- Schematic of the events leading to I/R-induced cell death
I/R results in a complex series of events that can lead to cellular damage, and ultimately cell death, if the myocyte is unable to mount an appropriate prosurvival stress response.

Ischemia results in the collapse of oxidative phosphorylation (OxPhos)[2]. In response, the myocyte can, for short periods of time, rely on anaerobic glycolysis to provide small quantities of ATP. However, anaerobic glycolysis does not generate enough ATP to maintain normal cardiomyocyte function. As a result, ATP is rapidly depleted following the onset of ischemia[3]. A consequence of resorting to glycolysis is the accumulation of lactic acid. The lack of blood flow to the area also results in an accumulation of CO_2 from the waning OxPhos activity. This contributes to a decrease in the pH of the myocyte, a condition known as acidosis[4]. A more acidic environment, by itself, may prime the cell for apoptosis through activation of proapoptotic Bcl family members, such as Bnip3, and activation of caspase 3 via cytochrome c release from the mitochondria[5]. However, acidosis is also a contributing factor to the misregulation and accumulation of calcium in the ischemic myocyte.

The calcium concentration of the intracellular environment, and between the different organelles within the myocyte, is controlled by a number of channels, pumps, and transporters that can all be affected by ischemia. As protons accumulate during ischemia (acidosis), the H^+/Na^+ antiporter drives an accumulation of intracellular Na^+ which, in turn causes the Na^+/Ca^+ antiporter to pump calcium into the cell, resulting in increases in cytosolic calcium[6, 7]. The depletion of ATP following the collapse of OxPhos further contributes to the increase in intracellular calcium concentration. The sarcolemmal calcium

ATPase pumps calcium across the plasma membrane and out of the cell. The

sarco/endoplasmic reticulum ATPases (SERCA's) regulate calcium within the

sarcoplasmic and endoplasmic reticulum (SR and ER, respectively), and their

activities are vital to the cycle of calcium induced calcium release that

regulates contraction. The failure of the cell to provide enough ATP during

ischemia to power these pumps exacerbates the accumulation of calcium

during ischemia[3]. The result of this unregulated increase in intracellular

calcium is commonly referred to as calcium overload.

Calcium overload is a significant contributing factor to myocyte death

during an MI[8]. Calcium overload has direct effects on mitochondria where it

can cause swelling of the organelle and mitochondrial permeability transition

pore opening (MPTP)[9]. This can result in release of cytochrome c and

activation of the apoptosome resulting in cleavage and activation of caspases.

Increased cytosolic calcium can also activate calpains, a group of cysteine

proteases[10]. Active calpains can cleave and activate proapoptotic proteins,

such as Bax and Bid[11]. Calpains have also been found to directly target

structural and cell cycle proteins and may work in conjunction with effector

caspases during apoptosis.

Regulation of calcium is important to all cells, but the regulation of

calcium in myocytes is even more important, since myocytes rely on strictly

controlled calcium levels to regulate contraction, as well as intracellular

signaling pathways[12]. Contraction is a calcium regulated event that relies on

the binding and cleavage of ATP by the myosin head to provide the energy necessary to complete a contraction event. During ischemia, the combination of low levels of ATP and high levels of calcium results in an unregulated, sustained contraction of the sarcomeres referred to as contracture[8]. During contracture the intense, sustained forces result in damage and unfolding of the sarcomeric proteins. Stretch sensors associated with the sarcomeres, such as muscle LIM protein, can activate apoptosis in response to this type of damage. For myocytes that do not enter apoptosis, this damage can hinder their ability to create contractile force, which can severely reduce the heart's ability to pump blood.

Hopefully, an MI patient survives the initial blockage of the arteries and has blood flow restored to the ischemic tissue through angioplasty, bypass, or thrombolytic drugs[13]. However, they are now faced with the so-called "Oxygen Paradox" [14]. The return of blood flow brings much needed oxygen and nutrients to the previously ischemic tissue, but at the same time results in a burst of damaging reactive oxygen species (ROS) during the first few minutes of reperfusion.

This burst of reactive oxygen species can be partially attributed to calcium overload of the mitochondria during ischemia. The elevated calcium levels experienced during ischemia inhibit dephosphorylation and subsequent inactivation of NADH dehydrogenase in the mitochondria (Complex I) while also inhibiting ATP inhibition of cytochrome c oxidase (Complex IV)[15]. As a

result, when OxPhos is restarted following reperfusion, the respiratory chain is primed to operate at a very high (Complex I), but not very efficient (Complex IV) level, the result is a rapid rise in mitochondrial membrane potential that leads to increased ROS production.

Superoxide anion is the primary ROS species that is generated during reperfusion, and it is quickly converted to H_2O_2 by superoxide dismutases (SOD's)[16]. Hydrogen peroxide can, in turn, react with ferric ion via the Fenton reaction, to generate hydroxyl radicals, strong oxidants with the potential to cause significant cellular damage[3]. Reactive oxygen species have the potential to damage every major macromolecule in the cell [17]. They create DNA adducts and strand breaks which hinder replication and transcription and may lead to mutation. These oxidants result in lipid peroxidation, which can alter the fluidity and function of membranes. They also attack peptide bonds and reactive amino acid side chains of proteins, altering or even destroying protein structure and function. These damaging effects can lead to cellular dysfunction and, ultimately, to cell death through either necrotic or apoptotic pathways[9, 18].

B. The Cardiomyocyte Response to I/R Injury

Cardiomyocytes subjected to I/R will either die by necrosis or apoptosis or they will survive and recover. In order to survive I/R the myocytes must mount an extremely complex stress response in an attempt to restore homeostasis.

1. Antioxidants

The heart requires large amounts of ATP to continuously circulate blood throughout the body. As such, cardiomyocytes are packed with mitochondria; but, being a cell type with such a high metabolic rate means cardiomyocytes must constantly manage ROS. Even when the myocyte is not, stressed up to 2-5% of oxygen consumed by OxPhos results in ROS generation[15, 19]. However, when the cells are stressed during I/R, the rate of ROS release can increase 7.5 fold[20]. In order to prevent ROS-induced cell death under both basal and stressed conditions it is imperative that the cardiomyocyte possess a robust network of antioxidants [21] (**Figure 2)**.

a. Superoxide dismutase

Superoxide anion is the primary source of ROS generated from the mitochondria under both basal metabolic conditions and during, stresses such as I/R[15]. When compared to other ROS species, superoxide anion, by itself, is relatively slow to react with other cellular components. However, it still needs to be metabolized rapidly in order to prevent damage and subsequent generation of other ROS species. This is accomplished by a group of enzymes that convert the superoxide anion to hydrogen peroxide as the first step in detoxifying ROS.[17] There are two classes of superoxide dismutases (SOD) that are defined by their associated metals and are segregated into different cellular compartments. Copper/Zinc superoxide dismutase (SOD1)
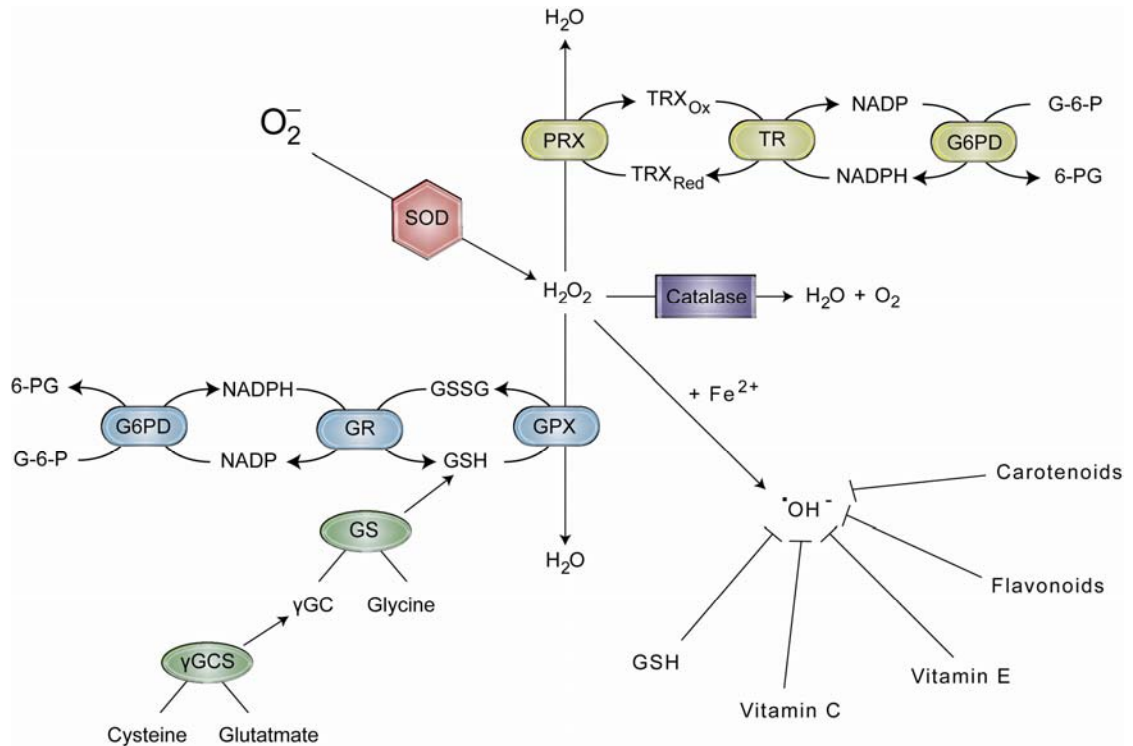
Figure 2- The major antioxidant systems of the heart
 The heart detoxifies ROS by utilizing a robust network of antioxidants. Glutathione is considered the first line of defense from ROS-related damage and exists as a redox couple between its reduced and oxidized forms. The redox potential of the glutathione couple is primarily maintained by the glutathione recycling enzymes glucose-6-phosphate dehydrogenase (G6PD) and glutathione reductase (GR). Thioredoxin (TRX) is utilized in a similar manner to glutathione and is also recycled in a similar manner by thioredoxin reductase (TR). Catalase can directly convert H_2O_2 to H_2O and O_2 by way of a very elegant detoxifying reaction. Catalase has a very high specific activity, however, it is found in low quantities in the heart. The ROS scavengers, such as vitamins C and E and the carotenoids and flavonoids are the last line of defense against ROS-induced damage. γ-glutamyl cysteine synthase (γGCS), glutathione synthase (GS), glutathione peroxidase (GPX), superoxide dismutase (SOD), peroxiredoxin (PRX)

exists in the cytoplasm, while manganese superoxide dismutase (SOD2) is found exclusively in the mitochondria. Overexpression by transgenisis or viral transfer of either form of SOD has been found to provide protection against I/R injury in the heart[22, 23].

b. Glutathione and associated antioxidant enzymes

Glutathione is considered the first line of defense against ROS damage[24]. Alterations in glutathione levels cause significant changes in the hearts ability to respond to I/R. The glutathione synthesis inhibitor, buthionine sulphoxamine, has been used to deplete the glutathione pool of hearts prior to I/R challenge, and results in reduced functional recovery when compared to untreated hearts[25, 26]. Depletion of glutathione has also been shown to trigger loss of mitochondrial membrane potential and mitochondrial membrane potential fluctuations, as well as ROS release from mitochondria in guinea pig hearts even without I/R challenge[19]. In contrast, oral supplementation of GSH in exercise-trained rats has been found to protect hearts against I/R[27].

Glutathione is synthesized from cysteine, glutamate, and glycine in a two step process by the enzymes γ-glutamylcysteine-synthetase (rate limiting) and glutathione synthetase[28]. Glutathione is ubiquitous within tissues and is also found circulating in the blood and in intercellular spaces. In the heart glutathione exists in concentrations of up to 8mM [29]. This places the heart

toward the upper end of tissue glutathione concentrations which are generally found at a concentration of 1-10 mM [30].

Glutathione exists in a reduced form, GSH, and its oxidized form, GSSG. Under basal conditions, 95% of cellular glutathione exists in the reduced form, resulting in a high ratio of GSH:GSSG [31]. During stress, such as I/R, when increased amounts of ROS are being generated, the ratio of GSH:GSSG falls as a result of glutathione being utilized to detoxify oxidants, as the cell tries to prevent cellular damage from occurring. Glutathione is primarily utilized to detoxify the hydrogen peroxide that results from the dismutation of the superoxide radical that is generated by the mitochondria during I/R. This reaction is catalyzed by glutathione peroxidase, or peroxiredoxin and results in hydrogen peroxide being reduced to water and GSSG [28, 29].

When glutathione is oxidized, either through direct interaction with an oxidizer or through its participation in an enzyme regulated redox reaction, GSSG is formed. At high concentrations, GSSG becomes toxic, as it can form mixed protein disulfide through disulfide exchange. These mixed protein disulfides can then react with themselves to form protein-protein thiols which results in loss of protein function and a decrease in protein stability that can lead to aggregation [32].

There are two routes through which GSSG can be eliminated. The first is by exporting GSSG out of the cell by way of the multi drug resistance

associated protein (MRP1) [33]. However, this results in a reduction of the glutathione pool and a diminished capacity to detoxify ROS[25, 26]. The second route is by recycling GSSG back to GSH [34]. In order to recycle GSSG, the cell relies on NADPH produced by glucose-6-phosphate dehydrogenase (G6PD). NADPH is utilized by glutathione reductase (GR) to catalyze the reduction of GSSG resulting in the reduced form of glutathione, GSH, and $NADP^+$.

Alterations in the levels and activities of the glutathione associated antioxidant enzymes have been shown to alter the abilities of cells to respond to oxidative stress. For example, inhibiting the activity of GR in cardiomyocytes results in a 50% decrease in intracellular glutathione [28]. Similarly, mice harboring a mutation in the 5' untranslated region of G6PD, which causes an 80% reduction in G6PD activity, exhibit significantly lower glutathione levels compared to wildtype controls[35]. Consistent with this reduction in glutathione levels, the same study showed hearts from these G6PD deficient mice are more susceptible to I/R injury. On the other hand, overexpression of G6PD has been shown to be effective in preventing glutathione depletion during oxidative stress [36]. Mice with deficiencies in SOD and GPX exhibited an increased sensitivity to ROS when compared to control animals, which results in premature death when animals are challenged with paraquat[37]. In fact, SOD knockout has been demonstrated to be lethal, with animals dying shortly after birth [38]. In contrast, transgenic

mice that overexpress either GPX or SOD have been found to be protected from I/R injury [39, 40]. Furthermore, administration of ebselen, a glutathione peroxdase mimic, reduced infarct size in a canine model of I/R [41]. Injection of adenoviral constructs expressing SOD, following coronary artery occlusion in rat hearts, resulted in a 50% reduction in infarct size [23].

c. Other enzymatic antioxidants

There also exists a set of enzyme antioxidants that do not rely on glutathione to detoxify ROS; catalase is one such enzyme. The catalase monomer is roughly 500 amino acids and contains a heme group. The assembled catalase enzyme is a tetramer with an Fe^{3+} center that detoxifies 2 molecules of H_2O_2 at a time to produce 2 molecules of H_2O and one molecule of O_2 [42]. The reaction involves two steps, whereby the first molecule of H_2O_2 enters the active site and one oxygen is transferred to the iron center and H_2O is released. When a second molecule of H_2O_2 enters the active site it reacts with the oxygen in the Fe^{3+} core resulting in the release of H_2O and O_2. Catalase not only performs a very elegant reaction to detoxify hydrogen peroxide, but also performs this reaction at an extremely high rate [43]. In fact, catalase may have the highest catalytic turnover of any enzyme and is believed to operate near the limit of enzymatic efficiency. However, the contribution of catalase to detoxifying H_2O_2 has been shown to be much lower than that of glutathione peroxidase [29]. However, hearts from transgenic mice

that overexpress 60-fold more catalase than wild type animals were found to be protected from I/R, due to an increased ability to detoxify ROS [44].

Thioredoxin is an encoded small protein that acts as an antioxidant and an important signaling molecule [45]. Thioredoxin is a ubiquitously expressed, 12 kilodalton protein with a redox reactive core containing two cysteine residues, that when oxidized, form a disulfide bridge. Utilizing NADPH as an electron donor, oxidized thioredoxin is recycled back to the reduced form by thioredoxin reductase. *In vivo* models have demonstrated that thioredoxin can protect the heart against I/R injury, even by simply administrating an intraperotoneal injection of thioredoxin prior to coronary artery ligature[46-50]. Thioredoxin also acts as a signaling molecule and may be used as a sensor of redox status. In its reduced form, thioredoxin binds to and inhibits apoptosis regulating kinase 1 (ASK1) [51]. Upon oxidation of thioredoxin, ASK1 is released, resulting in the activation of downstream kinases such as p38 and JNK. This regulation of signaling molecules may be an important contributing factor in myocardial hypertrophy and the progression of heart failure [52].

d. Non-enzymatic antioxidants

Non-enzymatic antioxidants are generally ROS scavengers derived from nutritional sources and include vitamin E and vitamin C, flavonoids and carotenoids [16]. Vitamin E is the major lipid soluble antioxidant, and is believed to play a role in protecting membranes from ROS induced damage.

Vitamin C is a small soluble antioxidant that acts both as a ROS scavenger and a reducing agent for oxidized vitamin E. Studies in mice and rats have demonstrated that manipulating levels of vitamin E and/or vitamin C through supplementation can result in a significant reduction in I/R associated damage in both *in vivo* and *ex vivo* models [53-55]. However, human studies using large groups of participants have found mixed results regarding the protective effects of antioxidant supplementation [56, 57].

2. Signaling pathways activated by I/R injury

Following an I/R injury, myocytes progress through three temporally separate, but related phases, which have distinct signal transduction profiles [58]. The first phase occurs minutes to hours following the onset of I/R and is concerned with survival of the myocyte. The second phase, occuring hours to days later, concerns recovery of the myocyte from injury. The third phase, which takes place over days, months and even years following I/R, concerns the myocytes role in the remodeling of the myocardium in response to injury.

The scope and coordination of the myocyte response to I/R will probably not be truly understood until the promise of systems biology is fulfilled. Nonetheless, significant progress has been made in understanding the pathways utilized by these specialized cells to survive I/R and help maintain organ function. The activation or deactivation of signaling pathways in response to I/R results in up and downregulation of gene expression,

reorganization of signaling complexes and changes in their activity, alterations in contraction, activation of both cell death and survival signaling, metabolic changes, and changes in communication with surrounding cells, just to name a few. We probably only understand a few of the possible outcomes for activating or deactivating any given pathway and even less about the extent of cross talk between the different signaling pathways.

For the purposes of this dissertation, I will touch briefly upon a couple of the major signaling pathways involved in survival signaling during the acute response to I/R. I will then elaborate further on the mitogen activated protein kinase family as this group of proteins is directly related to the subject of this dissertation.

a. PI3K/AKT signaling pathway

The PI3K/AKT signaling pathway is one of the most well studied signal transduction pathways in the heart, and plays a role in regulating growth, survival, and metabolism. I/R activates PI3K which results an increase of phosphoinositol 3,4,5 triphosphate; this, in turn, results in the recruitment of AKT to the plasma membrane [59]. At the plasma membrane AKT is activated via dual phosphorylation by PDK1 on threonine 308, followed by a second phosphorylation on serine 473, by a currently unknown kinase. Once activated, AKT translocates away from the membrane into the cytosol and nucleus, where it phosphorylates target proteins.

Activation of AKT through extracellular signaling molecules such as IGF-1, or drugs such as vanadate and isoflourane, is associated with protection of myocytes against IR damage[60-62] [63, 64]. Constitutive activation of AKT in the hearts of transgenic mouse models through the addition of a myristoyl moiety, which targets AKT to the plasma membrane, has been shown to reduce infarct size and inhibit apoptosis in hearts exposed to I/R[65].

AKT acts on a number of targets both in the cytosol and the nucleus to provide protection against I/R [59]. AKT inhibits apoptosis through phosphorylation of the proapoptotic Bcl-2 family member, BAD, resulting in its sequestration in the cytosol. Phosphorylation of pro-caspase 9 by AKT blocks its activation and prevents apoptosis. AKT also results in the activation of prosurvival transcription factor nuclear factor kappa B (NFκB) and stabilization of the antiapoptotic transcription factor β-catenin. AKT is also involved in cross talk with a number of other signaling pathways that are activated during I/R.

b. Protein kinase C

There are four isoforms of protein kinase C (PKC) expressed in the heart (α,β,δ,ε) [66]. PKC activation occurs in a two step process requiring phosphorylation within the activation loop and subsequent interaction with the second messengers Ca^{2+} and diacylglycerol. Upon activation the different isozymes are targeted to unique subcellular locations by interactions with

RACK (receptors for activated C-kinase) proteins [67]. Synthetic peptides that mimic the interaction sites of the RACK proteins can be used to activate PKC. Conversely, synthetic peptides that mimic the RACK interaction domain of PKC can be used to block PKC activation. These strategies have been used extensively to elucidate the different roles of PKCδ and PKCε.

Activation of PKCδ and PKCε seem to play opposing roles during I/R [68, 69]. Following activation, PKCδ translocates to the mitochondria and increases apoptosis. Inhibition of PKCδ results in significant reduction in cellular damage following I/R, and drastically reduces infarct size [70, 71]. Conversely, activation of PKCε by perfusion of synthetic peptides that mimic the PKC binding site of the RACK proteins results in a significant decrease in infarct size and cellular damage due to I/R [72-75].

c. Mitogen activated protein kinases

The mitogen activated protein kinases (MAPK's) are a group of protein kinases that are activated during stress. The three MAPK signaling pathways are named for the end effector kinase which they activate. The MAPK signaling pathways have been implicated in a number of different cellular processes including growth, development, inflammation and survival [76-78]. As such their activation during I/R and its consequences for the heart have been, and continue to be, an area of intense interest among cardiovascular researchers **(Figure 3)**.

i. ERK

Extracellular signal regulated kinase (ERK) is classically activated by extracellular mitogens, such as growth factors and hormones [77]. ERK is activated via the ras-raf-MKK1/2 signaling pathway culminating in dual phosphorylation of theronine 202 and tyrosine 204 of ERK1 or threonine 185 and tyrosine 187 of ERK2. ERK has not been found to be activated by ischemia alone, but it has been found to be activated by I/R[79]. The upstream activator of ERK, MKK2, was also found to be activated by I/R. Further studies by Li et al. demonstrated that nitric oxide (NO) produced during reperfusion may be responsible for ERK activation [80]. ERK has also been shown to be activated in an oxidative stress dependent manner by the PKC-mediated activation of RAF [81].

Multiple studies have demonstrated that ERK plays a protective role in the heart during I/R. Perfusing hearts with the NO donor SNAP demonstrated that NO mediated activation of ERK resulted in increased functional recovery in an *ex vivo* model of I/R[80]. Inhibition of ERK activation via the MEK inhibitor PD98059 results in increased apoptosis in cardiomyocytes exposed to hypoxia followed by reoxygenation, an *in vitro* mimic of I/R injury [82]. In the same study, PD98059 treatment also resulted in reduced functional recovery of hearts subjected to *ex vivo* I/R. In contrast, activation of ERK1/2 in the heart by a number of factors either prior to or during I/R have been shown to

provide protection resulting in reduced apoptosis in cell culture models and

reduced cell death and improved functional recovery in both *ex vivo* and *in*

vivo models of I/R [79].

ERK can directly inhibit caspase 3 activation, however; ERK facilitates

protection from I/R at a higher level by inhibiting the activity of a number of the

pro-apoptotic bcl-2 family members [77, 81]. For example, ERK

phosphorylates BAD and BAX, allowing 14-3-3 to sequester these

proapoptotic proteins in the cytosol, thus preventing them from translocating to

the mitochondria and causing cytochrome c release. ERK activation also

down regulates expression of PUMA, another proapoptotic Bcl-2 family

member [83]. Deletion of PUMA has been demonstrated to attenutate

cardiomyocyte death due to I/R [84]. ERK acitvtion also leads to the

upregulation of Mcl-1 which is known to inhibit the activity of proapoptotic Bcl-2

proteins PUMA, BIM, and BAK [83].

ii. JNK

There are three c-jun n-terminal kinase (JNK) encoding genes (JNK1-

3), JNK 1 and JNK 2 are expressed ubiquitously while expression of JNK 3 is

confined to the brain and testes [85]. Due to alternative splicing, the three

JNK genes give rise to 10 different isoforms of JNK. JNK is known to be

activated in response to stress but in contrast to ERK, JNK activation, with a

few exceptions, has generally been associated with activation of apoptosis and cell death.

JNK is activated by extracellular signals by the ras-raf-cdc42 signal transduction pathway, which, in turn, activate MAP Kinase Kinase Kinase's (MKKK) 1and 4 as well as ASK1 [78] [86]. The MKKK's, in turn, activate MKK4 and MKK7, resulting in activation of JNK [87]. JNK activation has been reported following I/R and coincides with increased ROS generation [76, 88, 89]. Hypoxia and reoxygenation of neonatal rat ventricular cardiomyocytes (NRVCM's) resulted in increased JNK activation and this JNK activation could be mimicked through the use of electron transport chain inhibitors that are known to increase mitochondrial ROS generation [90]. Alternatively, in a rat model of myocardial infarction, antioxidant treatment attenuated JNK activation in sections of the myocardium that were outside the infarct zone [89]. ASK 1, an upstream activator of MKK4 and MKK7, is inactive under basal conditions due to binding of reduced thioredoxin [86]. Under oxidative stress, such as during I/R, ASK1 becomes activated as the level of reduced thioredoxin is depleted due to a significant increase in cellular oxidants. This ROS-related activation of ASK1 via depletion of reduced thioredoxin also plays a role in activation of the related MAPK, p38 [88].

JNK activation during I/R generally seems to be associated with activation of apoptosis. JNK inhibition by AS601245 results in a significant reduction of infarct size in rats subjected to LAD occlusion for 30min followed

by 3 hours of reperfusion [91]. Similar results were found in mice with genetic ablation of either JNK1 or JNK2 or transgenic mice that expressed dominant negative JNK1/2 [92]. Conversely, expression of a constitutively active mutant of MKK7 in adult mice through a very elegant tamoxifen-activated cre-lox system resulted in JNK activation, as well as a progressive cardiomyopathy [93]. JNK may contribute to increased apoptosis by destabilizing the 14-3-3 BAD complex which keeps the proapoptotic BAD inactivated and sequestered in the cytosol[94]. Activation of JNK is associated with activation and translocation of BAD to the mitochondria. The mechanisms underlying JNK activation of BAD is somewhat controversial with differing reports regarding phosphorylation of BAD directly or phosphorylation of 14-3-3 causing destabilization of the Bad-14-3-3 complex and subsequent activation of apoptosis [94-97]. Jnk has also been shown to directly phosphorylate and inhibit anti-apoptotic Bcl-2 family members [85].

iii. p38

Like the other members of the MAPK family, p38 exists as several isoforms [76, 98]. Two of the isoforms, p38α and p38β, are ubiquitously expressed. The third isoform, p38 γ is found in skeletal muscle. The final isoform, p38 δ is found expressed in the kidney, pancreas and small intestine. In the heart it appears that p38α is generally associated with the activation of apoptosis while p38β is linked to hypertrophy and survival signals. The two
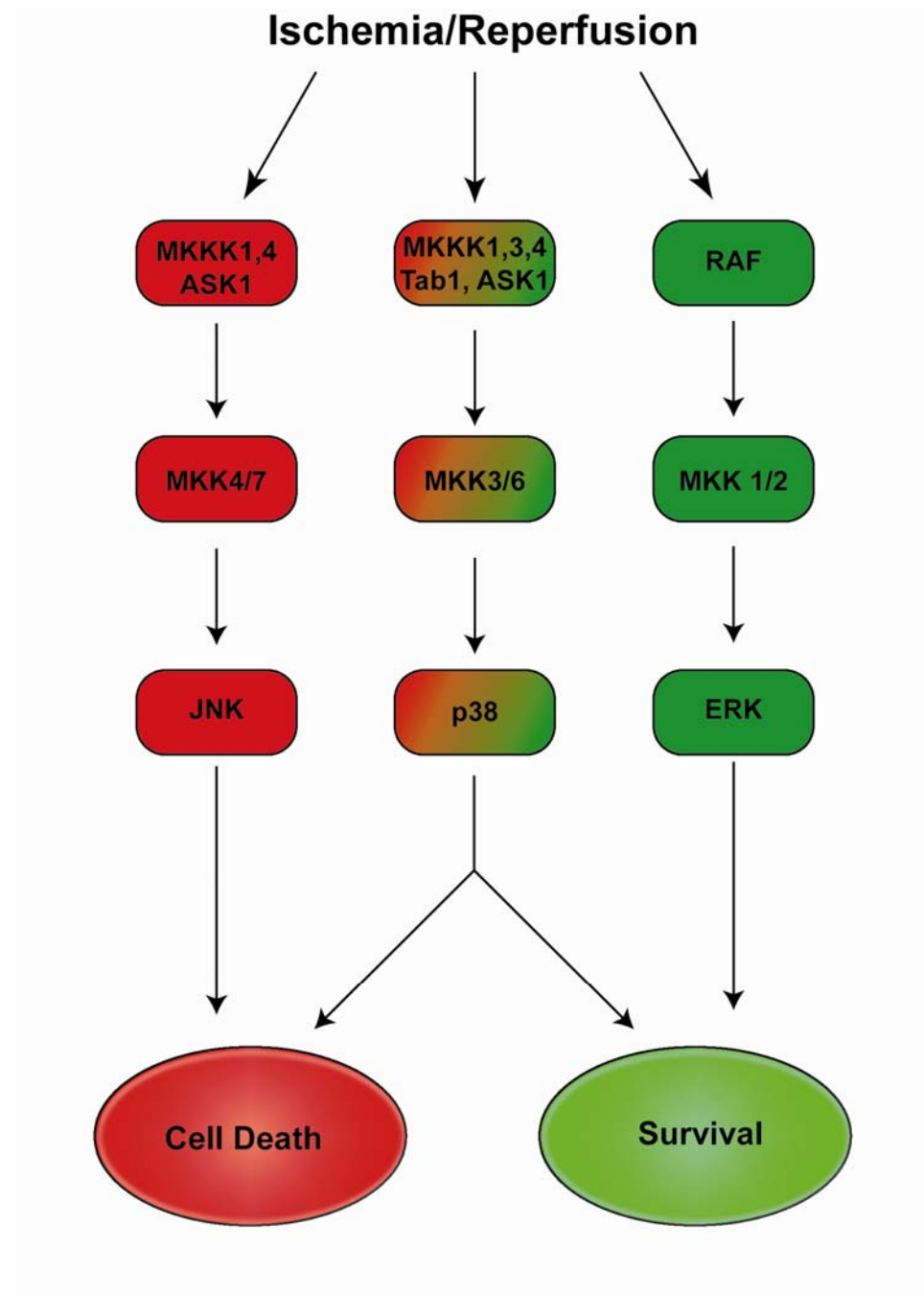
Figure 3- The MAPK signaling pathways in I/R injury
Activation of the different MAPK pathways influence cell fate during I/R.
Extracellular regulated kinase (ERK), Jun N-terminal kinase (JNK), mitogen
activated protein kinase kinase (MKK), mitogen activated protein kinase
kinase kinase (MKKK), apoptosis regulated kinase-1 (ASK1), TAK1 binding
protein (TAB).

isoforms are expressed at different levels with more p38α present in the heart than p38β [99].

The upstream kinases, MKK3 and MKK6, are responsible for the activation of p38 [100, 101]. MKK3 appears to be a specific activator of p38α while MKK6 is more promiscuous and phosphorylates both forms of p38 found in the heart. p38 activation is complex, with extra cellular signals such as growth factors and inflammatory cytokines able to activate p38 through ASK1 or the TAB1-TAK1 complex [102, 103]. p38 can also be activated via MTK1, MLK3, and ASK1 by stress, such as I/R . The consequences of p38 activation during I/R are extremely controversial, which may be due to an inability to distinguish between activation of the different isoforms.

Activation of p38 during I/R has been shown to induce apoptosis and exacerbate injury in a number of different models. For instance, inhibition of p38 via perfusion of pig myocardium with SB203580 results in decreased infarct size following LAD occlusion [104]. Similar observations have been made in rat models of I/R [105] [88]. It has also been demonstrated that p38 activation can reduce contractility of the heart via a negative inotropic effect which can be blocked by expression of dominant negative p38 or administration of SB203580 [106, 107]. Treatment of NRVCM's with the phosphatase inhibitor vanadate, leads to increased cell death following hypoxia and reoxygenation and is associated with increased p38 activation

[108] . In this case increased cell death was abrogated by treatment with SB203580.

On the other hand, there are studies that demonstrate a protective role for p38 activation. In an *ex vivo* mouse model of I/R, perfusion with SB203580 prior to I/R resulted in reduced functional recovery and increased tissue damage [109]. Similarly, transgenic mice which overexpress the upstream activator MKK6 were found to be protected from I/R injury [99, 110]. Several studies have also found a requirement for p38 activation in ischemic preconditioning [101, 111].

The mechanisms by which p38 activation may protect the myocardium are wide ranging, since p38 seems to participate in a number of diverse pathways. MAPK activated protein kinase 2 (MAPKAPK2) is a downstream target of p38 [112]. Activation of MAPKAPK2 leads to phosphorylation of the small heat shock proteins alpha B-crystallin (αBC) and HSP27. Phosphorylation of both of these proteins is known to provide protection against a number of different stresses. Activation of p38 was also shown to regulate expression levels of αBC [99]. p38 activation is a requirement for NFκB activation, as well as IL-6 release in NRVCM's, both of which foster protection from stress [113]. Transgenic mice which overexpress MKK6 demonstrate a significant reduction in a number of electron transport genes [110]. This results in reduced metabolic activity and reduced ROS generation which may help to protect these mice from I/R injury. Activation of p38 has

also been shown to be required for insulin, opioid and adenosine receptor mediated protection of the heart as well as beta adrenergic preconditioning [111, 114, 115].

d. Chaperones

Chaperones are a class of proteins whose primary role is to protect proteins from misfolding and aggregating [116]. Most chaperones are stress inducible and fall in to the HSP family of proteins [117]. There are two instances when the presence of chaperones is vital [116]. The first is during folding of newly synthesized polypeptide chains. The exit pore of the ribosome is believed to only be 15 A° wide, limiting protein folding within the ribosome to the creation of α-helices. Therefore, the majority of protein folding occurs either in the cytosol or the lumen of the endoplasmic reticulum where crowding and molecular compaction can result in misfolding and aggregation of the nascent polypeptide before it can establish its native conformation. The presence of chaperones, such as HSP70, chaperonin, and protein disulfide isomerases, prevent this aggregation from occurring by recognizing and binding to exposed hydrophobic residues and unstructured backbone regions.

Chaperones are also required during stress to help proteins maintain their native structure and prevent aggregation. During I/R, oxidative stress and increases in intracellular calcium can lead to protein unfolding and aggregation. Chaperones have been demonstrated to be important in

preventing myocardial damage during I/R. Knockout of inducible HSP70 in the heart results in reduced functional recovery and increased creatine kinase release, a marker of necrosis, following *ex vivo* I/R on a Langendorff apparatus [118]. Knockout of HSP40, a eukaryotic homolog of the prokaryotic DNAJ, results in severe mitochondrial dysfunction that leads to dilated cardiac myopathy in mice, even in the absence of stress [119].

Conversely, overexpression of chaperone proteins results in protection of myocytes from stress induced cell death. Mitochondrial associated HSP70 is one such example, overexpression of HSP70 in NRVCM's results in reduced cell death compared to control cells following simulated ischemia [120]. Further examples of protection from I/R by overexpression exist. For example, overexpression of GRP 78, an ER stress inducible chaperone, has been shown to protect NRVCM's simulated ischemia induced cell death [121]. Likewise, overexpression of either HSP60 or HSP10, components of the chaperonin complex have been shown to protect NRVCM's from apoptosis induced by simulated ischemia[122].

One group of chaperones that have been demonstrated to play a vital role in protecting cardiomyocytes from cell death during stresses, such as I/R, are the family of small heat shock proteins (sHSP's). Small heat shock proteins are a diverse group of proteins; however, they do share several distinctive traits [123]. The members of the family range in size from 12-43Kd, the defining characteristic of sHSP's is the presence of the conserved α-

crystallin domain. Several studies have demonstrated an important role for sHSP's in protecting cardiomoycytes during stress.

Hearts from transgenic mice which oxerexpress HSP20 demonstrate increased functional recovery and decreased apoptosis in *ex vivo* Langendorff studies [124]. Similarly, transgenic overexpression of HSP27 in mouse hearts is associated with increased functional recovery and reduced cell death following I/R [125]. The role of another member of the sHSP family of protein, alpha B- crystallin (αBC), in protecting the myocardium from I/R injury has been firmly established over the last two decades and is reviewed in the following sections.

C. Alpha B-Crystallin

Alpha crystallin was first isolated and recognized as one of the major protein components in the lens of the eye in 1927[126]. By the 1970's, alpha crystallin had been found to be composed of the subunits alpha A-Crystallin (αAC) and alpha B-Crystallin and both were found to play a role in inhibiting cataractogenesis[127]. By 1985, both alpha crystallin genes had been cloned[128]. However, it wasn't until 1989 that αBC was reported to be expressed in tissues other than the lens[129]. Besides the lens, αBC is expressed in the lung, brain, kidney, skeletal muscle and the heart. In the heart, αBC is expressed at extremely high levels and is believed to make up as much as 3-5% of the total protein[130]. This discovery led to a flurry of

interest in the role of αBC in the heart. Nearly two decades of study regarding

αBC and the myocardium have established it as an important cardioprotective

protein. The consensus in the current literature is that αBC acts through many

different mechanisms to protect cardiomyocytes from stress related injury.

However, many of the mechanisms of αBC mediated protection remain ill-

defined. Here, I review the existing literature regarding αBC in the heart,

highlighting currently postulated mechanisms of αBC mediated

cardioprotection.

1. Gene structure and regulation of expression

Alpha B-Crystallin is located on chromosomes 8, 9 and 11 in the rat,

mouse and human genome, respectively [131, 132]. In humans, transcription

of the αBC gene produces an mRNA of 712 base pairs, which is translated

into a 175 amino acid protein with a molecular weight of approximately 22

kilodaltons (**Figure 4**). The αBC gene is oriented in a head-to-head manner

with the structurally related sHSP, HSPB2, with a shared promoter lying

between the two genes [133]. The promoter region drives preferential

activation of αBC transcription in the heart, consistent with the relative

expression levels these two genes. Early DNA foot printing experiments

identified 5'-enhancer elements in the αBC promoter that are required for

expression of αBC in the heart[134]. Further studies have revealed that these

elements contain at least one serum response element and two heat shock elements[112].

Although αBC is expressed at very high levels in the heart, very little is understood about the regulation of cardiac αBC gene expression. Studies have identified several transcription factors that have been shown to influence αBC expression. Upstream stimulatory factor (USF) has been shown to bind to the muscle specific enhacer of αBC in nuclear extracts from NRVCM's[134]. The transcription factor, HSF1, has been shown to be an important regulator of αBC expression. Hearts from mice lacking the HSF1 gene express 40% less αBC protein compared to control animals[122]. In response to stress it has been shown, both *in vitro* and *in vivo,* that activation of MKK6 can upregulate the αBC gene[110, 112]. This may occur through p38 mediated phosphorylation of ATF6. Other transcription factors that have been shown to regulate αBC expression in other cell types but are also found in cardiomyocytes include SP1, glucocorticoid receptor and HMGA1[135-137].

2. Protein Structure and Function

Alpha B-crystallin protein is considered to have three main regions, the N-terminal region, the core, alpha crystallin domain, and the C-terminal extension **(Figure 4)**.   The core alpha crystallin domain is conserved between sHSP's[123]. Alpha B-crystallin exists in oligomers ranging in size from 200-800 kilodaltons. In the heart these structures may or may not be homogenous

oligomers of αBC. Alpha A-crystallin is not expressed in the heart however; αBC has been shown to interact with a number of related sHSPs including HSP25/27, HSP22 and HSP20, all of which have been shown to exist as multimers. Studies examining the oligomerization of αBC, using either point mutations or peptide binding studies, have identified sequences within all three regions of the αBC protein that affect oligomerization[138-141]. Oligomerization state can also be affected by post translational modifications, most notably phosphorylation [142, 143]. For instance, mimicking phosphorylation at all three serine residues of αBC is associated with oligomers of a reduced size[144].

Alpha B-crystallin is a robust chaperone *in vitro* for a number of diverse target proteins under several denaturing conditions. Several groups have tried to use point mutation to identify amino acids and sequences within the αBC gene that are required for chaperone activity[140, 145-149]. Point mutations at various sites result in reduced chaperone ability, which may suggest that αBC interacts with target proteins at multiple sites. Most of the mutations that affect chaperone activity occur in the C-terminal extension and in the alpha crystallin domain. However, two sites within the N-terminal region stand out for their ability to affect chaperone activity. Serines 45 and 59 represent two of the three phosphorylation sites on the αBC protein and are known to be phosphorylated via MAPK pathways[112, 150, 151]. The mutation of serines 45 and 59 of αBC to alanine results in a 30% reduction in chaperone activity.

Conversely, mimicking phosphorylation of αBC by mutating the serines to aspartate enhances it chaperone ability[144].

3. Post-translational modifications

Post translational modifications are changes to the protein that can affect structure and function in either a reversible on non-reversible manner. Many different post translational modifications exist, some of which are controlled enzymatically such as phosphorylation or glycosylation, and some occur non-enzymatically, such oxidation. Several different post translational modifications of αBC have been identified.

Phosphorylation is by far the most well studied of the post translational modifications of αBC. Phosphorylation occurs at serines 19, 45, and 59 in response to various stresses [151]. Stresses such as heat shock or arsenite treatment, result in phosphorylation at all three sites, however, stresses such as H_2O_2 or sorbitol only result in phosphorylation of two of the three sites. This suggested that phosphorylation of each site was controlled by a separate signaling pathway. The ERK pathway was found to be responsible for phosphorylation at serine 45, while the p38 pathway was found to be responsible for phosphorylation of serine 59 [112, 150]. The signaling pathway that results in phosphorylation of serine 19 is currently unknown.
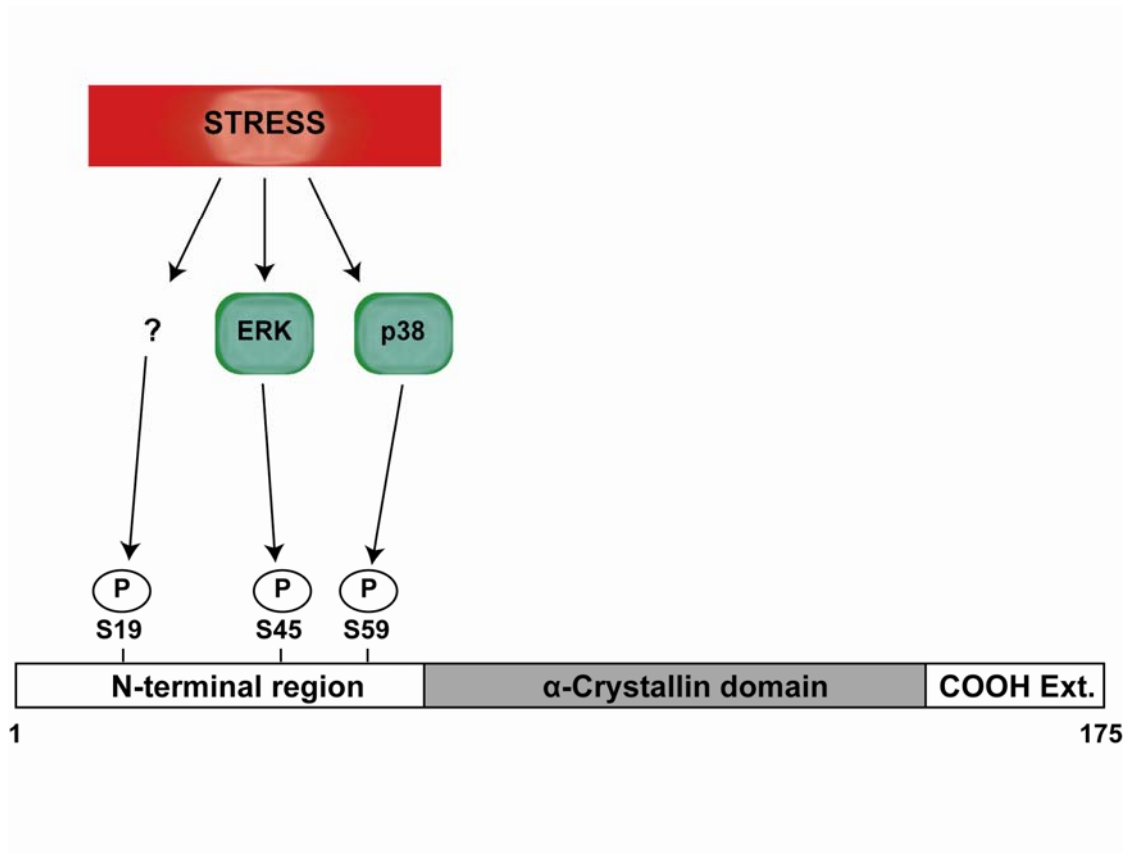
Figure 4- αBC protein structure and phosphorylation by MAPK pathways
Alpha-BC is a 175 amino acid protein that contains three domains. The N-terminal region contains the three serines that are phosphorylated in response to stress. The α-crystallin domain is a conserved domain present in all small heat shock proteins and is believed to be important for chaperone activity. The C-terminal extension has been demonstrated to be involved in chaperone activity and oligomerization.

Mimicking phosphorylation via mutation of the serine residues to aspartate or glutamate has provided a wealth of information regarding the consequences of αBC phosphorylation and its effect on αBC structure and function. By utilizing mimics of phosphorylation, serine 59 was identified as being necessary and sufficient for αBC to protect cardiomyocytes from stress [152]. Consistent with these findings are the observation that phosphorylation of αBC serine 59 is increased in the heart in response to I/R [99, 109]. Protection mediated by phosphorylated αBC may be due in part to an enhanced chaperone ability [153-155]. A number of groups utilizing mutants of αBC that mimic phosphorylation at all three sites have demonstrated an enhanced chaperone ability when compared to wild type αBC.

Phosphorylation is a reversible modification that can be used to regulated protein structure and function. The phosphorylation status of any given protein is controlled by the balance of kinase and phosphatase activity. There have not been any phosphatases identified that act specifically on αBC. However, in cardiomyocytes treatment with the phosphatase inhibitor calyculin A was associated with increased αBC phosphorylation and protection from ischemia induced cell death [156].

Several other lesser studied modifications of αBC have also been identified. One interesting modification that may play a role in diabetic associated cardiomoypathy is glycation of αBC. The appearance of advanced glycation end products (AGE) can result as an end product of oxidative

modification of proteins[157]. In the lens, accumulation of AGE modified αBC is common with aging and is seen during cataractogenesis [149]. AGE modification of αBC results in a dramatic reduction of chaperone ability which may explain its correlation with the development of cataracts. Diabetics are especially susceptible to AGE-modification of proteins in an around the heart [158].. However, currently there are no reports of AGE modified αBC in the heart

Deamidated αBC has been observed in the heart under basal conditions and rapidly disappears following I/R [140, 159]. Deamidation of glutamine and aspartate within proteins is irreversible suggesting that the disappearance of deamidated αBC is facilitated by rapid degradation of the protein. Deamidation results in reduced αBC chaperone activity which may be why the cell rapidly eliminates deamidated αBC during stress [140].

O-linked GlcNAcylation of αBC has also been observed in non-stressed rat hearts [160]. Interestingly, the same study showed glycosylated αBC was never been found to be phosphorylated, and phosphorylated αBC has never been found to be glycosylated. Furthermore, pulse-chase studies in astroglioma cells found that turnover of the αBC protein was many times slower than turnover of the carbohydrate moiety suggesting that αBC is glycosylated in a reversible manner. These results suggest that glycoslyation may play a role in regulating αBC in some manner, however, there haven't been any reports to demonstrate that this occurs in the heart.

4. Protection of the heart during I/R

Alpha B-crystallin has emerged as an important protector of the myocardium during stress. In nearly every report concerning αBC, the heart, and I/R injury, αBC has been shown to promote myocyte survival and preservation of cardiac function. While a complete understanding of the mechanisms of αBC mediated protection is elusive, several mechanisms have been postulated.

a. Functional studies in mouse models

Some of the most compelling evidence for a cardioprotective role for αBC comes from studies that have utilized transgenesis and knockout technology to create mouse models that facilitate study of the whole heart. The first published study to use such a model came from Wolfgang Dillmann's lab, where they created a line of mice that overexpressed wildtype αBC [161]. These transgenic mice expressed roughly 7 times more αBC protein in the heart compared to control animals. Using an *ex vivo* working heart model, hearts from both control and transgenic animals were exposed to I/R injury. The hearts of transgenic mice demonstrated a significantly better functional recovery as well as reductions in infarct size and apoptosis compared to control hearts.

A compliment to the αBC transgenic study was published by our lab shortly thereafter [162]. This study utilized αBC knockout mice that had

originally been derived by Eric Wawrousek's lab in order to dissect the roles of αAC and αBC in the lens of the eye [163]. Mice with a deletion of the αBC gene develop normally however, they do die prematurely due to malnutrition primarily due to development of megaesophagus around 10 months of age. The heart, however, appears normal up till and including death. Hearts from these animals were subjected to I/R using an *ex vivo* Langendorff perfusion apparatus. Hearts from knockout animals showed significantly reduced functional recovery compared to hearts from control animals. This reduced functional recovery coincided with significantly higher levels of apoptois and necrosis in the knockout animals when compared to hearts from control animals.

One other αBC mouse model has been generated and provides an insight in to the importance of αBC's function in the heart. The existence of the R120G mutation of αBC was first brought to light in a genetic analysis of a French family with a large number of cases of cardiomoypathy and cataracts [164]. Follow up studies on the effect of R120G on αBC's structure and function found that this mutation inhibited chaperone activity and resulted in the formation of desmin rich aggresomes.

Mice harboring the R120G mutation recreate the phenotype seen in the family members, where the mutation was originally described [165]. R120G mice demonstrate aggregate formation within myocytes accompanied by gross disorganization of the myofilaments. Cardiac function was significantly

compromised, and by 6 months, the mice were in the advanced stages of heart failure. Taking into account results from *in vitro* studies that demonstrate that recombinant αBC-R120G is severely compromised in its chaperone activity, these results suggests that the chaperone activity of αBC is vital to maintaining homeostasis within the cardiomyocyte [166].

b. Translocation to the sarcomeres in response to I/R

Translocation of αBC out of the cytosol in response to stress was first reported in guinea pigs hearts subjected to ischemia [167]. The hearts were fractionated to produce a soluble and insoluble pellet. The insoluble pellet contained the cytoskeletal and sarcomeric proteins. Under basal conditions αBC was found exclusively in the cytosol but following ischemia αBC was detectable in the insoluble fraction. A similar phenomenon has been reported in several different cell lines in response to heat stress [168].

These studies, while intriguing, where only a small step in determining targets for αBC during stress.   However, they did provide a clue as to where to start looking for targets of αBC. Golenhofen *et al.* were the next to address translocation of αBC out of the cytosol, this time using left coronary artery occlusion (LAD), an *in vivo* model of myocardial infarction, in pigs [169]. Golenhofen *et al.* utilized immunocytofluoresence to visualize the location of αBC under basal and stress conditions. Under basal conditions αBC was cytosolic and showed very little association with the cytoskeleton or

sarcomeres. However, following LAD there was a striking increase in αBC association with the z-lines of the sarcomeres. Two dimensional gel analysis of tissue samples demonstrated the appearance of acidic forms of αBC following LAD suggesting that translocation of αBC to the sarcomeres coincided with phosphorylation of αBC. Many studies have followed that examine the interaction of αBC with sarcomeric proteins, in particular proteins that make up the z-lines[169-174]. To date αBC has been shown to interact with actin, myosin, desmin, titin and vimentin.

The protective effect of αBC translocation to the sarcomere was analyzed using papillary muscles from the hearts of αBC knockout mice[175]. Under basal conditions, the papillary muscles from αBC knockout and control mice produced equivalent contractile force. In response to ischemia the papillary muscles of αBC mice went in to contracture much sooner and experienced much higher tensile force during contracture when compared to control animals. These results demonstrated an increased stiffness in αBC knockout mice in response to stress.

Elasticity within the sarcomeres is maintained, in part, by the structural support of titin[176]. Titin spans half the distance of a sarcomere, connecting the m-line and z-disc. Titin, as would be expected for the largest known protein, has a complex structure, the region of titin that spans the I-band has a number of globular domains with no well-defined secondary structure. The

regions can stretch and unfold, but by maintaining interactions within a globular structure, these regions provide elasticity to the sarcomeres.

Using immunogold labeling, αBC was shown by electron microscopy to localize to the I-band portion of the sarcomeres [172]. Binding assays using recombinant fragments of the different regions of titin confirmed that αBC binds to the globular regions of titin. Together these results suggest that αBC may help maintain sarcomeric elasticity during stress by binding to the globular regions of titin [170]. By maintaining sarcomeric elasticity αBC helps maintain sarcomeric structure during ischemia when sustained unregulated contration applies mechanical strain to neighboring sarcomeres. These data are further supported by electron micrographs of the sarcomeres of αBC knockout mice which demonstrate larger I-band regions when compared to control animals, suggesting that the sarcomeres are being stretched apart [162].

c. Anti-apoptotic effects

The old axiom that the heart is a terminally differentiated organ and that there is no turnover of myocytes, has gone by the wayside. However, hearts that experience severe I/R injury generally replace dead myocytes with scar tissue and eventually progress to failure [177]. As such, it appears that the regenerative ability of the native stem cell population within the heart is insufficient to replace the large numbers of myocytes lost to a major ischemic

injury. In order for the heart to demonstrate functional recovery following an ischemic event, and avoid failure in the future, there must be a preservation of the myocyte population. Cell death as a consequence of I/R injury occurs through both necrotic and apoptotic pathways[177]. Direct inhibition of apoptosis using caspase inhibitors has been shown to reduce infarct size in *in vivo* models of I/R [178]. This suggests that inhibiting apoptosis may be an effective strategy for preserving heart function following I/R. In response to I/R, numberous anti-apoptotic signaling pathways are activated including PI3K/AKT, PKCε, ERK, and p38. Alpha B-crystallin lies downstream of both ERK and p38, and has been demonstrated to be an anti-apoptotic protein.

Overexpression of wildtype αBC, and especially αBC phosphorylated on serine 59, in various cell culture models prevents apoptosis due to oxidative, hyperosmotic, and hypoxic stresses [152]. Transgenic mice that over express αBC were found to have fewer TUNEL positive cells than control animals following I/R [161]. Conversely, hearts from αBC knockout mice exposed to I/R had increased DNA laddering compared to control hearts [162]. Examination of the mechanisms by which αBC confers protection from apoptosis demonstrate that it interacts with and inhibits the activities of several pro-apoptotic proteins.

In response to stress proapoptotic members of the Bcl-2 family such as Bad, Bax, Bim, and Bcl-X$_s$, translocate to the mitochondria, where they induce apoptosis by facilitating release of cytochrome c from the mitochondria [179].

Overexpression of αBC inhibited translocation of both Bax and Bcl-X$_s$ from the

cytosol to the mitochondria in response to staurosporine treatment [180].

Furthermore, it was shown that overexpression of αBC was sufficient to

prevent caspase 3 activation and DNA laddering following staurosporine

treatment. Alpha B-crystallin was shown by immunoprecipitation from lens

epithelial cell extracts to interact with the pro-apoptotic Bcl-2 family members

Bax and Bcl-X$_s$ [180]. These results suggest that αBC can prevent apoptosis

at the level of the mitochondria by preventing cytochrome c release, perhaps

by keeping the mitochondrial permeability transition pore closed. Consistent

with these results are reports that demonstrate incubating recombinant αBC

with isolated mitochondria prevent calcium induced swelling, and that

overexpression of αBC in NRVCM's helps maintain mitochondrial membrane

potential during oxidative stress[109].

In addition to preventing apoptosis at the level of the mitochondria, αBC

can also prevent-receptor mediated activation of apoptosis through inhibiting

TRAIL signaling [181]. More interestingly however, is the ability to prevent

pro-caspase 3 maturation which was first observed in differentiating myoblasts

[182]. In a follow up study, it was shown that αBC can prevent capase 3

activation, regardless of whether apoptosis was activated by the intrinsic or

extrinsic pathways[183]. This study also found that pro-caspase 3 co-

immunoprecipitated with αBC suggesting that capase 3 maturation is inhibitied

by direct interaction with αBC. Taken together, these studies suggest that

αBC may be able to regulate apoptosis from the earliest apoptotic signals all the way through the end events of cell death.

D. Hypothesis

The literature clearly demonstrates that αBC plays a role in protecting the myocardium from I/R injury. It does this through a number of different mechanisms that help to preserve cell structure and function, while also directly inhibiting cell death. However, several observations suggest that there may be a number of unexplored mechanisms by which αBC provides protection against I/R.

Expression of related sHSP's such as HSP25/27 have been shown to influence redox status in a number of different cell lines. Expression of αBC in L929 fibroblasts was shown to provide protection against hydrogen peroxide but the mechanism of protection was never explored. Hearts from mice overexpressing αBC, that were submitted to I/R injury, were found to contain significantly less malondialdehyde, a product of lipid peroxidation, when compared to control animals. Hearts from mice which lack the transcription factor, HSF1, were found to express 40% less αBC and were found to have a lower reduced glutathione to oxidized glutathione ratio, when compared to hearts from control animals. However, given this collection of observations, an analysis of the effects of αBC expression on the redox status of the heart, and the mechanisms by which αBC may influence redox status has never been performed.

Alpha B-crystallin has been demonstrated to be a potent inhibitor of apoptosis, and has been shown to inhibit apoptotic signaling at several points

within the apoptotic signaling pathways. This includes, interactions with proteins that translocate to the mitochondria to induce apoptosis such as Bax and BclX$_s$, and effector proteins that are activated by events regulated by the mitochondria such as caspase 3. Recently, several studies form our own lab, have demonstrated that αBC translocates to the mitochondria during I/R and that its presence there may inhibit events that lead to apoptosis. However, these studies have not yet examined the nature of the translocation event, the targets of αBC at the mitochondria, or the mechanism by which the presence of αBC at the mitochondria may affect apoptotic signaling.

Therefore, taking into account the current literature regarding αBC, I propose the following as the overarching hypothesis of this dissertation:

Alpha B-crystallin is an important protective protein within the myocardium and acts in several different ways to preserve the structure and function of the heart. The mechanisms of protection by αBC include, but are not limited to, stabilizing sarcomeric and cytoskeletal structure, preventing apoptosis, maintaining a favorable redox environment, and protecting the integrity and function of the mitochondria (**Figure 5**).

This hypothesis will be addressed in the following three chapters. The first chapter will address the mechanisms by which αBC may prevent apoptotic signaling by interacting with the mitochondria. The second chapter explores the effect of αBC on the redox status of the heart and demonstrates how αBC can influence intracellular glutathione levels. The third

chapter introduces a novel protein transduction tool that could be utilized as a delivery vehicle for the use of αBC as a therapeutic for treating MI. This work respresent the preliminary stages of development for a novel drug delivery platform that could be developed, not only to deliver αBC, but also to deliver other protein and small molecule drugs.

Figure 5- Hypothesis
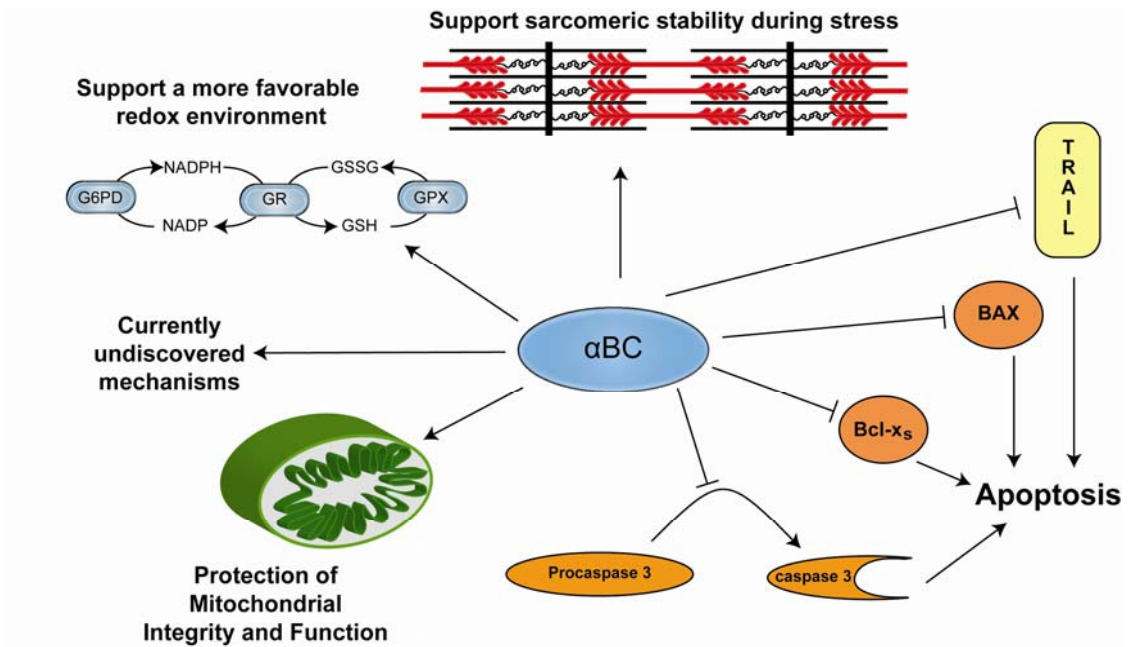 Alpha B-crystallin is an important protective protein within the myocardium and acts in several different ways to preserve the structure and function of the heart. The mechanisms of protection by αBC include, but are not limited to, stabilizing sarcomeric and cytoskeletal structure, preventing apoptosis, maintaining a favorable redox environment, and protecting the integrity and function of the mitochondria.

Chapter Two

1. Introduction

Ischemia/reperfusion (I/R) in the heart leads to the generation of mitochondrial-derived reactive oxygen species (ROS), which can contribute to myocardial damage [8, 13]. A number of signaling cascades are activated in the heart during I/R, including the mitogen activated protein kinases (MAPKs), which have been associated with both tissue damage and protection [77, 81, 103, 104, 111, 113, 184-187]. Activation of p38 MAPK leads to the phosphorylation of numerous proteins, including the small heat shock protein (sHSP), alpha B-crystallin (αBC), one of the ten-member sHSP family of molecular chaperones [112, 150]. αBC is expressed in several tissues, including the lung, eye, brain, and skeletal muscle; however, its expression in the heart is particularly noteworthy, since it comprises 3-5% of the total protein in the myocardium [123, 129, 171].

Numerous studies have established a role for αBC in protecting the myocardium from I/R. In response to stress αBC is phosphorylated on serine-59, in a p38 dependent manner. Mimicking the phosphorylation of αBC at serine-59 was shown to enhance the ability of αBC to protect cells against several different stresses that imitate I/R [152]. Like other members of the sHSP family, it is through its molecular chaperone activity that αBC is thought to exert most of its protective functions [169, 181-183]. Because of its cardioprotective effects, identifying the cellular binding partners of αBC and examining the functional consequences of αBC binding to those targets, have

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been the emphases of numerous studies; in fact, it has been proposed that post-translational modification of αBC, specifically phosphorylation, may augment its chaperone activity [188].

Transgenesis and gene-targeting have provided additional evidence that αBC plays an important protective role in the mouse heart, *in vivo*. For example, it was demonstrated that compared to wild type mouse hearts, the hearts from transgenic mice that overexpress αBC in a cardiac-specific manner exhibited less tissue damage and preserved contractile function when subjected to *ex vivo* I/R [161]. It was also shown that when subjected to *ex vivo* I/R, the hearts from mice lacking αBC were more susceptible to injury than hearts from wild type mice [162].

In terms of its subcellular localization, αBC is very dynamic. Several studies have demonstrated that in the heart, stress induces αBC to translocate from a diffuse cytosolic locale to sarcomeres, where has been hypothesized to stabilize myofilament structure [168-170, 189]. In mouse hearts subjected to *ex vivo* I/R, αBC translocates to mitochondria, where it was postulated to bind to key mitochondrial proteins and to preserve mitochondrial function [99, 109]. Moreover, the level of phospho-αBC-S59 associated with mitochondria was increased upon I/R. Inhibiting p38, which decreased mitochondrial phospho-αBC-S59, also increased myocardial damage in an *ex vivo* model of I/R. These findings support the hypothesis that p38, which is activated in the heart during ischemia and reperfusion, leads to increased accumulation of

phosphorylated αBC in mitochondrial fractions, and that the resulting

mitochondrial phospho-αBC-S59 may exert a protective function by interacting

with and affecting the function of specific mitochondrial proteins. The results

of the present study are consistent with this hypothesis, and they suggest that

mitochondrial αBC may interact with the voltage-dependent anion channel

(VDAC), which resides on the outer mitochondrial membrane and plays a

central role in preserving mitochondrial function [190-192]. Furthermore,

consistent with maintaining mitochondrial function during I/R, αBC may also

interact with key components of the electron transport chain.

2. Materials and Methods

A. Animals- Approximately 100, 10-14 week-old C57/BL6 mice (*Mus musculus*) were used in this study. All procedures involving animals were performed in accordance with institutional guidelines. The animal protocol used in this study was reviewed and approved by the San Diego State University Institutional Animal Care and Use Committee.

B. *Ex Vivo* Ischemia-Reperfusion- Global no-flow *ex vivo* I/R was performed on a Langendorff apparatus, as previously described [162]. Briefly, mice were treated with 500 U/kg heparin 10 min prior to administration of 150 mg/kg of pentobarbital, both via intaperitoneal injection. Animals were then sacrificed, hearts were quickly removed and placed in ice-cold modified Krebs Henseleit buffer. The aorta was then cannulated and the heart was mounted on a Langendorff apparatus and perfused with oxygenated Krebs Henseleit buffer at a constant pressure of 80 mmHg. The left atrium was removed and a water-filled balloon connected to a pressure transducer was inserted into the left ventricle and inflated in order to record left ventricle developed pressure (LDVP). Hearts were electrically paced at 8.7 to 9.2 Hz via an electrode placed on the right atrium. Following a 30-minute equilibration period, hearts were subjected to varying times of ischemia $\pm$ subsequent reperfusion. Hearts were submerged in buffer at 37°C at all times. Following the appropriate treatment times, hearts were quickly removed from the apparatus, the right

atria and any remaining vessels and connective tissue were removed, and hearts were flash frozen in liquid nitrogen. Hearts were stored at -80°C until processed.

    C. Preparation of Subcellular Fractions- Frozen hearts were pulverized while in dry ice and then homogenized in 1 ml of isolation buffer (70mM sucrose, 190mM mannitol, 20mM HEPES, 0.2 mM EDTA, 200 µM sodium orthovanadate, 10 µg/ml aprotinin, 10 µg/ml leupeptin, 0.5 mM PNPP, 1 mM PMSF). Following homogenization 70 µl of the homogenate were added to 400 µl of RIPA buffer (150 mM NaCl, 20 mM Tris-HCl pH 7.5, 0.1%SDS, 1% Triton X-100, 1 mM EDTA, 200 µM sodium orthovanadate, 10 µg/ml aprotinin, 10 µg/ml leupeptin, 0. 5mM PNPP, 1mM PMSF), this whole homogenate fraction was saved for immunoblot analysis. The remainder of each homogenate was centrifuged at 600 x g for 10 minutes to remove nuclei and myofibrils. The resulting supernatant, containing mitochondria and membrane fractions, was centrifuged at 5,000xg for 15 min. producing a pellet containing the mitochondria and a supernatant containing the cytosol and the endoplasmic reticulum. The pellet was washed twice with 1 ml of isolation buffer and resuspended in 400 µl of RIPA buffer to produce the mitochondrial fraction. The supernatant was centrifuged at 100,000xg for 60 min. The resulting supernatant was the cytosolic fraction.

D. Mitochondrial Trypsin Protection Assay- Mitochondria were isolated as described above; 200 µg of isolated mitochondrial protein were resuspended in 200 µl of isolation buffer and treated $\pm$ trypsin (1 mg/ml) for one hour on ice. Mitochondria were then collected by centrifugation at 5,000 x g for 15 min, washed twice with isolation buffer containing soybean trypsin inhibitor (1 mg/ml), and then resuspended in 100 µl of RIPA buffer, also containing soybean trypsin inhibitor (1 mg/ml).

E. Cytochrome C Release Assay- Neonatal rat ventricular myocyte cells (NRVMC) were cultured, as previously described [112]. NRVMC were infected with recombinant adenovirus strains encoding either no αBC (control), wild type αBC, or a mutant form of αBC which mimics phosphorylation at serine 59 while blocking phosphorylation at serines 19 and 45 (αBC-AAE). Forty eight hours after infection, cultures were placed in minimal media containing 200µM H_2O_2 and incubated for one hour, after which cells were harvested by trypsinization, collected by centrifugation, and mitochondria isolated, as described above.

F. Immunoprecipitation- NRVCM's were infected with recombinant adenovirus encoding αBC-AAE with an N-terminal HA tag. Forty eight hours after infection, cultures were exposed to 200 µM H_2O_2 in minimal media for 1

hour. Cells were released from culture dishes by trypsinization, collected by

sedimentation, and fractionated by differential centrifugation, as described

above, to obtain mitochondrial-enriched fractions. Mitochondria were then

homogenized in RIPA buffer, as described above. For each

immunoprecipitation, 30 µg of mitochondrial protein were incubated for

approximately 16h at 4°C in a total volume of 500 µl with either HA antibody,

or control mouse IgG antibody, both conjugated to agarose beads. Following

incubation, beads were washed twice with PBS-T (PBS with 0.05% Tween-

20), followed by 3 washes with PBS. Antibody-protein complexes were

released from the agarose beads by boiling samples in SDS sample buffer.

Samples were then fractionated by SDS-PAGE and subjected to

immunoblotting for αBC, adenine nucleotide translocase (ANT) or voltage-

dependent anion channel (VDAC).

Immunoprecipitations from mouse heart mitochondria were performed

in a similar manner however, endogenous protein was immunoprecipitated

using sepharose beads and anti-αBC antibody.

G. Proteomic analysis (MudPIT)- Proteomic analysis of

immunoprecipitation samples was performed as previously described [110].

3. Results

To examine the kinetics of αBC movement to mitochondria, mouse hearts were subjected to *ex vivo* ischemia (I) or ischemia/reperfusion (I/R), for various times **(Figure 6A)**, followed by homogenization and subcellular fractionation. Immunoblotting for the myofibril, cytosol and mitochondria marker proteins, α-actinin, GAPDH, and VDAC, respectively, in subcellular fractions prepared from a control heart showed that the mitochondrial fractions were free from contamination from cytosolic and myofibril proteins **(Figure 6B)**, which can potentially contain large quantities of αBC.

Whole heart homogenates were examined by immunoblotting; examples of blots prepared from homgenates of hearts subjected to 5 or 30 min of I, or to 30 min of I followed by 10 or 60 min of R are shown in (**Figure 7A**). An antibody that cross-reacts with all forms of αBC was used to measure total αBC in homogenates. Compared to control hearts, the levels of total αBC in hearts subjected to I or I/R did not change significantly **(Figure 7A, Total αBC)**. Quantification of immunoblots performed at all of the times shown in Figure 1A further demonstrated that compared to perfusion time-matched controls, there was no significant loss of αBC after any of the I or I/R times **(Figure 7B, Total αBC, squares)**. In contrast, antibodies specific for αBC phosphorylated on serine-59 (phospho-αBC-S59) showed that the level of phospho-αBC-S59 in whole heart homogenates reached a maximum of 8-fold over control after 30 min of I followed by 60 min of R **(Figure 7A, Phospho-αBC-S59; Figure 7B, triangles)**. Since the phosphorylation of αBC on

A. Time Course of I/R
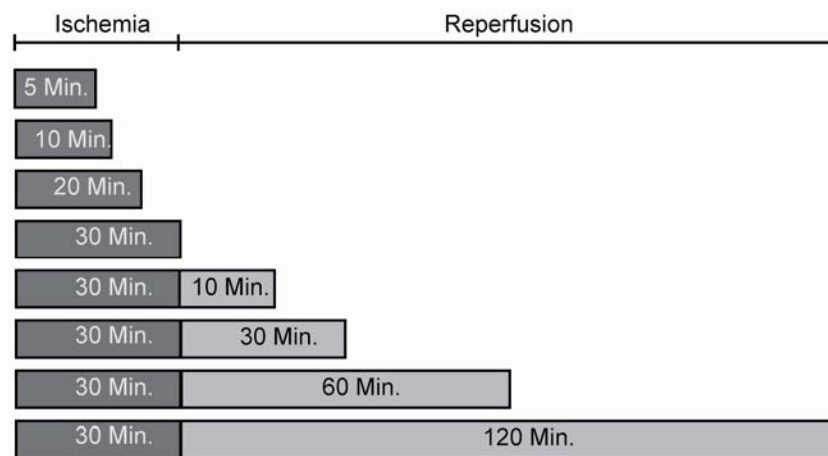
B. Subcellular fractionation
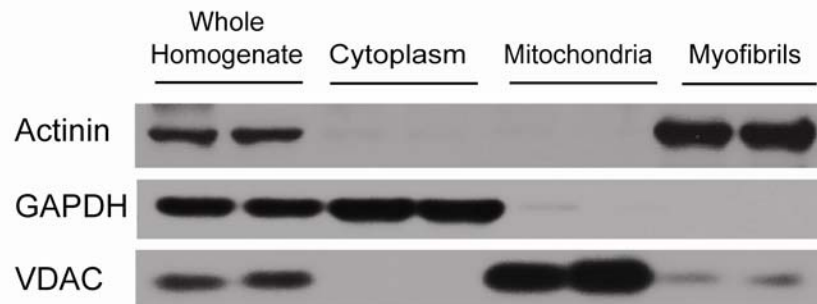
Figure 6- Diagram of the I/R protocol used in this study and analysis of the subcellular fractionations for organelle contamination:

Panel A- Following 30 minute of equilibration mouse hearts were subjected to various times of ischemia and reperfusion on a Langendorff apparatus (n=3 mouse hearts/time) as shown. A continual perfusion control that matched each I and I/R time was also generated.

Panel B- After each treatment, hearts were homogenized and, after removal of a sample for later analysis, homogenates were subjected to subcellular fractionation. The tissue was later homogenized and fractionated by differential centrifugation and examined by immunoblot for typical subcellular fraction marker proteins.

Figure 7- Effect of ischemia or ischemia/reperfusion on αBC, phospho-αBC-S59, p38 and Phospho-p38 in whole heart homogenates

Prior to subcellular fractionation, samples of the whole heart homogenates from the 8 experimental samples shown in Figure 6A, and 8 time-matched controls (n = 3 hearts per time), were subjected to immunoblot analysis.

Panel A- Samples from 4 of the time points were analyzed by immunoblotting for Total αBC, Phospho-αBC-S59, p38, Phospho-p38 and GAPDH.

Panel B- The immunoblots of all samples for Total αBC (squares) and Phospho-αBC-S59 (triangles) were quantified; values are shown as fold over control hearts for each time point normalized to GAPDH.

Panel C- The immunoblots of all samples for Total p38 (squares) and Phospho-p38 (triangles) were quantified; values are shown as fold over control hearts for each time point normalized to GAPDH. All values = Mean $\pm$ SEM, n=3 for each time point. All values for phospho-αBC-S59 and phospho-p38 are significantly greater than control perfusion values ($p < 0.05$).

A. Sample Blots

(blot labels: Control 5 Min. I   Control 30 Min. I   Control 30I/10R   Control 30I/60R)

- Total αBC
- Phospho-αBC-S-59
- Total p38
- Phospho-p38
- GAPDH

B. αBC

- ■ Total αBC
- △ Phospho-αBC-S59

C. p38

- ■ Total p38
- △ Phospho-p38

(y-axis: Fold Over Control)

(x-axis: 5 I, 10 I, 20 I, 30 I, 30I/10R, 30I/30R, 30I/60R, 30I/120R)
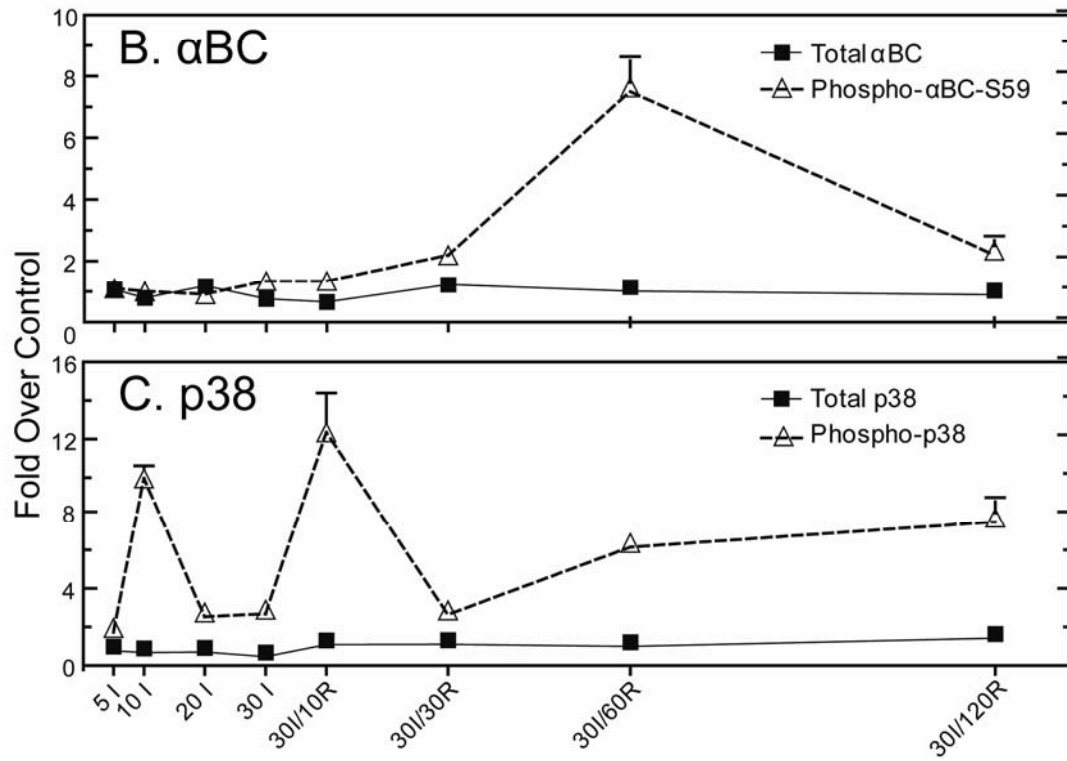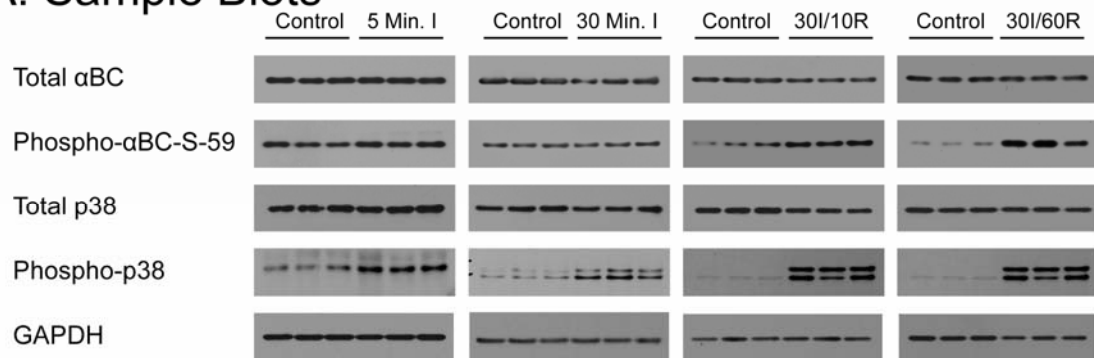
serine-59 depends on p38 activation, the levels of total and phosphorylated p38 (P-p38; active) were also examined. As expected, total p38 did not change as a function of I/R **(Figure 7A, total p38; Figure 7C, total p38 squares)**. However, the relative levels of phospho-p38 increased transiently, to about 10-fold over control within 10 min of I, and then decreased to about 3-fold over control at the longer I times **(Figure 7C, Phospho-p38 triangles)**. Phospho-p38 exhibited a second transient increase of 12-fold over control after 30I/10R, which was followed by more modest increases to about 6- and 8-fold over control after 60 and 120 min of R, respectively. This profile of p38 activation is consistent with the transient activation of p38 during ischemia and reactivation again during reperfusion, the latter of which requires mitochondrial-derived ROS. These results demonstrate that αBC phosphorylation on serine-59 occurred primarily during R when whole heart homogenates were examined; however, dramatically different results were observed in the subcellular fractions. For example, αBC left the cytosol rapidly following the onset of I, by 30 minutes of I there was in a complete loss of total αBC and phospho-αBC-S59 **(Figure 8, 5-30 Min I)**. This loss of αBC persisted throughout all reperfusion times examined **(Figure 8, 30I/10-120 Min. R)**. Thus, during I, αBC quantitatively moved from the cytosol to other subcellular fractions and remained at these other cellular locations throughout the reperfusion time points examined.
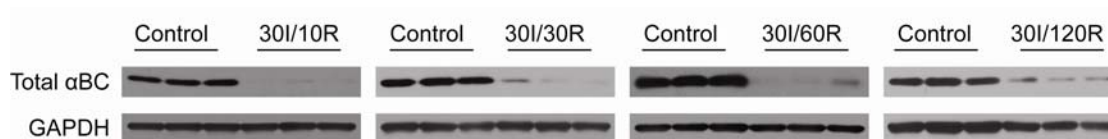
A. Ischemia
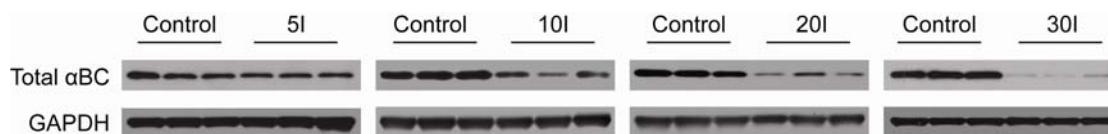
B. Reperfusion

Figure 8- **Effect of ischemia and ischemia/reperfusion on αBC in cytosolic fractions**

Immunoblotting was performed on cytosolic fractions from all the time points to examine total αBC and GAPDH.

We recently reported that phospho-αBC-S59, plays important roles in protecting mitochondria during stress. However, after examining the whole heart homogenates in the current study, it appeared that αBC phosphorylation might occur too late to provide significant protection of mitochondria from reperfusion damage. Accordingly, mitochondrial fractions were examined. Examples of blots prepared from hearts subjected to I or I/R as in Figure 2A are shown in **Figure 9A**. In contrast to whole homogenates, total αBC in mitochondrial fractions (total αBC) increased primarily during I **(Figure. 9A, Total αBC)**. Quantification of sample blots from all times shown in Figure 1A demonstrated that total mitochondrial αBC increased by about 4-fold after as little as 10 min of I, reaching a maximum of 7-fold over control by 20 min of I and subsequently declining throughout the remaining times of I and I/R **(Figure 9B, Total αBC squares)**. The level of mitochondrial phospho-αBC-S59 was also maximal during I, but exhibited delayed kinetics compared to that of total mitochondrial αBC **(Figure 9A, and 9B, P-αBC-S59 triangles)**. In fact, mitochondrial phospho-αBC-S59 was maximal after 30 min of I, 10 min after the maximum for total mitochondrial αBC. At longer R times, the level of mitochondrial phoshpo-αBC-S59 declined steadily to about 4-fold over control after 120 min of R, the last time point examined **(Figure 4B, Phospho-αBC-S59)**.

Since it appeared as though αBC phosphorylation occurred after translocation to mitochondria, and since p38 activation is required for this

Figure 9- Effect of ischemia and ischemia/reperfusion on αBC, Phospho-αBC-S59, p38, and Phospho-p38 in mitochondrial fractions

After subcellular fractionation, samples of the mitochondrial fractions from the 8 experimental samples shown in Figure 1A, and 8 time-matched controls (n = 3 hearts per time), were subjected to immunoblot analysis.

Panel A- Samples from 4 of the time points were analyzed by immunoblotting for Total αBC, Phospho-αBC-S59, p38, Phospho-p38 and VDAC.

Panel B- The immunoblots of all samples for Total αBC (squares) and Phospho-αBC-S59 (triangles) were quantified; values are shown as fold over control hearts for each time point normalized to VDAC.

Panel C- The immunoblots of all samples for Total p38 (squares) and Phospho-p38 (trangles) were quantified; values are shown as fold over control hearts for each time point normalized to VDAC. All values = Mean $\pm$ SEM, n=3 for each time point. All values are significantly greater than control perfusion values ($p < 0.05$)

A. Sample Blots

B. αBC

C. p38
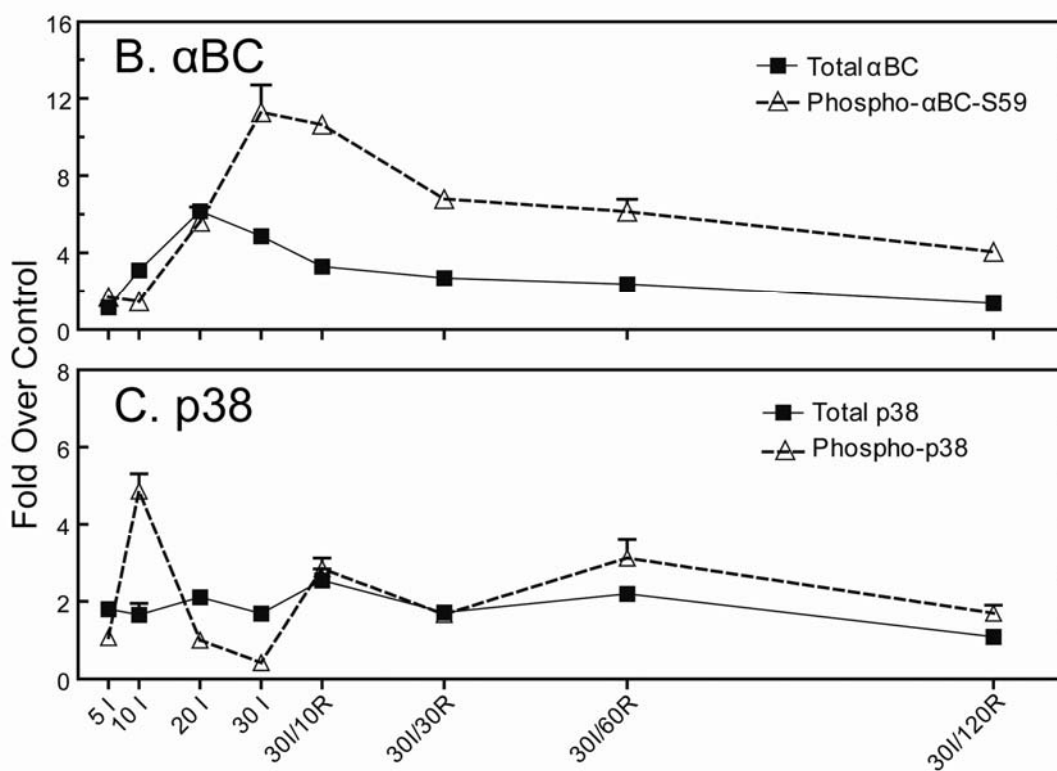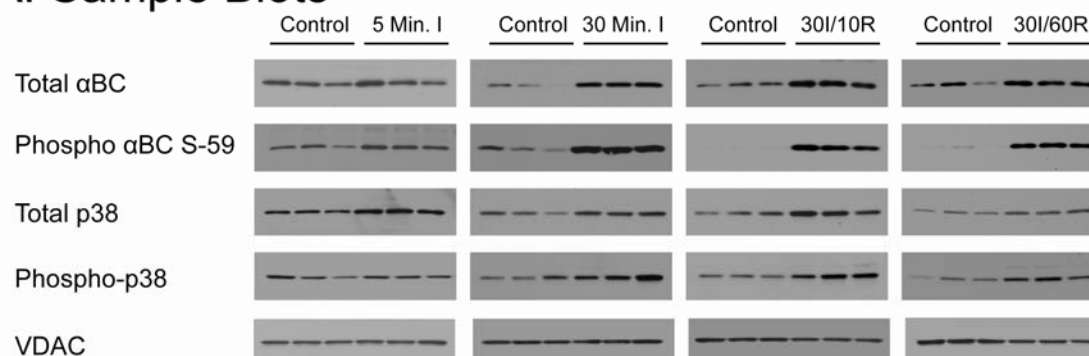
Figure 10- Effect of Trypsin on Mitochondrial αBC

Hearts (n=3) were subjected to 30 min of *ex vivo* ischemia followed by 10 minutes of reperfusion, and then homogenized and mitochondria isolated as described in Materials and Methods. 200 µg of isolated mitochondrial protein were then treated with 1mg/ml trypsin for 1 hour at 4˚C.

Panel A- Immunoblots for Total αBC, Phospho-αBC-S59, TOM20 and COX4 before and after incubating isolated mitochondria with trypsin. TOM20 was used as a marker for proteins located outside the mitochondrial outer membrane and COX4 was used as a marker for proteins located within the outer mitochondrial membrane.

Panel B- Immunoblots for Total αBC, Phospho-αBC-S59, TOM20 and COX4 after mitochondrial membrane disruption by sonication before trypsin treatment.

Panel C- Quantitation of the fraction of Total αBC, Phospho-αBC-S59. COX4 and TOM20 remaining after trypsin treatment of intact mitochondria. Values are represented as mean percent remaining $\pm$ SEM, n=3.

A. Protease protection assay

Control   Trypsin

αBC Total

αBC Phospho-59

TOM 20

COX IV

B. Control Digest

CTL TRP

αBC Total

αBC Phospho-59

Tom 20

COX IV
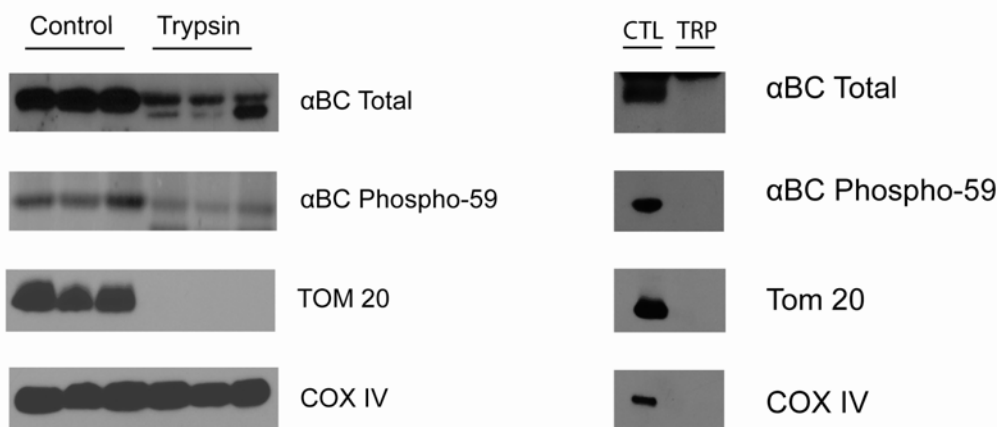
C. Quantitation
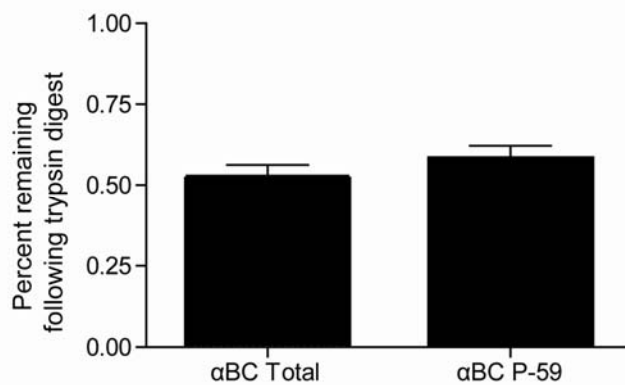
phosphorylation, the levels of p38 in the mitochondrial fractions were analyzed. Total p38 in mitochondrial fractions increased by about 2-fold over control within 5 min of the onset of I **(Figures 9A and 9C, Total p38, squares)**. Total mitochondrial p38 remained at this level through 60 min of R, and then declined somewhat thereafter. The levels of phospho-p38 in the mitochondrial fractions exhibited a transient increase to about 5-fold over control within 10 min of I, which was followed by a decline to nearly control levels of phosphorylation at the later I times **(Figure 9A, Phospho-p38; Figure 9B open triangles)**. Mitochondrial phospho-p38 levels increased to about 2.5-fold over control upon 10 min of R, and remained at this level through 60 min of R, and then declined to control levels by 120 min or R. Taken together, these results indicate that in contrast to the whole heart homogenates, the increases in mitochondrial total αBC and phospho-αBC-S59 occurred primarily during I. Moreover, the timing of the increases in phospho-p38 in mitochondrial fractions provided support for the idea that αBC is phosphorylated in a p38-dependent manner, and that at least in part, this phosphorylation takes place after its translocation to mitochondria.

To examine the nature of the association of αBC with mitochondria, a protease protection experiment was carried out. After treatment of isolated mitochondria with trypsin, total αBC and phospho-αBC-S59 were decreased, but only by about 50% **(Figures 10A and C)**, suggesting that about half of the αBC may be localized to regions of mitochondria that are protected from

Figure 11- Effect of wild type αBC-AdV and αBC-AAE-AdV on H$_2$O$_2$-induced Cytochrome C release

Overexpression of wild type αBC or the phosphorylation mimic, αBC-AAE, in neonatal rat ventricular cardiomyocytes was accomplished via infection with adenovirus (AdV) harboring the appropriate expression construct, or no αBC expression construct (Con-AdV). Cells were treated with 200 μM H$_2$O$_2$ for 1 hour, followed by subcellular fractionation and cytochrome c immunoblots on the cytosolic fraction.

Panel A- Immunoblots of cytochrome c and GAPDH in the cytosol.

Panel B- Quantitation of cytochrome c in cytosol normalized to GAPDH. Values represent average $\pm$ SEM, ** and †† are different from other groups by ANOVA with Student Newman Keuls *post hoc* test, p< .05.

Panel C- Immunoblot for HA-tagged, AdV-encoded wild type αBC, or αBC-AAE in mitochondrial fractions isolated from cultured cardiac myocytes.

A. Cytochrome C western blot

IB: Cytochrome C

IB: GAPDH

Adv-GFP   Adv-GFP   Adv-αBC   Adv-AAE

H2O2
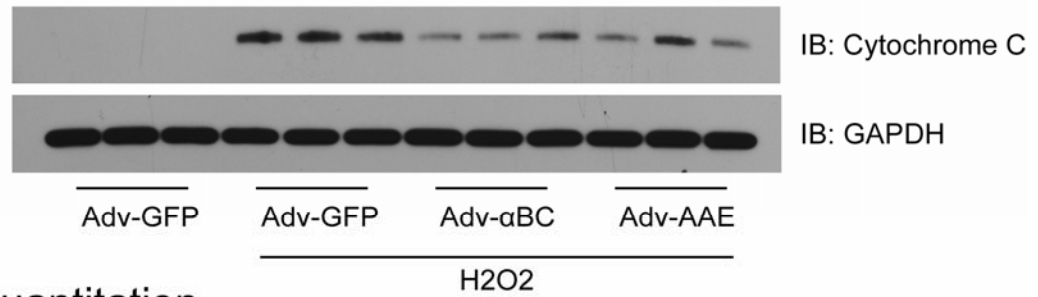
B. Quantitation
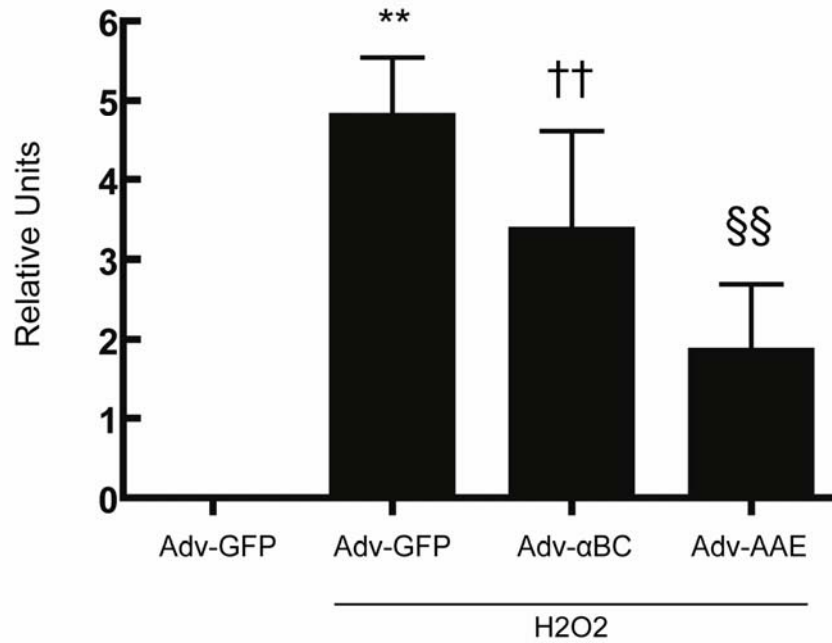
C. HA western blot of mitochondrial fraction

IB: HA

Adv-GFP   Adv-αBC   Adv-AAE
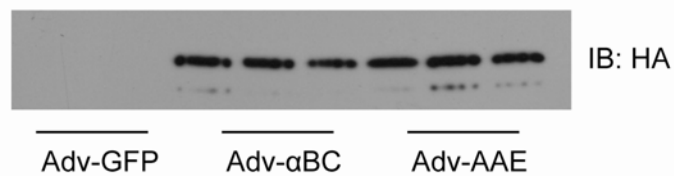
trypsin. In contrast, TOM 20, a protein that resides on the cytoplasmic face of mitochondria, was completely digested by trypsin, while COX4, which resides in the mitochondrial matrix, was completely resistant to protease treatement **(Figure 10A, TOM20 and COX IV)**. To address the formal possibility that a certain portion of αBC could not be degraded by trypsin, mitochondrial integrity was compromised by sonication prior to protease treatment, and it was found that αBC, phospho-αBC-S59 and COXIV were completely degraded. These results suggest that a portion of the mitochondrial αBC and phospho-αBC-S59 reside on the surface, while the remainder may be localized within the outer mitochondrial membrane.

To examine possible functional roles for mitochondrial αBC, the effects of overexpressing wild type αBC, or αBC-AAE, the latter of which mimics phosphorylation at serine-59, in cultured cardiac myocytes were assessed. When cultures were infected with the control adenovirus, Con-AdV, but not subjected to oxidative stress, there was no detectible release of mitochondrial cytochrome c, as expected **(Figure 11A, lanes 1-3; Figure 11B, bar 1)**. However, when Con-AdV-infected cultures were treated with H_2O_2, the release of mitochondrial cytochrome c increased dramatically **(Figure 11A, lanes 4-6; Figure 11B, bar 2)**. In contrast, when cultures were infected with αBC-AdV or αBC-AAE-AdV, H_2O_2-activated cytochrome c release decreased by about 25 and 50%, respectively **(Figure 11A, lanes 7-12; Figure 11B, bars 3 an 4)**, consistent with the hypothesis that mitochondrial αBC protects mitochondria

Figure 12- Co-immunoprecipitation of αBC and VDAC from neonatal rat cardiomyocyte mitochondria and mouse heart mitochondria

Panel A- Immunoprecipitations were performed on mitochondrial extracts using either anti-HA antibody conjugated to agarose beads or control non-immune mouse antibody conjugated to agarose beads. Immunoprecipitations were then separated by SDS-PAGE and analyzed by western blotting for the presence of ANT, VDAC and αBC. Lanes containing control beads did not demonstrate any staining. Lanes containing HA-beads stained positive for both αBC and VDAC.

Panel B- Immunoprecipitations were also performed on mitochondrial extracts purified fom mouse hearts that had been exposed to 30I/10R on a Langendorff apparatus. Anti-αBC antibody was used to precipitate endogenous αBC. The immunoprecipitations were separated by SDS-PAGE and analazed for the presence of αBC and VDAC. The control lanes did not demonstrate any significant staining. However, anti-αBC lanes stained positive for both αBC and VDAC.

A. Immunoprecipitation from NRVCM mitochondria

IB: ANT

IB: VDAC

IB: HA

Input 2ug   IP: Control   IP:Anti-HA
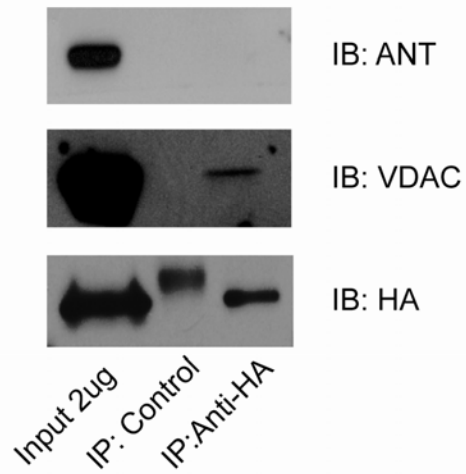
B. Immunoprecipitation from mouse heart mitochondria

IB: VDAC

IB: αBC

Input 5ug   Preclear Beads   IP: Control   IP:Anti-αBC
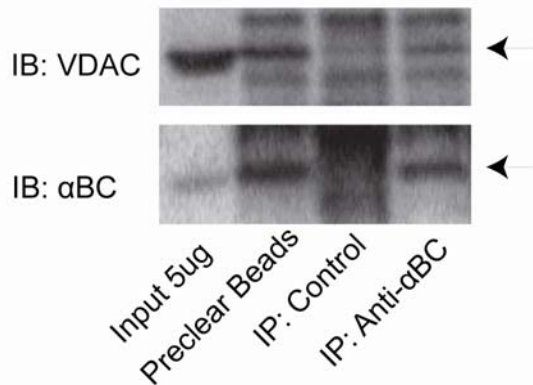
from oxidative damage, and that phosphorylation of αBC on serine-59 may enhance these protective effects. These results are also consistent with our previous findings that αBC-AdV or αBC-AAE-AdV both reduced H_2O_2-mediated loss of mitochondrial membrane potential in NRVMC[109]. Immunoblots showed that HA-αBC and HA-αBC-AAE were both associated with mitochondria **(Figure 11C)**, consistent with the hypothesis that while the association of non-phosphorylated αBC with mitochondria may provide some protection, phospho-αBC-S59 provides maximal protection.

Since HA-αBC-AAE was associated with isolated mitochondria, immunoprecipitation experiments were carried in attempts to identify putative mitochondrial αBC binding partners. Accordingly, HA-αBC was immunoprecipitated from mitochondria isolated from αBC-AAE-AdV-infected cultured cardiac myocytes. Immunoprecipitates were then analyzed by immunoblotting for several potential outer mitochondrial membrane binding partners thought to participate in cytochrome c release, adenine nucleotide translocase (ANT) or voltage-dependent anion channel (VDAC). While the blot for ANT did not reveal evidence of binding **(Figure 12A, IB: ANT lane 3)**, the blot for VDAC demonstrated that this protein bound to HA-αBC **(Figure 12A, IB: VDAC, lane 3)**, consistent with a role for VDAC in mediating the protective effects of αBC at mitochondria. To examine is this interaction occurs *in vivo*, αBC was immunoprecipitated from mitochondria isolated from mouse hearts that had been treated with 30I/10R prior to subcellular

fractionation. Consistent with the cell culture findings, VDAC co-immunoprecipitated with αBC (**Figure 12B**).

To identify other targets of αBC at the mitochondria, αBC was again immunoprecipitated from mouse heart mitochondria isolated following treatment with 30I/10R on the Langendorff apparatus. These samples were separated on an SDS-PAGE gel and silver stained. Silver staining revealed several unique bands resulting from αBC immunoprecipitation when compared to the control (**Figure 13A, Arrows**). In order to identify, these targets αBC and control immunprecipitations from mitochondrial fractions from hearts treated with either 30I/10R were submitted to MudPIT (Multidimensional Protein Identification) analysis. MudPIT analysis identified 8 mitochondrial proteins as potential targets of αBC during ischemia (**Figure 13B**).

4. Discussion

The results of this study show that in the mouse heart, ischemia causes a striking quantitative disappearance of αBC from the cytosol **(Figure 8)** that occurs on a timeframe coordinate with its appearance in other subcellular fractions, including mitochondria. Although the mechanism responsible for ischemia-mediated translocation of αBC to mitochondria is not known, previous studies on myocardial and other cell types suggest that αBC's propensity to bind to other proteins as a chaperone is likely to be at least partly responsible. For example, αBC has been shown to bind to numerous sarcomeric proteins, where it is believed to contribute to maintaining contractile element integrity during stress [169-173, 189, 193]. In human lens epithelial cells αBC has been shown to bind to the BH3-only proteins, Bax and Bcl-x$_s$ in the cytosol; since αBC remains in the cytosol in this cell type, this binding inhibits translocation of these proteins to mitochondria, thereby inhibiting apoptosis [180]. Perhaps the translocalization of αBC to mitochondria in cardiac myocytes may involve its binding to proteins like Bax and/or Bcl-x$_s$, an interaction which, in contrast to epithelial cells, may not inhibit the migration of these Bcl family members to mitochondria. In this case, it may be possible that αBC modulates Bax and/or Bcl-x$_s$ pore-formation at the mitochondrial outer membrane (MOM), which would inhibit cytochrome c release and decrease mitochondrial-dependent apoptosis. It is also possible that the unfolding of MOM proteins attracts chaperones, like αBC. For

A. Silver Stain of αBC immunoprecipitations
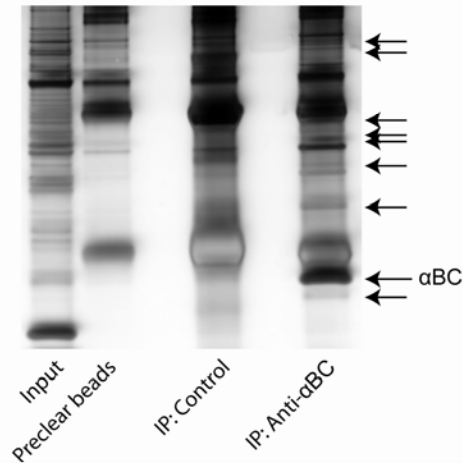
B.MudPIT results for αBC immunoprecipitations

IP: Control	IP:anti-αBC	Protein Identified	Function
0	18	Alpha B-Crystallin	Chaperone
0	9	Acyl-CoA medium chain specific	β-oxidation of fatty acids
4	26	Hydroxyacyl-Coenzyme A Dehydrogenase	β-oxidation of fatty acids
1	6	Mitochondrial 2-oxoglutarate/malate carrier protein	Inner mitochondrial membrane transport
1	6	NADH-ubiquinone oxidoreductase MLRQ subunit	Electron transport chain
0	5	Nicotinamide nucleotide transhydrogenase	Mitochondrial proton pump
0	5	ATP Synthase delta	Electron transport chain
15	73	ATP Synthase alpha	Electron transport chain

Figure 13- Proteomics analysis of interactions between αBC and components of the mitochondria

Panel A- Immunoprecipitations using anti-αBC and control antibodies were performed on extracts of mouse heart mitochondria that had been isolated following 30I/10R on a langendorff apparatus. Immunoprecipitations were separated by SDS-PAGE and silverstained. Uniques bands resulting from αBC immunoprecipitation are indicated by arrows.

Panel B- Results of the MudPIT analysis revealed a number of proteins that were immunoprecipitated in a specific manner.

example, the MOM protein, TOM20, is prone to unfolding during myocardial ischemia, and other chaperones are known to bind to TOM20 under these conditions [194]. Additionally, it has been postulated that mitochondrial permeability transition pore (MPTP) proteins unfold during oxidative stress, and that modulating this unfolding through the binding of chaperones may also modulate permeability transition and the associated cell death [191].

In the present study, the kinetics of ischemia-mediated αBC translocation and phosphorylation were consistent with the possibility that at least a portion of αBC phosphorylation occurred in the mitochondrial fraction. The known dependence of αBC phosphorylation on serine-59 by p38-mediated activation of MAP kinase activated protein kinase-2 (MK2) is consistent with the findings of the current study that during ischemia, activated p38 also translocated to mitochondria **(Figure 9C)** [112]. Previous studies showing that both p38 and MAPKAP-K2 translocate to mitochondria during stress, and that αBC can form a complex with p38 and MAPKAP-K2, suggest that αBC might translocate to mitochondria as part of a p38 signalsome [195].

The nature of the physical association of αBC with mitochondrial fractions was also examined in the present study. It was shown that at least half of the phospho-αBC-S59 was susceptible to proteolytic degradation, indicating that at least a portion of it localizes to the surface of mitochondria during ischemia, while the remainder, being protease resistant, may be intramitochondrial **(Figure 10)**. The potential functional consequence of the association of αBC

with mitochondria was examined in cultured cardiac myocytes, where it was shown that αBC-AAE, which mimics phospho-αBC-S59, decreased the oxidative-stress dependent release of cytochrome c **(Figure 11A and 11B)**, consistent with our previous observation that αBC-AAE reduces mitochondria-dependent apoptosis. Finally, the co-immunoprecipitation of αBC-AAE and VDAC from NRVCM's **(Figure 12A**) as well as the co-immunoprecipitation of endogenous αBC and VDAC support the hypothetical physical association between αBC-AAE and a key MOM protein known to regulate mitochondrial function during oxidative stress.

What role does VDAC play and how could the interaction of αBC with VDAC during ischemia modulate its role in a manner consistent with protection? VDAC was once thought to be a component of the MPTP, which opens during reperfusion leading to mitochondrial uncoupling, decreased ATP synthesis and eventual necrosis or apoptosis. However, more recent studies showing continued function of the MPTP in mitochondria that lack all 3 isoforms of VDAC convincingly demonstrated that VDAC is not an integral part of the MPTP [196]. Nonetheless, VDAC lies at the convergence of cell survival and death pathways, and has been described as a global regulator of mitochondrial function [190, 192]. VDAC plays an important role in ion and metabolite transport into and out of mitochondria, and its ability to close during ischemia may contribute to the global mitochondrial suppression thought to be key to survival during reperfusion [191]. VDAC is also thought to form

channels through which cytochrome c can be released from stressed mitochondria. Moreover, the binding of various pro- and anti-apoptotic proteins to VDAC, such as tBID, Bax, and Bcl-x_L, regulate VDAC opening and, thus, VDAC-dependent cytochrome c release [190]. Accordingly, it is possible that αBC modulates mitochondrial cytochrome c release through either direct or indirect binding to VDAC. In fact, since αBC has already been shown to bind to Bax, which binds to VDAC, it is reasonable to hypothesize that αBC may exist in a complex with BAX and VDAC in a manner that regulates pore formation.

Furthermore, the preliminary MudPIT studies provide evidence that αBC may interact with certain metabolic proteins, including members of the electron transport chain (**Figure 13B**). These findings are consistent with the results of the protease protection experiment (**Figure 10**) that suggest a portion of the αBC found at the mitochondria exists within the MOM. Moreover, they are also consistent with mitochondrial respiration studies performed on isolated mitochondria from the hearts of αBC/HSPB2 double knockout mice that show reduced complex I activity [197]. These findings strongly suggest an entirely new role for αBC in regulating metabolism.

Until recently, the conventional wisdom regarding αBC within cardiomyocytes was that, in response to stresses, such as heat shock or I/R, αBC translocates out of the cytosol and moves to the sarcomeres. While, the sarcomeres are an important target of αBC during stress, this study and

previous studies from our lab during the last several years have established that mitochondria are an equally important target [99, 109]. Consistent with our previous findings, we have demonstrated that the presence of αBC at the mitochondria is important for maintaining mitochondrial function and integrity. Furthermore, we have identified physical interactions between αBC and several key components of the mitochondria, however, the current list should in no way be considered all inclusive and undoubtedly many more interactions will be uncovered (**Figure 14**).

Importantly, this study revealed that αBC translocates to the mitochondria and is phosphorylated within a time frame consistent with protecting mitochondrial function and integrity during I/R and prevents cytochrome c release. Given previously published studies, it appears that αBC can regulate early apoptotic signaling through interactions with pro-apoptotic Bcl family members, prevent cytochrome c release from mitochondria, and inhibit activation of effector caspases. Therefore, the findings presented bolster the role of αBC as a powerful, anti-apoptotic protein and suggest that αBC is a key regulator at all levels of mitochondrial dependent apoptosis signaling.
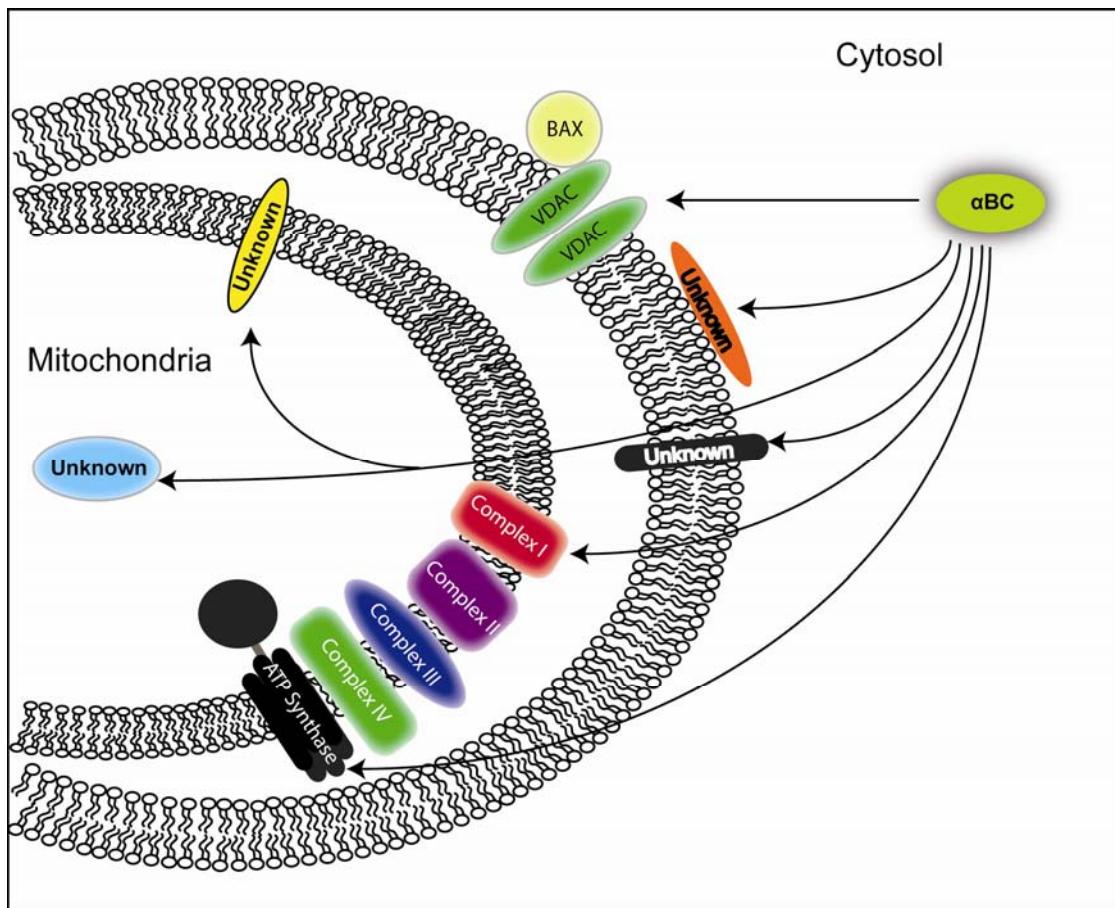
Figure 14- Summary of findings regarding αBC translocation to the mitochondria

αBC was found to translocate to the mitochondria in response to I/R and prevent cytochrome c release. Further examination revealed a portion of αBC may exist within the MOM. Consistent with this finding, αBc was found to interact with a number of proteins that exist both outside and within the MOM.

Chapter Three

1. Introduction

Following an MI, blood flow must be restored to the ischemic region of the heart if the tissue is to survive. However, while reperfusion is a requirement for survival of the tissue, paradoxically, it also results in significant tissue damage [14]. This is due to the burst of ROS generation that follows reperfusion of the ischemic tissue. This generation of ROS can rapidly overwhelm cellular antioxidant systems, resulting in widespread cellular damage that can lead to cell death [31, 81]. Current clinical protocols for treating MI focus primarily on restoring blood flow to the ischemic tissue and provide no protection against ROS related reperfusion injury [198]. Therefore, antioxidant systems and the signaling pathways that control their activities have been a subject of numerous recent studies.

Members of the sHSP family of proteins have been shown to enhance antioxidant activity and prevent oxidative stress associated cellular damage and cell death[199-203]. The most well studied sHSP in this regard is HSP 25/27. Expression of HSP 27 has been shown to increase intracellular glutathione levels in several cell lines, including L929 (fibrosarcoma cell line) and C2C12 (skeletal muscle satellite cell line) [202, 204]. This increase in glutathione was associated with reduced protein oxidation, lipid peroxidation and cell death when cells were challenged with either H_2O_2 or tumor necrosis factor alpha (TNFα). Examination of the mechanism by which HSP25/27

elicits this effect has shown that HSP 27 expression can increase G6PD activity, which enhances glutathione recycling, and helps to preserve intracellular levels glutathione[201, 202].

Several studies examining the protective effects of αBC against I/R damage have provided results suggesting that, like HSP 27, αBC may also influence antioxidant systems[201, 202]. The HSP 27 studies referenced above were some of the earliest to suggest that αBC could provide protection against oxidative stress. In parallel experiments the same group created a stably transfected line of L929 cells that expressed αBC. These cells were more resistant to ROS induced damage and exhibited lower cell death when challenged with TNFα[201]. Using the same stably transfected cell line, the same group also demonstrated that αBC could increase intracellular glutathione and protect against H_2O_2-induced cell death[202].

Studies utilizing transgenic and knockout mice have suggested that αBC plays a role in protecting the heart from oxidative stress. Hearts from mice with a cardiac-specific overexpression of αBC were shown to have greater functional recovery and reduced cell death following *ex vivo* I/R[161]. Consistent with this was the finding that following I/R, the αBC overexpressing hearts were found to contain lower levels of malondialdehyde, a product of lipid peroxidation, compared to hearts from control animals [161]. Conversely, the hearts of HSF1 knockout mice were found to express 40% less αBC and 72% less HSP25 (HSP25 is the equivalent of HSP 27 in mice), compared to

control animals [122]. The hearts from these animals were found to have a significantly lower reduced glutathione to oxidized glutathione ratio and increased levels of protein carbonyls when compared to control animals, even in the absence of any stressors. Taken together these results suggest that αBC may protect hearts from oxidative stress by influencing the glutathione system.

There have been no reports exploring the mechanism(s) by which αBC influences the glutathione system. Furthermore, there have been no published studies examining the effects of αBC on glutathione levels, or on the activities of glutathione-related enzymes in the heart. Therefore, this study was undertaken to examine the effects of αBC expression on glutathione levels in the heart, and to determine the mechanism(s) by which αBC may provide protection against oxidative stress.

2. Materials and Methods

A. Animals- Approximately 40, 10-20 week-old 129SVEV$_{TAC}$ and αBC/HSPB2 double knockout mice (*Mus musculus*) were used in this study. All procedures involving animals were performed in accordance with institutional guidelines. The animal protocol used in this study was reviewed and approved by the San Diego State University Institutional Animal Care and Use Committee.

B. Measurement of reduced and oxidized glutathione- Measurement of reduced glutathione and glutathione disulfide were performed as previously described, with modifications for use in a 96 well plate format [30].

C. Measurement of glucose-6-phosphate dehydrogenase activity- Glucose-6-phosphate dehydrogenase activity was measured as described previously [205] ,with modifications for use in a 96 well plate format. Briefly, 10 ug of sample was added to reaction buffer 1 (86.3 uM triethanolamine buffer pH 7.6, 6.7uM MgCl2, 100uM NADP, 200uM glucose-6-phosphate, 200uM 6-phosphgluconate) and to reaction buffer 2 (86.3 uM triethanolamine buffer pH 7.6, 6.7uM MgCl2, 100uM NADP, 200uM 6-phosphgluconate) to start the reactions which were followed at 340 nm for 5 min at 22°C (VersaMax microplate reader, Molecular Devices). Glucose-6- phosphate activity was determined by subtracting the activity of reaction 2 from reaction

1. nMoles NADPH were calculated using a molar extinction coefficient of 6250 $M^{-1}cm^{-1}$.

D. Measurement of glutathione reductase activity- Glutathione reductase activity was measured as previously described with modifications for use in a 96 well plate format[200]. Briefly, 50-100ug of sample were added to reaction buffer (0.1M potassium phosphate buffer pH 7.4, 0.6mM EDTA, 2mM GSSG) to a total volume of 237.5ul and allowed to incubate at 22°C for 10 min. The reaction was started by the addition of 12.5 ul of 2mM NADPH and was followed at 340 nm for 3 min. nMoles of NADPH were calculated using a molar extinction coefficient of 6250 $M^{-1}cm^{-1}$.

E. Measurement of caspase 3 activity- Following transfection, HeLa cells were plated at a density of $0.5X10^6$ cells per well in 6 well plates and allowed to recover overnight. The cells were then treated with 200uM hydrogen peroxide in minimal media for 8 hours. Following the incubation, cells were washed gently with ice cold 1X PBS and scraped in caspase assay buffer (50mM HEPES pH 7.4, 0.1% CHAPS, 0.1mM EDTA), sonicated briefly, then centrifuged at 15,000g for 10 minutes at 4°C. Fifty ul of sample per well was loaded into a black 96-well plate and combined with 10ul of assay buffer and 45ul of reaction buffer (50mM HEPES, 0.1% CHAPS, 0.1mM EDTA, .01mM DTT, 40uM N-Acetyl-Asp-Glu-Val-Asp-7-amido-4-trifluomethyl-coumarin).

The reaction was incubated in the dark at 37°C for 1 hour. Following the incubation, period the plate was read on a 96 well fluorimeter with the excitation and emission wavelengths of 400nm and 505nm, respectively. Background fluorescence was determined using a separate reaction where the caspase 3 substrate in the reaction buffer was replaced with dimethyl sulfoxide.

F. Co-Immunoprecipitation of αBC and glutathione reductase- HeLa cells were transfected with a plasmid enconding wildtype αBC with an N-terminal HA tag and allowed to recover on a 150mm dish for 24 hours. HeLa cells were then scraped in RIPA buffer, briefly sonicated, and centrifuged at 15,000g for 10 minutes. For each immunoprecipitation 30 ug of HeLa cell extracts were incubated overnight at 4°C in a total volume of 500ul with either HA antibody (Santa Cruz, sc-7392-AC) or control mouse IgG antibody (Santa Cruz, sc-2343-AC) conjugated to agarose beads. Following incubation, beads were washed twice with PBS-T (1XPBS with .05% Tween 20) followed by 3 washes with 1XPBS. Antibody-protein complexes were released from the agarose beads by boiling samples in laemelli buffer. Samples were then separated by SDS-PAGE and analyzed by western blotting.

G. Statistics- Data were analyzed by students t-test or ANOVA followed by Newman-Keuls post hoc, where appropriate. Data are presented as means +/- standard error of the mean (SEM).

3. Results

Alpha-BC knockout mice were created by Dr. Eric Wawrousek's lab by deleting part of the promoter region and a portion of exon 1 of the αBC gene [163]. However, this strategy created mice that also harbor a knockout of the neighboring gene, HSPB2. These mice (αBC/HSPB2-KO) were previously demonstrated to be more susceptible to I/R damage and were used in this study to examine the effect of αBC expression on glutathione levels in the heart [162]. To analyze glutathione levels, hearts were quickly excised from anesthetized animals, weighed and then homogenized in 5% sulfosalycilic acid. Glutathione levels were assessed using a glutathione reductase cycling assay. Total glutathione content in hearts from αBC/HSPB2-KO mice was found to be reduced 43% compared to hearts from control animals (**Figure 15A**). Conversely, the oxidized glutathione content of αBC/HSPB2-KO hearts was found to be 54% greater than that of control hearts (**Figure 15B**). This, in turn, resulted in a decrease in the reduced glutathione to oxidized glutathione ratio, from 56.79 ± 2.82 for hearts from control animals to 21.73 ± 0.818 for αBC/HSPB2-KO hearts, a reduction of more than 60% (**Figure 15C**).

Following analysis of the glutathione levels in hearts of αBC/HSPB2-KO, the activities of the enzymes associated with glutathione cycling were measured in whole heart extracts. Measurement of G6PD activity revealed no significant difference in activity between hearts from αBC/HSPB2-KO mice and control animals (**Figure 16A**). However, measurement of GR activity

revealed a 23% reduction in activity in hearts from αBC/HSPB2-KO mice when compared to control animals (**Figure 16B**). Deletion of the αBC and HSPB2 genes had no effect on protein expression levels of either G6PD and GR (**Figure 16C**). This suggested that αBC does not influences protein turnover but may affect GR activity through either direct or indirect interaction with GR.

To recreate this phenomenom *in vitro*, HeLa cells were chosen as a model system to further examine the effects of αBC expression on glutathione levels and to explore how αBC interacts with glutathione cycling enzymes. HeLa cells are an excellent complement to the αBC-KO hearts (**Figure 17A)** as they express neither αBC nor HSPB2 (**Figure 17B,lanes 1-3**). Importantly, transfection of HeLa cells with a plasmid that expresses αBC did not change the levels of the related sHSP, HSP27, which is known to influence glutathione levels and glutathione cycling enzyme activity (**Figure 17B, lanes 4-6**).

Glutathione levels in HeLa cells were measured using the same glutathione reductase cycling assay that was used to assess glutathione levels in hearts. Under basal conditions, αBC expression had no effect on total glutathione levels (**Figure 18A**). Oxidized glutathione levels were slightly lower in cells expressing αBC, nevertheless, this difference did not reach statistical significance (**Figure 18B**). However, the expression of αBC did result in a 48% higher ratio of reduced glutathione to oxidized glutathione (**Figure 18C**).

Figure 15- **Measurement of total glutathione, oxidized glutathione, and reduced glutathione to oxidized glutathione ratios in hearts from αBC/HSPB2 knockout mice.** *This data was previously published:* Morrison LE, Whittaker RJ, Klepper RE, Wawrousek EF, Glembotski CC. *Roles for alphaB-crystallin and HSPB2 in protecting the myocardium from ischemia-reperfusion-induced damage in a KO mouse model.*   Am J Physiol Heart Circ Physiol. 2004 Mar;286(3):H847-55.

Panel A: Total glutathione was measured in hearts from wildtype and αBC/HSPB2 KO mice using a glutathione reductase cycling assay (n=3 for each group). *= $p < .05$ by Students T-test

Panel B: Oxidized glutathione was measured in the same samples following derivitization of reduced glutathione with 2-vinylpyridine. (n=3 for each group). *= $p < .05$ by Students T-test

Panel C: Ratio of reduced glutathione to oxidized glutathione; reduced glutathione was calculated from total and oxidized glutathione measurements. *= $p < .05$ by Students T-test

A. Total Glutathione

B. Oxidized Glutathione

C. GSH/GSSG Ratio
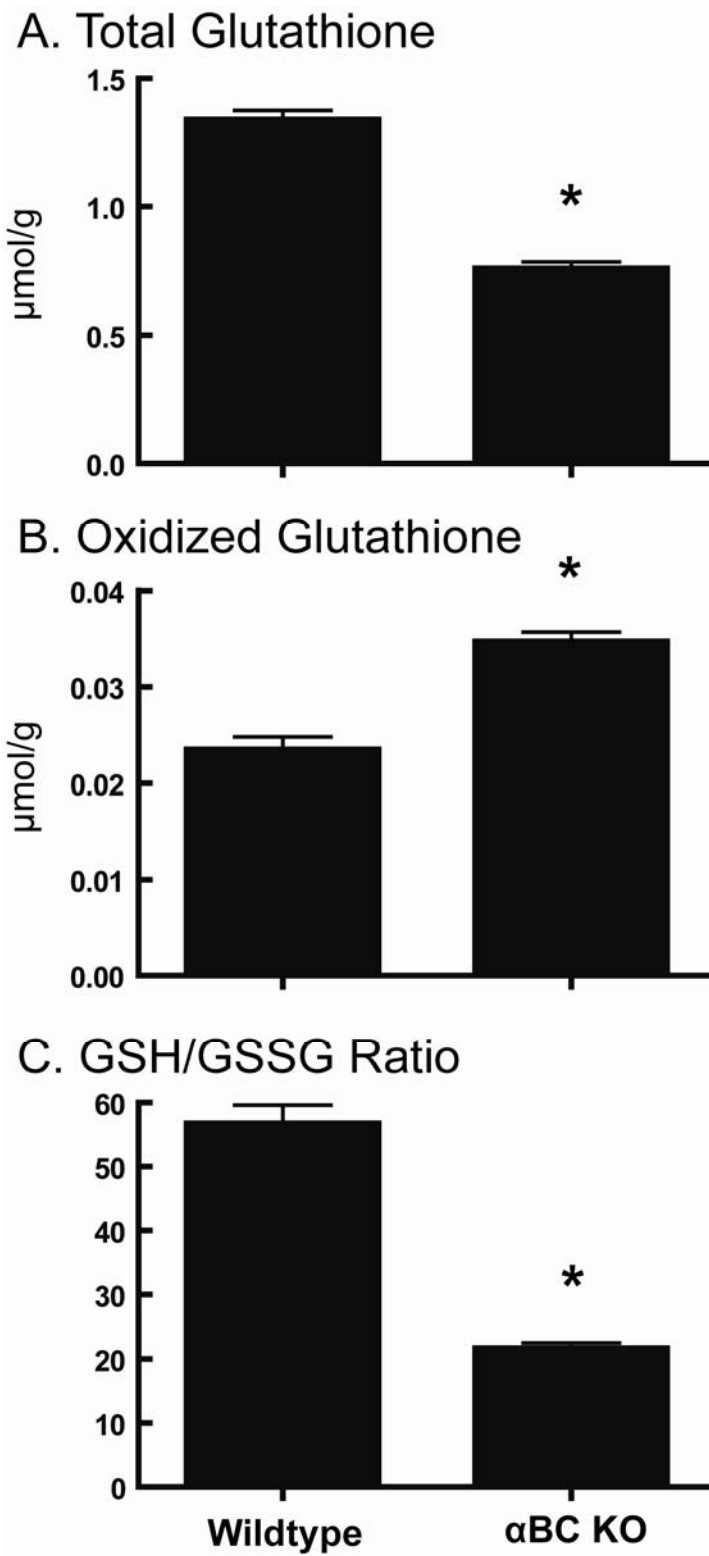
Figure 16- G6PD, GR activity and protein levels in αBC/HSPB2 knockout mouse hearts.

Panel A: G6PD activity was measured in hearts from wildtype and αBC/HSPB2 KO mice (n=3 for each group).

Panel B: GR activity was measured in hearts from wildtype and αBC/HSPB2 KO mice (n=3 for each group). *= p<.05 by Students T-test

Panel C: Protein levels of both G6PD and GR were analyzed by western blotting in hearts from wildtype (lanes 1-3) and αBC/HSPB2 KO (lanes 4-6) hearts.

A. G6PD Activity
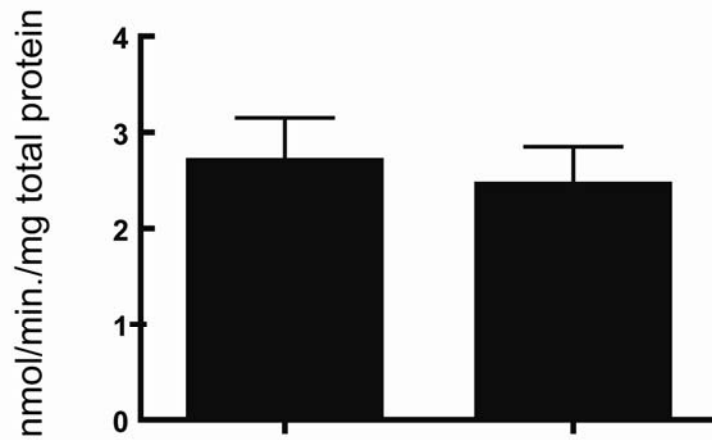
B. Glutathione Reductase Activity
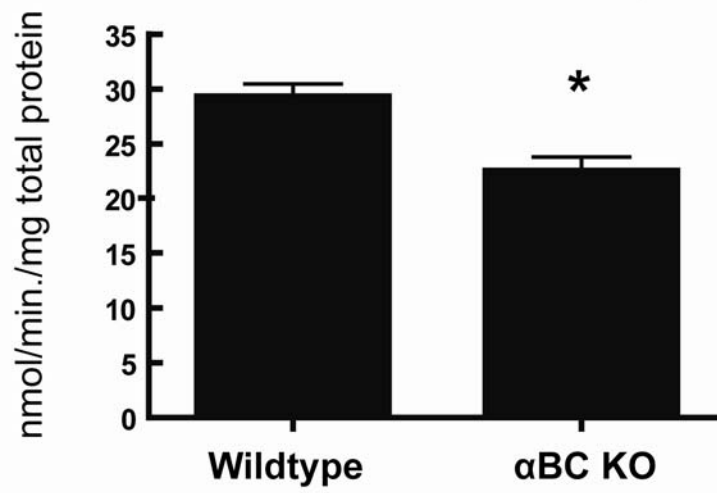
C. Protein Levels
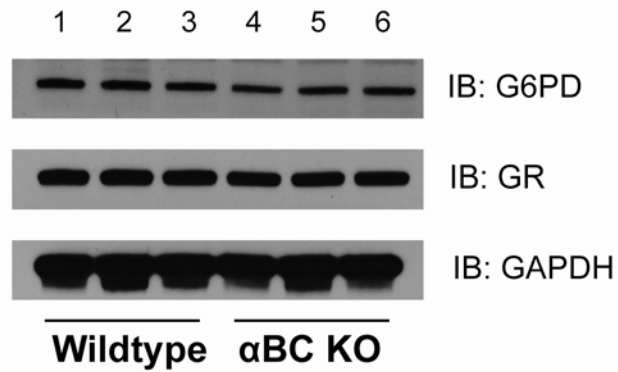
To examine if expression of αBC prevented loss of glutathione during stress HeLa cells were treated with 200uM H_2O_2. Following a 90 minute treatment with H_2O_2, total glutathione levels were found to be significantly higher in HeLa cells transfected with αBC when compared to cells transfected with control plasmid (**Figure 19A**). Conversly, oxidized glutathione levels were found to be significantly reduced in HeLa cells transfected with αBC (**Figure 19B**).   This resulted in a 230% higher reduced glutathione to oxidized glutathione ratio in cells transfected with αBC compared to cells transfected with control plasmid (**Figure 19C**). This suggested that the presence of αBC may support enhanced glutathione recycling.

Therefore, the effect of αBC expression on the activity of glutathione cycling enzymes was assessed in the HeLa cells. Expression of αBC had no effect on the activity of G6PD in HeLa cells (**Figure 20A)**. However, transfecting HeLa cells with αBC resulted in a 63% increase in GR activity when compared to cells transfected with control plasmid (**Figure 20B**). Protein levels of G6PD and GR were assessed by western blot analysis. Expression of αBC had no effect on protein levels of either G6PD or GR (**Figure 20C**). This again suggested that the presence of αBC enhances GR activity.

To determine if the enhanced glutathione levels had made the cells more resistant to oxidative stress, capase 3 activity was analyzed in cells transfected with or without αBC and treated with H_2O_2. Consistent with

enhanced glutathione levels providing protection against oxidative stress, caspase-3 activity was found to be reduced following treatment with H_2O_2 in cells transfected with αBC, when compared to cells transfected with control plasmid (**Figure 21**).

To determine if αBC interacted either directly or as part of a complex that included GR, immunoprecipitations were performed using cell extracts from HeLa cells that had been transfected with αBC plasmid. Immunoprecipitations were performed using either agarose beads with non-immune mouse antibody, as a control, or agarose beads with an anti-HA antibody. Control immunoprecipitations were negative for both αBC and GR by western blot analysis. However, using anti-HA beads resulted in co-immunoprecipitation of αBC and GR (**Figure 22A**). To better understand the nature of the interaction between αBC and GR, the enzyme activity of purified GR was analyzed *in vitro* in the presence and absence of αBC. Compared to a BSA control, αBC increased GR activity by a statistically significant 18% (**Figure 22B**).
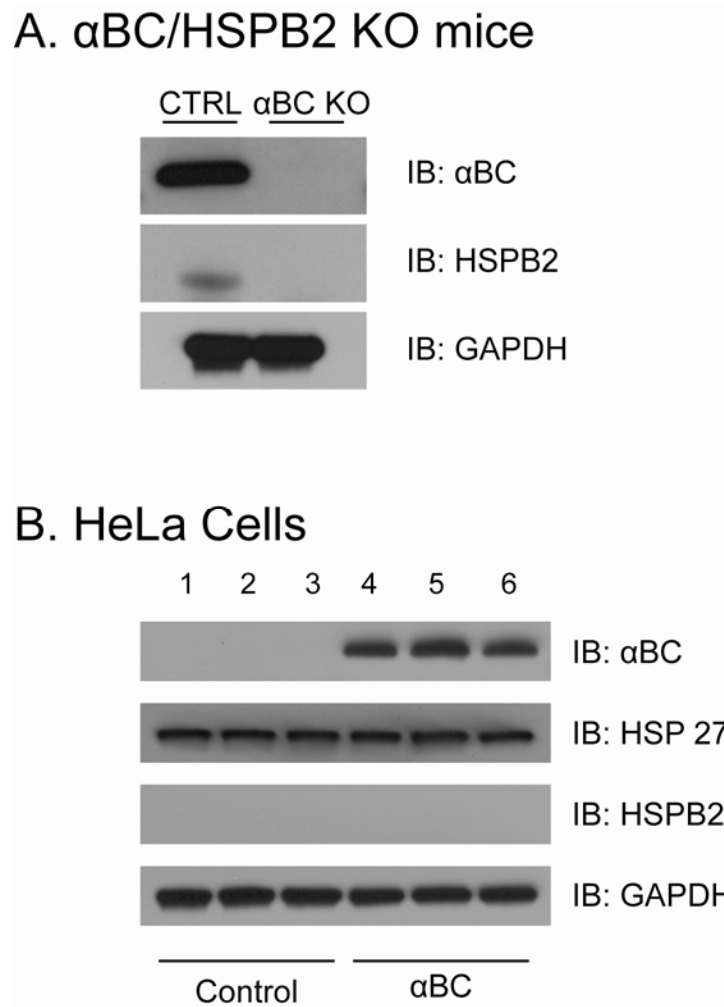
Figure 17- Comparison of protein expression in αBC/HSPB2 KO hearts and HeLa cells.

Panel A: Western blots to examine expression of αBC and HSPB2 in hearts from wildtype and αBC/HSPB2 KO mice.

Panel B: Western blots to examine expression of αBC, HSP27, and HSPB2 in HeLa cells transfected with either a control plasmid (lanes 1-3) or a plasmid which expresses αBC (lanes 4-6).

<u>Figure 18</u>- Measurement of total glutathione, oxidized glutathione and reduced glutathione to oxidized glutathione ratios in HeLa cells transfected with αBC plasmid.

Panel A: Total glutathione was measured in HeLa cells transfected with either control plasmid or and plasmid which expresses αBC using a glutathione reductase cycling assay (n=27 for each group).

Panel B: Oxidized glutathione was measured in the same samples following derivitization of reduced glutathione with 2-vinylpyridine. (n=27 for each group).

Panel C: Ratio of reduced glutathione to oxidized glutathione; reduced glutathione was calculated from total and oxidized glutathione measurements. *= $p < .05$ by Students T-test

A. Total Glutathione

B. Oxidized Glutathione

C. GSH/GSSG Ratio
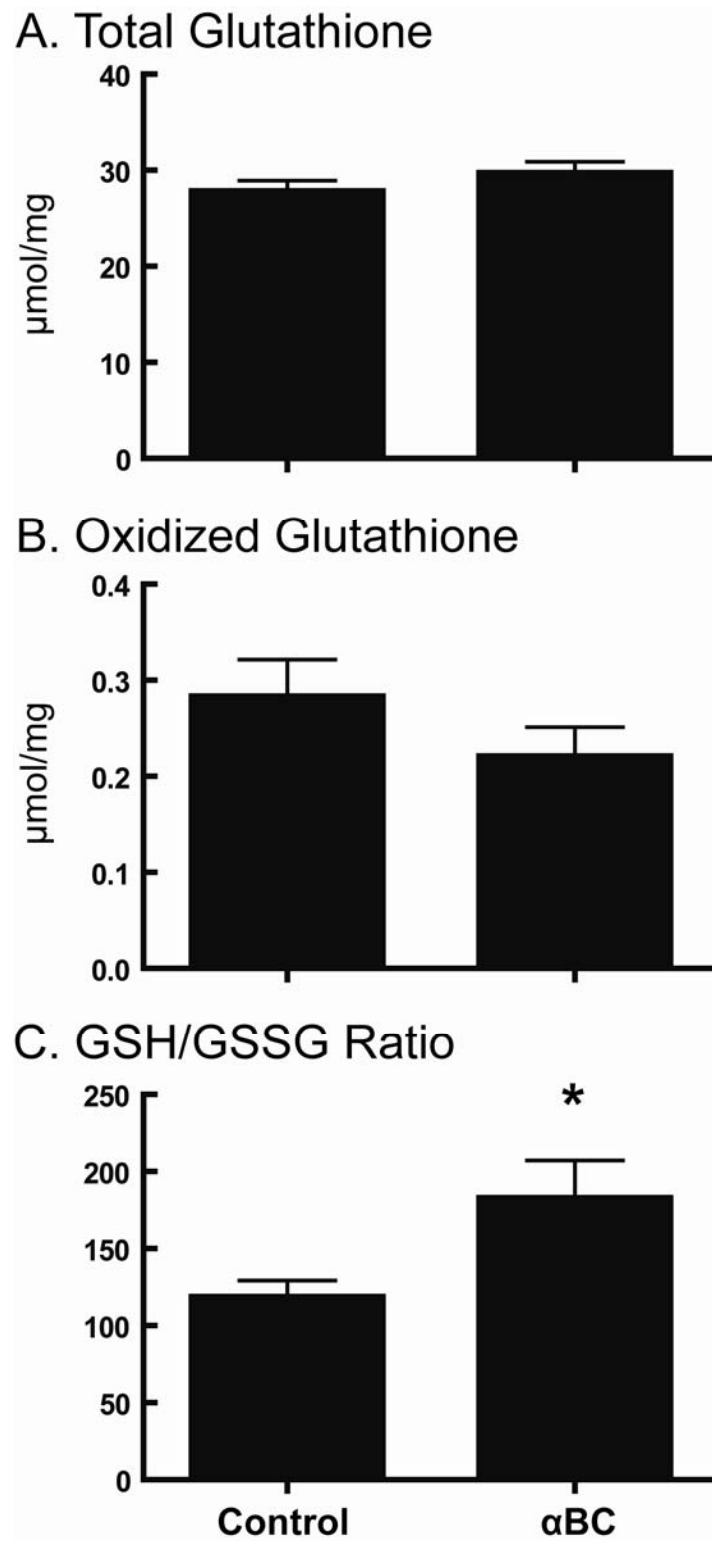
Figure 19- **Measurement of total glutathione, oxidized glutathione and reduced glutathione to oxidized glutathione ratios in HeLa cells transfected with of withour αBC plasmid and treated with 200μM H$_2$O$_2$ for 90 minutes.**

Panel A: Total glutathione was measured in HeLa cells transfected with either control plasmid or and plasmid which expresses αBC using a glutathione reductase cycling assay (n=27 for each group). *= p<.05 by Students T-test

Panel B: Oxidized glutathione was measured in the same samples following derivitization of reduced glutathione with 2-vinylpyridine. (n=27 for each group). *= p<.05 by Students T-test

Panel C: Ratio of reduced glutathione to oxidized glutathione; reduced glutathione was calculated from total and oxidized glutathione measurements. *= p<.05 by Students T-test

A. Total Glutathione

B. Oxidized Glutathione

C. GSH/GSSG Ratio
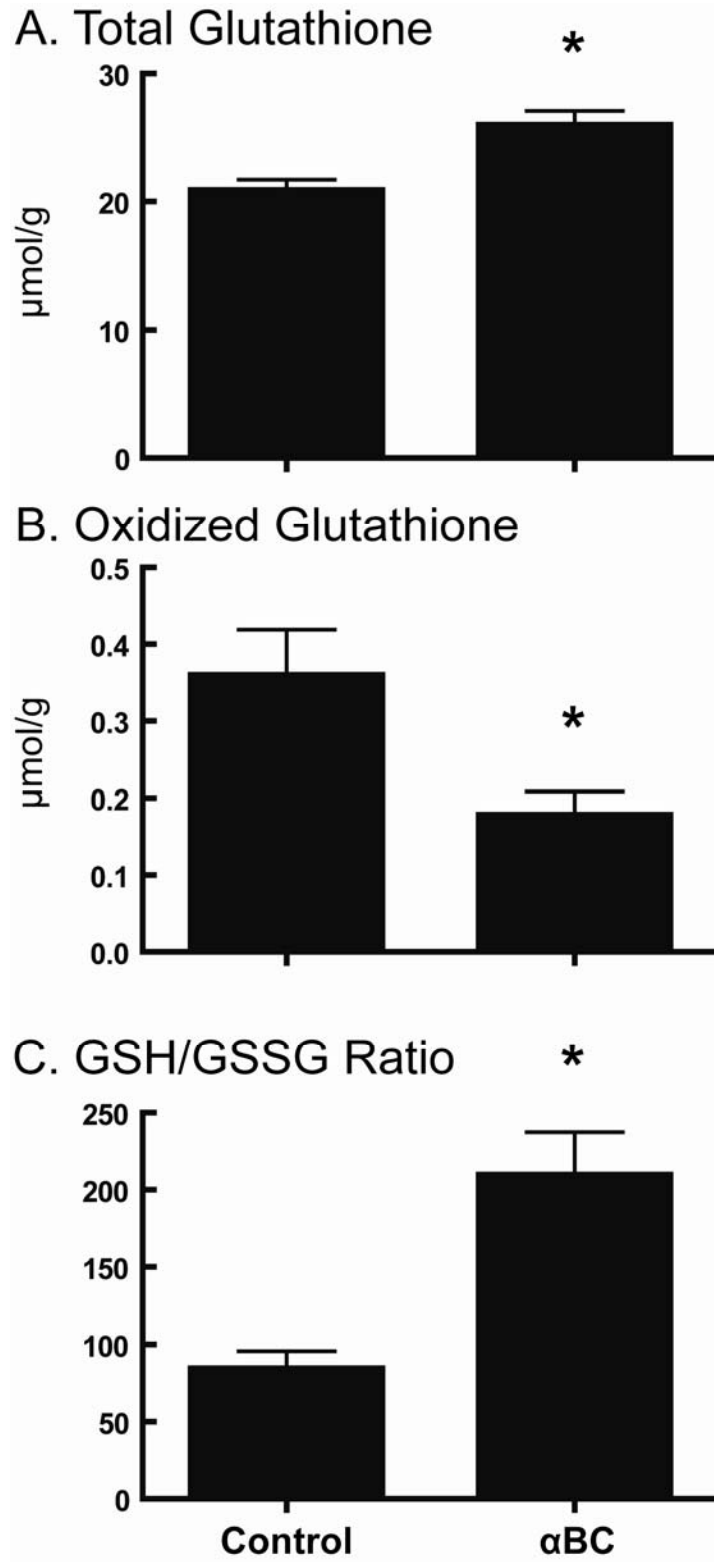
<u>Figure 20</u>- G6PD, GR activity and protein levels in αBC/HSPB2 knockout mouse hearts.

Panel A: G6PD activity was measured in HeLa cells transfected with either control plasmid (n=15) or a plasmid which expresses αBC (n=18).

Panel B: GR activity was measured in HeLa cells transfected with either control plasmid or a plasmid which expresses αBC (n=13 for each sample). *= p<.05 by Students T-test

Panel C: Protein levels of both G6PD and GR were analyzed by western blotting HeLa cells transfected with either control plasmid (lanes 1-3) or a plasmid which expresses αBC (lanes 4-6)

A. G6PD Activity
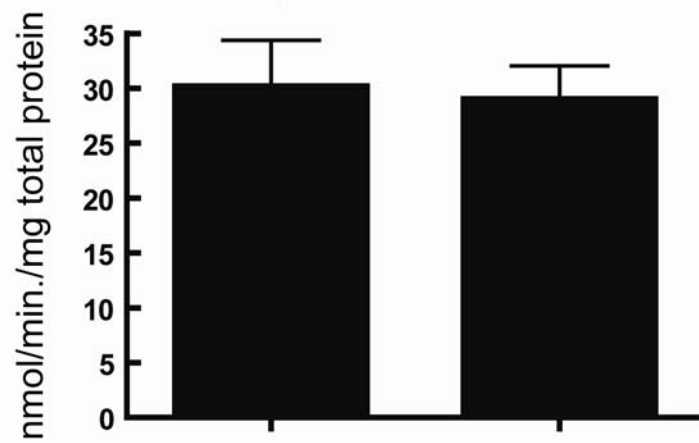
B. Glutathione Reductase Activity
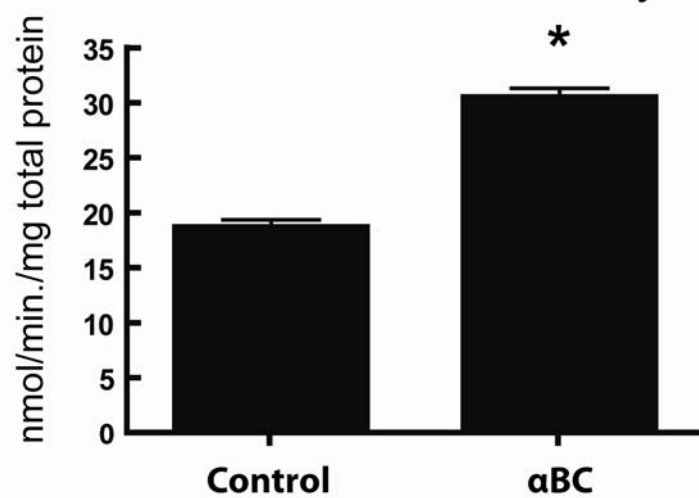
C. Protein Levels
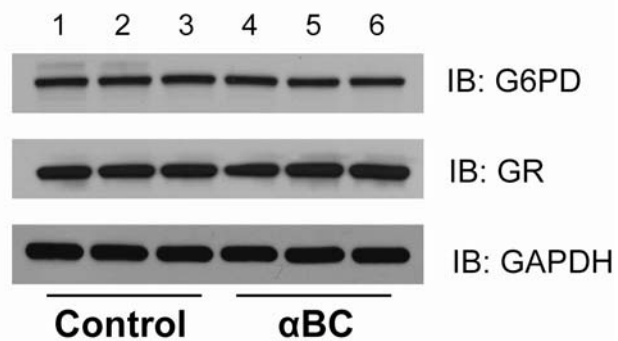
A. Caspase 3 Activity
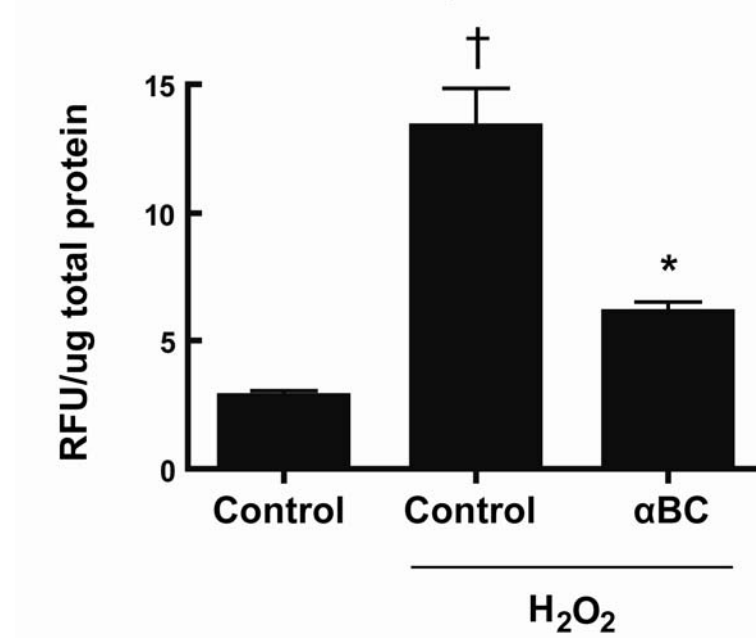
Figure 21- **Protection against hydrogen peroxide induced activation of apoptosis by αBC expression.**

Caspase 3 activity was measured using caspase 3 substrate that fluoresces upon cleavage of the target sequence. Activation of caspase 3 was measured in cell extracts from cells transfected with either control plasmid or αBC plasmid treated +/- 200μM H_2O_2 for 8 hours in minimal media. †= different from other groups by ANOVA, $p<.05$, *= different from other groups by ANOVA, $p<.05$,

4. Discussion

Cardiomyocytes require large amounts of ATP in order to contract and contribute to the pumping action of the heart. In order to meet this energy requirement mitochondria make up nearly 40% of the volume of the cardiomyocyte [206]. Even under basal conditions, approximately 2-5% of the oxygen metabolized by cardiomyocyte mitochondria result in ROS generation [14]. However, during the first few minutes of reperfusion following an extended period of ischemia, ROS generation increases nearly 7.5-fold over basal levels [20]. This rapid increase in ROS generation results in the cardiomyocyte entering oxidative stress, a condition where the cell's antioxidant systems are no longer able to detoxify ROS species at the same rate as they are being generated.

During oxidative stress, the cell is extremely susceptible to damage as ROS can attack nearly every component of the cell [17]. Understanding how antioxidant systems are controlled will help lead to antioxidant therapies to be dispensed as adjuvants to the reperfusion therapies currently administered to patients diagnosed with MI. In the present study, we found that αBC gene disruption reduced glutathione recycling in the mouse heart, resulting in lower levels of total glutathione and a lower reduced glutathione to oxidized glutathione ratio. This most likely arises from the significant decrease in GR activity seen in the knockout mice. Taken together, these results suggest that loss of αBC leaves the heart with a reduced ability to detoxify ROS. This is

A. Immunoprecipitation of αBC

IB: Glutathione Reductase

IB: αBC

Input   IP: Ctrl   IP: HA
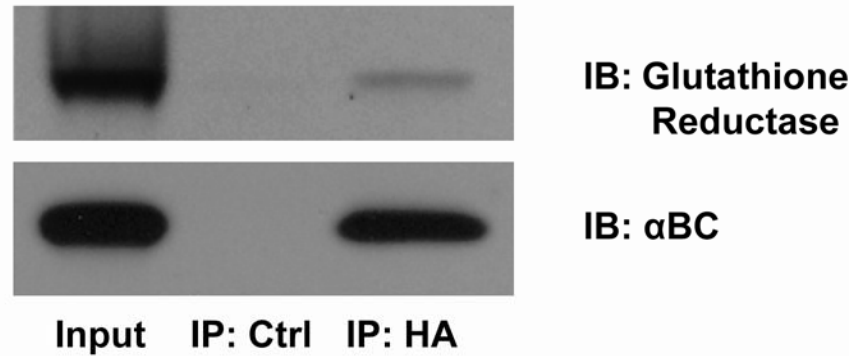
B. *in vitro* Gluathione Reductase Activity
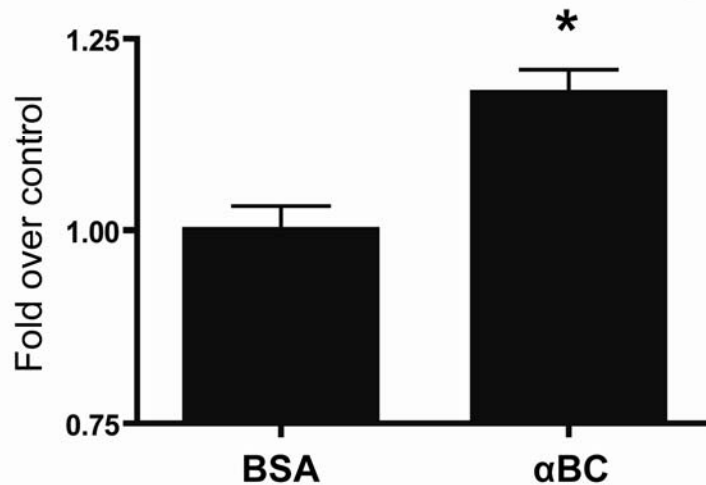
Figure 22- Co-immunoprecipitation of αBC and GR and enhancement of GR activity *in vitro* by purified αBC.

Panel A: Immunoprecipitation from whole cell cell extracts of HeLA cells transfected with αBC plasmid using either control or anti-HA antibodies, followed by western blotting for αBC and GR.

Panel B: *in vitro* GR activity assays using purified GR and purified αBC, BSA is used as a control protein (n=12 for each group). Proteins were added at a 1:4 molar ratio to GR.

consistent with our previous report that demonstrated that loss of αBC renders hearts more susceptible to I/R damage [162].

However, our mouse model is not perfect, the strain of mice that we studied also harbor a deletion of the related HSPB2 gene. Even though HSPB2 is expressed at much lower levels than αBC, it is formally possible that it contributes to enhancing glutathione recycling. Fortunately, HeLa cells provide an easily manipulated cell culture model that mimics the αBC and HSPB2 deletions of the knockout mice. Transfection of HeLa cells with a plasmid which expresses αBC results in an increase in the reduced glutathione to oxidized glutathione ratio. When the transfected cells were treated with H_2O_2 the cells transfected with αBC demonstrated significantly higher levels of total glutathione compared to control transfected cells. Following H_2O_2 treatment, levels of oxidized glutathione were significantly reduced in cells transfected with αBC and contributed to a much higher reduced glutathione to oxidized glutathione ratio in these cell. These changes are associated with an increase in GR activity observed in cells transfected with αBC.

Generally, a higher reduced to oxidized glutathione ratio suggests a cell is better able to detoxify ROS and prevent injury. Consistent with this theory, cells transfected with αBC experienced less and apoptosis following H_2O_2 treatment compared to cells transfected with control plasmid.

The question that remains, however, is why expression of αBC did not increase glutathione levels under basal conditions the same way it did in the heart? More than likely, this is due to several differences between the models. The first arises due to the cell physiology; HeLa cells are not packed with mitochondria the way that cardiomyocytes are, therefore the endogenous ROS production of HeLa cells is presumably much lower and places a lower demand on the antioxidant systems. The second reason stems from differences between studying cell cultures and tissues. Cells in culture experience a much higher oxygen tension than cells within the organ; therefore, they may be "pre-adapted" to growing in an environment that would stimulate more ROS generation.

Nonetheless, the results demonstrate that αBC can protect cells from ROS damage by increasing the efficiency of glutathione recycling. To understand the mechanism behind this protection, we explored the interaction of GR and αBC. Immunoprecipitation experiments confirm that these two proteins interact within the cell. However, co-immunoprecipiation tells us nothing about the nature of the interaction, specifically whether the two proteins interact directly or as part of a complex. To answer this question we explored the effects of including αBC in *in vitro* activity assays using GR. Compared to the BSA control, αBC increased GR activity 18%. While this might appear to be a rather modest increase, it is consistent with the 23% decrease in GR

activity seen in αBC/HSPB2 KO hearts. The increase is also noteworthy, considering all substrates where provided at saturating levels.

The nature of the interaction between the two proteins, and how that results in increased GR activity (**FIGURE 23**), remains to be determined. More than likely, the chaperone activity of αBC is somehow involved. Future studies using αBC mutants which lack or have reduced chaperone activity could provide insight in this regard. A requirement for the presence of a chaperone might suggest that GR is more active in an unstable conformation and that αBC helps it to maintain this conformation. Unfortunately, such hypotheses can't be explored readily without performing X-ray crystallographic studies. However, what is clearly apparent from this study is that enhancing glutathione recycling is yet another way that αBC can protect the heart from I/R.
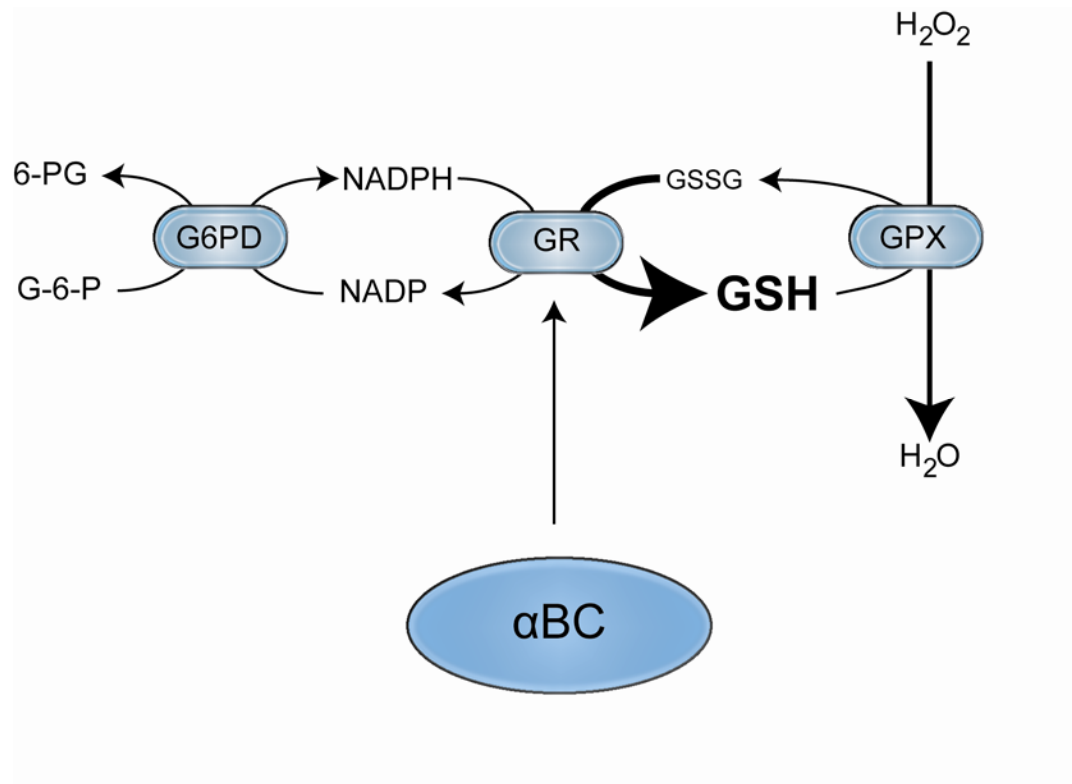
Figure 23- Summary of findings regarding the effects of αBC expression on gluthathione recycling

The results of this study demonstrate αBC can protect cells from oxidative stress by interacting with and increasing the activity of GR. This increases the ratio of reduced to oxidized glutathione and enhances the cells ability to detoxify ROS. This is consistent with the reduced caspase 3 activity seen in cells transfected with αBC prior to treatment with H_2O_2. 6-PG: 6-phospho-gluconate, G-6-P: glucose-6-phosphate, NADP(H): nicotinamide dinucleotide phosphate (reduced), GR: glutathione reducatse, GPX: glutathione peroxidase, GSH: reduced glutathione, GSSG: Glutahtione disulfide.

Chapter Four

1. Introduction

Cell penetrating peptides (CPP's) are a relatively new tool that may provide the answer to one of the most formidable obstacles in research and medicine, introducing exogenous hydrophilic molecules such as proteins into the cell without transgenesis [207]. The plasma membrane is perhaps the most complex organelle of the cell, but it's most basic functions, i.e. acting as a barrier and regulating import and export of molecules to and from the cell, provide a significant challenge to transferring what is learned in the lab to the clinical setting. The manipulations we make in the lab, such as introducing DNA or siRNA, cannot be easily or safely transferred to clinical practice. However, with the discovery of CPP's it may now be possible to not only transfer DNA and siRNA, but also proteins and small molecules across the plasma membrane and into cells [208].

Naturally occurring cell penetrating domains have been discovered in a number of proteins from a diverse array of organisms including, Drosophila, HIV-1 virus and yeast [209]. These cell penetrating domains have been shown to effectively cross cell membranes by themselves, as well as with attached cargo such as plasmids, proteins and small molecules. The term CPP encompasses both naturally occurring cell penetrating domains such as those found in HIV-tat protein and the drosophila antennapedia protein, and synthetic CPP's, such as arginine polymers [210] [207]. CPP's are generally

smaller than 30 amino acids and are rich in cationic residues.   Arginine appears to be the favored residue; the most efficient CPP's are generally rich in arginine residues. Interestingly, outside of a high proportion of arginine residues, there is little sequence or structural similarity between the various CPP's.

CPP mediated delivery of proteins has proven to be an effective way to deliver proteins into cells in a manner that preserves their biological activities. CPP's have been used effectively to deliver protective proteins, such as HSP27 and apoptosis repressor of caspase (ARC), to cardiomyocytes in culture and in *ex vivo* perfused hearts [211, 212]. Even more promising are studies that demonstrate CPP's can be used to deliver proteins to the hearts of mice *in vivo* [213].

CPP delivery of proteins potentially has several advantages over mouse models generated by genetic manipulations. Transgensis to generate over-expression models can result in undesirable transgene insertions, potentially creating genetic deletions that go undetected. Secondly, CPP delivery allows precisel control of the timing and the levels of the protein being introduced. Lastly, since it is unnecessary to breed out transgenic lines and maintain colonies, CPP delivery of proteins offer significant cost savings over traditional transgenic mouse models.

Importantly, beyond their use in the research laboratory, there is a very real potential for CPP's to become a vehicle for the delivery of therapeutics.

The sort of manipulations we can make in the laboratory, such as maintaining mitochondrial integrity during stress by expression of αBC phospho-mimetics, are not easily adaptable in the clinical setting. Furthermore, even if the sorts of genetic manipulations we perform in the lab were feasible in human subjects, long term, chronic over-expression of many proteins that are believed to protect the myocardium during I/R may not be desirable. Therefore CPP's, if they can live up to their expectations, may prove to be powerful tools for the development of the therapeutic potential of peptides.

For our work, we are interested in studying the effects of phosphorylation of αBC in protection of the myocardium during I/R. We decided that using CPP's to deliver different phosphomimetics of αBC might be a viable option that would allow us to manipulate not only the levels of the phospho-mimetics in the myocardium but also the timing of their presence in relation to the I/R progression. Therefore, in this study, we examined the use of CPP's to uncover the role of phosphorylation of αBC in protecting the myocardium from I/R related injury and whether this a viable approach to utilizing the protective effects of αBC.

2. Materials and Methods

A. Creation of tat-αBC expression constructs- Our lab had previously created mammalian αBC expression constructs for wildtype αBC and the phospho- mimetics, αBC-AAE and αBC-AAA in PCDNA3.1. The bacterial expression construct containing the Tat domain and a 6X histidine tag for purification was a generous gift from Dr. Steve Dowdy (UCSD). Wildtype αBC and the phospho-mimetics were excised from the PCDNA3.1 expression vectors by restriction digest with XhoI and were ligated into the Tat-expression construct and transformed into XL1-Blue cells (**Figure 24 A**). Clones were selected on agar plates with ampicillin. Clones with the αBC gene inserted in the right orientation were selected following sequencing and grown up to isolate plasmid DNA. BL-21 cells were transformed with the Tat-αBC constructs and utilized for expression and purification of the Tat-αBC proteins. Glycerol stocks of Tat-GFP and Tat-beta galactosidase to be used as control proteins were a generous gift from Dr. Roberta Gottleib (SDSU).

B. Purification of tat-αBC proteins- Tat-αBC proteins were purified as previously described [212]. Briefly 3 ml cultures were grown overnight @ 37°C with vigorous shaking in Luria broth (LB) with ampicillin. The following morning the 3ml cultures were added to 1L of fresh LB with ampicillin and allowed to incubate @ 37°C until they became cloudy, at this point, IPTG was added to a final concentration of 500µM. Incubation @ 37°C continued for 6

Figure 24- Cloning, expression and purification of tat-αBC proteins

Panel A: The tat-αBC proteins are fusion proteins that contain a 6X hisitidine tag for purification, the tat protein transduction domain, and an HA tag for detection.

Panel B: Several clones that expressed the tat-αBC proteins in an IPTG inducible manner were isolated.

Panel C: Purification on nickel-agarose columns resulted in isolated tat-αBC

protein that was essentially free of contaminating protein.

A. Tat-αBC construct design

6xHis	Tat-Domain	HA-Tag	αBC wildtype
6xHis	Tat-Domain	HA-Tag	αBC-AAE
6xHis	Tat-Domain	HA-Tag	αBC-AAA

B. Bacterial expression of Tat-αBC constructs
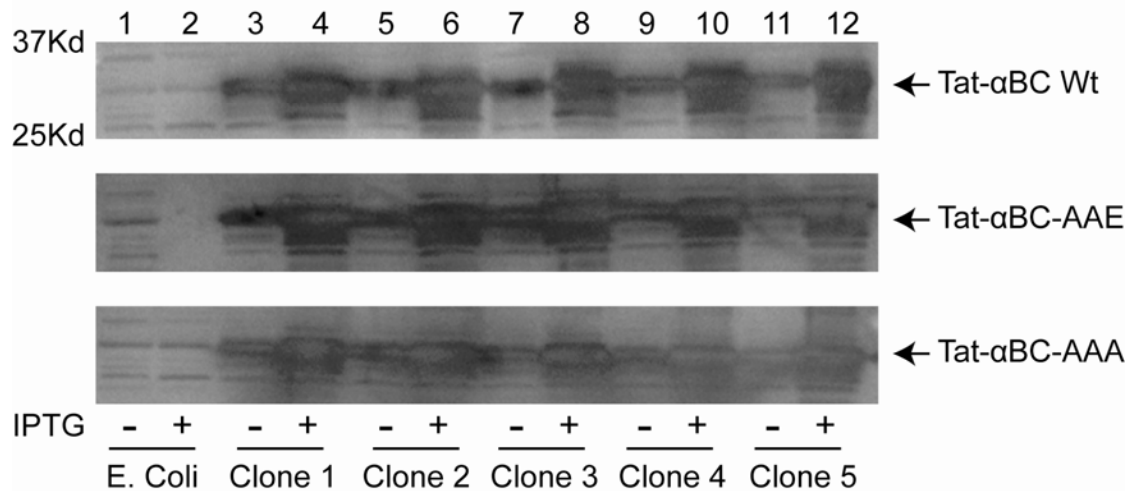
C. Purification of Tat-αBC constructs
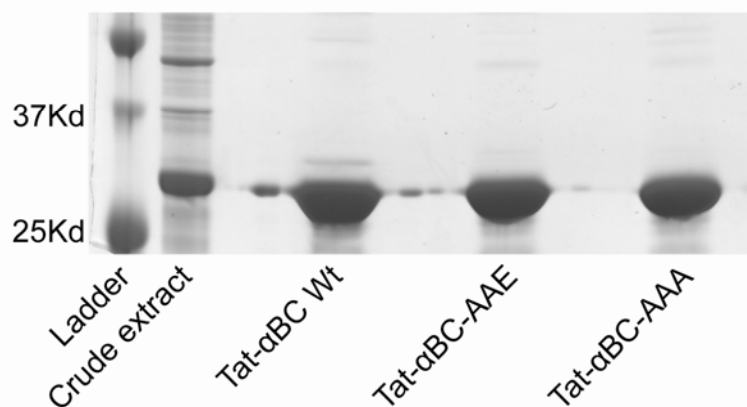
hours at which point the cultures were spun down, washed twice with 1xPBS and the pellets were frozen overnight. The next day the pellets were thawed and suspended in 10 mls buffer Z (8M urea, 100mM NaCl, 20mM HEPES pH 8.0). Cells were sonicated with 3, 20 second pulses with a minimum of 30 seconds on ice between pulses. The cells were then centrifuged at 20,000g for 20 min to pellet unbroken cells and cellular debris, the supernatant was collected. A 3ml Ni-NTA column is prepared and equilibrated with 50mls of buffer Z containing 10mM imidazole. The supernatant is applied to the column and allowed to flow through by gravity. The column is then washed with 50 mls of buffer Z with 10mm imidazole. The protein is eluted from the column with 10 mls buffer Z containing 250mM imidazole. The protein is refolded using PD-10 desalting columns per the manufacturer's directions. Purified protein is then concentrated using a Millipore Centricon 70.

C. Treatment of NRVCM's with tat-αBC proteins- NRVCM's were isolated as described in chapter one, and cultured in DMEM/F12 with 10% FBS for 2 days following isolation. On the third day tat-αBC proteins were added to the media in various concentrations. After one hour, the media was replaced with fresh media.

D. Confocal microscopy- Tat-αBC was conjugated to Texas-red fluorophore and re-purified on PD-10 desalting columns to exclude unconjugated label prior to being applied to NRVCM's. Following a 1 hour treatment with 200μM tat-αBC labeled with texas-red cells, were washed twice with 1XPBS and fixed in 4% formaldehyde in PBS. The cells were then stained for α-actinin using a FITC secondary and stained with TOPRO to identify nuclei. Images were captured on a Leica confocal microscope and associated software.

E. Heat shock Assay- Analysis of αBC translocation upon heat shock was performed as previously described[168]. Briefly, following incubation with purified tat-αBC proteins, NRVCM's were placed at 44°C 30 minutes. Cells were then trypsinized, collected and washed twice with 1X PBS. Cell pellets were resuspended in lysis buffer (10mM Tris pH 7.5, 10mM NaCl, 5mM $MgCl_2$, 0.5% Triton x-100, 1mM PMSF) and then centrifuged at 300g for 5 minutes. Supernatant was collected and the pellet is resuspended in 4X laemelli buffer. Equal proportions of each sample were seprated by SDS-PAGE and analyzed by western blotting.

F. ADH Aggregation assay- Examination of heat-induced denaturation of Alcohol dehydrogenase in the presence of various forms of αBC was

performed as previously described with modifications for use in a 96 well plate format [214].

G. Creation of αBC constructs for R9-C conjugation- αBC-AAE had been previously cloned into the PrsetA vector in order to purify it from E.Coli. The αBC-containing Prset plasmid was purified from an existing glycerol stock. The mutation to allow conjugation of the purified αBC-AAE to the R9-C peptides was added by utilizing mutagenesis with the primers: 5' gataaggatcgatgcggatccgagctcg 3' and 5' cgagctcggatccgcatcgatccttatc 3'. The mutants were transformed into BL-21 cells and purified in the same manner described above.

H. R9-C peptides- R9-C peptides were acquired at >95% purity from Simga Genosys.

I. Linkage of R9-C and αBC-AAE- Linkage of the modified αBC-AAE protein and the R9-C peptide were accomplished by incubation of the peptide and the protein in equimolar concentrations in 50mM Tris pH 7.0 for 1 hour at room temperature.

3. Results

Wildtype αBC, αBC-AAE and αBC-AAA were excised from previously created mammalian expression contructs and ligated into the ptat-expression construct, a generous gift from Dr. Steve Dowdy. These constructs produce protein products with a 6X histidine tag, a tat protein transduction domain, an HA-tag and either wildtype or one of the mutant forms of αBC (**Figure 24A**). BL-21 cells were transformed with the tat-αBC constructs. Clones were selected and analyzed for expression levels and IPTG inducibility (**Figure 24B**). All five clones of each tat-αBC construct were found to express protein at similar levels. One clone for each construct was selected and used throughout the remainder of the study. The tat-αBC proteins were purified using a nickel agarose column. Each of the tat-αBC protein preparations were shown to be nearly free of contaminating proteins by coomassie staining of SDS-PAGE gels (**Figure 24C lanes 4,6,8**).

Following purification, uptake of the tat-αBC proteins was tested in NRVCM's. Tat-αBC proteins were incubated with cells at 50 µM, 100µM and 200µM to examine dose-dependent uptake of the proteins. While all three proteins were taken up by the cells, only tat-αBC wildtype demonstrated a distinct dose-dependent uptake (**Figure 25A**). To ensure that the tat-αBC proteins were being internalized and were not just coating the surface of the cell, tat-αBC wildtype was visualized by confocal microscopy. Prior to being applied to the NRVCM's, Texas-red flourphore was covalently attached to the

tat-αBC wildtype fusion protein. Following a one hour incubation with the labeled tat-αBC wildtype, NRVCM's were fixed and stained for α-Actinin. TOPPRO was used to stain cell nuclei. The texas-red labeled tat-αBC wildtype protein was found throughout the cell, consistent with internalization of the protein (**Figure 25B**).

Following confirmation that the tat-αBC fusion proteins were being taken up into the cells, several functional assays were performed with cells that had been treated with tat-αBC proteins (data not shown). However, after experiencing mixed results from these experiments, the integrity of the protein once it entered the cell was in question. Therefore, several experiments were undertaken to determine if the tat-αBC proteins were behaving in a similar manner to endogenous αBC.

To test if the tat-αBC proteins distributed appropriately within the cell, and to see if they responded to stress in a manner similar to endogenous αBC, NRVCM's were incubated with the tat-αBC proteins and subjected to heat shock. Following heat shock, the cells were fractionated in buffer with mild detergent and centrifuged at 300g for 5 minutes. The soluble fraction (supernatant) and the insoluble fraction (pellet) were then analyzed for the presence of αBC by western blotting. Under non-stressed conditions endogenous αBC was found predominantly in the soluble fraction as expected (**Figure 26A lanes 1,5, and 9**). However, following heat shock, a majority of

A. Uptake of Tat-αBC protein by NRVCM's
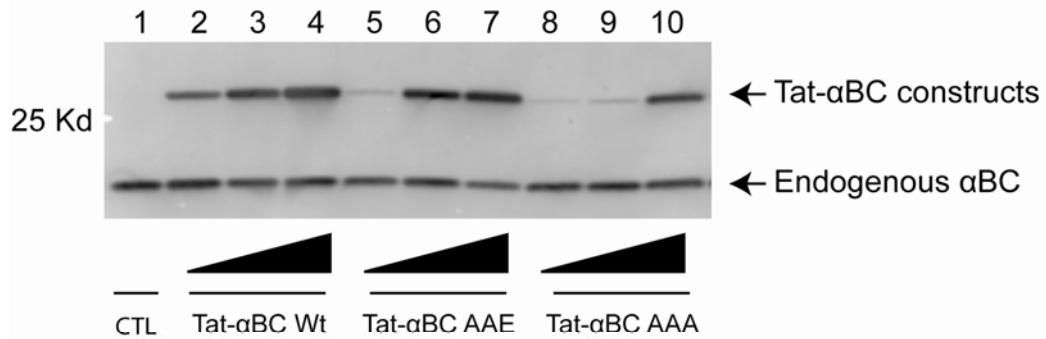
B. Confocal examination of Tat-αBC uptake
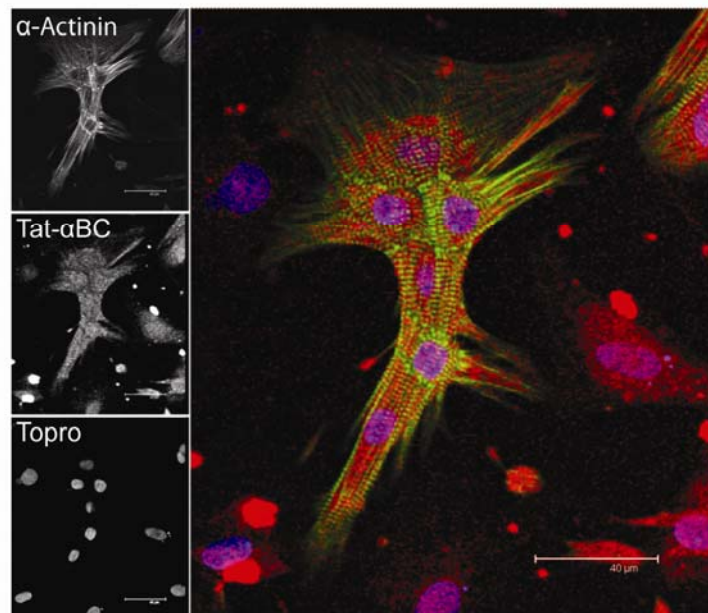
Figure 25- Uptake of tat-αBC protein by NRVCM's

Panel A: Western blots examining the uptake of tat-αBC fusion proteins by NRVCM's. NRVCM's were incubated with increasing concentrations of the fusion proteins ranging from 50nM to 200nM.
.
Panel B: Confocal analysis of tat-αBC uptake was performed using tat-αBC wildtype covalently linked to texas red flurophore.

endogenous αBC translocates to the insoluble fraction (**Figure 26A lanes 4,8, and 12**). On the other hand, the tat-αBC proteins were found exclusively in the insoluble fractions in both unstressed and stressed cells (**Figure 26A lanes 6,8,10, and 12**).

There have been quite a few reports that have shown that αBC can protect against the heat induced denaturation of proteins *in vitro* [154, 174, 214-216]. Therefore, the chaperone activity of the tat-αBC wildtype protein was assessed by examining its ability to prevent heat induced denaturation and aggregation of alcohol dehydrogenase (ADH), a protein that αBC has been previously shown to chaperone. ADH aggregation at 44°C was unaffected by either BSA (protein control) or tat-αBC wildtype (**Figure 26B**).

Since it appeared that fusing αBC to the tat domain had destabilized the protein and eliminated its chaperone activity a new plan was devised to develop a delivery platform based on CPP transduction which did not require the creation of fusion proteins or covalent linkages since appeared that the additional sequence was causing destabilization of the protien. Instead this new system relies on creating a disulfide bond between the CPP and the cargo protein. This disulfide bond should be fairly stable in the weakly reducing environments of cell media and blood. However, in the more strongly reducing intracellular environment it should be reduced resulting in release of the cargo protein from the CPP. This strategy has been used successfully with the tat transduction domain[217]. For this application a synthetic CPP,

R9-C, consisting of 9 arginines and a cysteine, to create the disulfide bond with cargo proteins, will be employed. The R9 motif was selected since it has been demonstrated to have one of the highest protein transduction efficiencies of all the known CPP's [218].

Since αBC-AAE is known to be the most protective form of αBC, it became the form that was focused on during the development of the αBC-R9-C delivery platform [152]. Alpha-BC does not contain any cysteins residues; therefore, in order make αBC-AAE compatible with this system a cysteine needed to be added to allow formation of the disulfide bond. In order to minimize structural changes that may occur by introducing a cysteine it was placed within the 9 amino acids that remain following enterokinase (EK) cleavage of the purified protein (**Figure 27A**). The R9-C peptide was synthesized and purified, *in vitro* (**Figure 27B**).

Linking the modified αBC-AAE protein and the R9-C peptide is accomplished by incubating the protein and the peptide in equimolar concentrations in 50mM Tris pH 7.4 at room temperature for 30 minutes (**Figure 27C and Figure 28A, lane 2**). To demonstrate that the linkage is sensitive to reduction, 5mM dithiotheritol (DTT) was added at the end of the reaction and resulted in elimination of the linked molecule (**Figure 28A, lane3**). Since it had been successfully demonstrated that the linkage between αBC-AAE and the R9-C peptide could be formed, the ability of the linked molecule to deliver αBC-AAE was tested in HeLa cells. Cells were incubated

with 100µM of the linked protein for 30 minutes, following the incubation cells

were trypsinized and collected. Delivery of αBC-AAE by the R9-C peptide was

confirmed by western blotting (**Figure 28B, lanes 5-7**). Delivery of αBC-AAE

was dependent on the presence of the R9-C peptide as shown by the failure

of the αBC-AAE protein alone to enter the cell (**Figure 28B, lanes 2-4**).

A. Heat Shock Induced Translocation of αBC

B. ADH Aggregation Assay
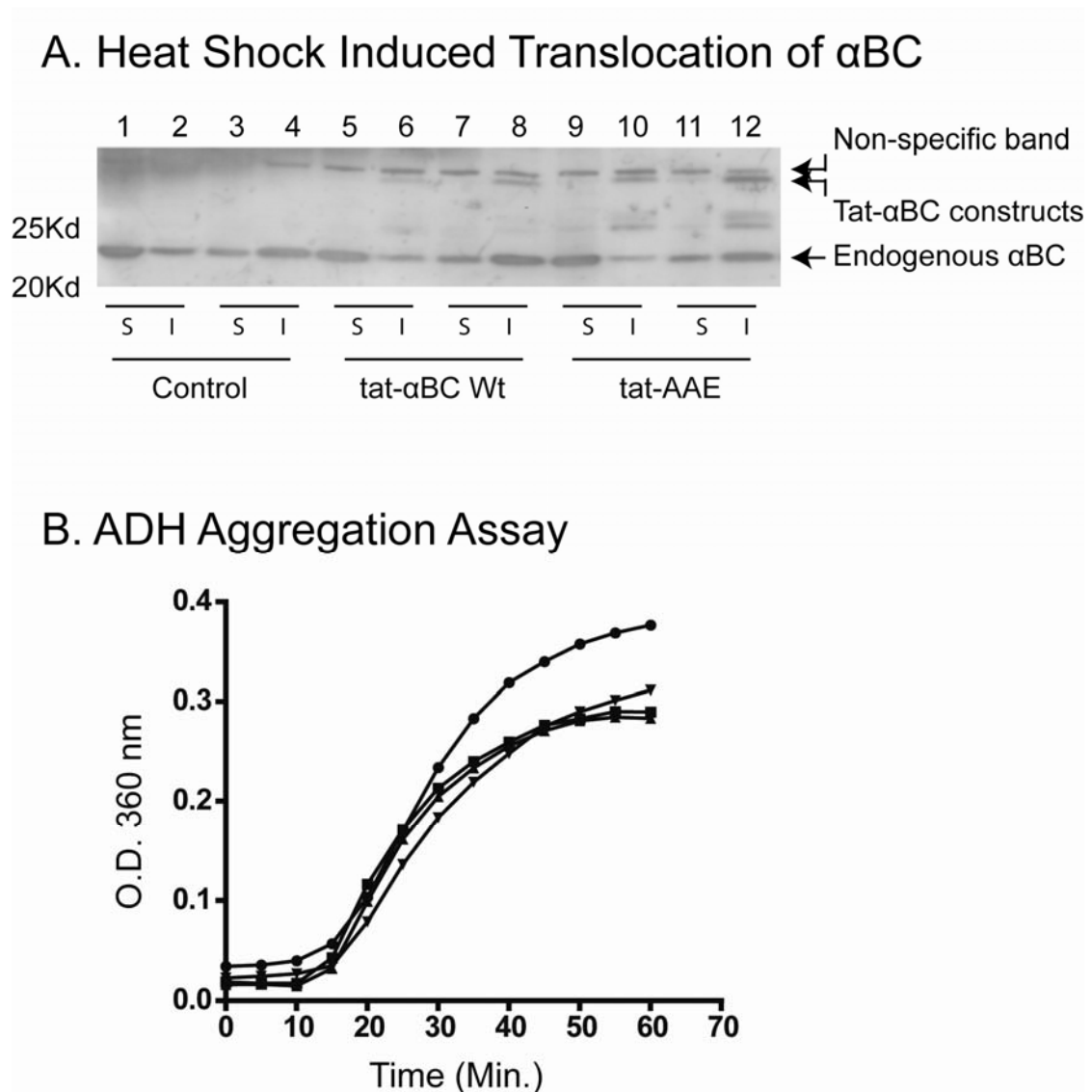
Figure 26- Inappropriate partitioning and instability of the tat-αBC proteins

Panel A: NRVCM's were incubated with tat-αBC constructs for 1 hour prior to heat shock. Following heat shock the cell extracts were separated into solube and insoluble fraction and analyzed by western blot for the presence of endogenous αBC and the tat-αBC constructs.

Panel B: ADH aggregation in the presence of either ADH Alone (squares), ADH and BSA (1:4 molar ratio, triangle), ADH and tat-αBC (1:2, upside down triangle) or ADH and tat-αBC (1:4,circles)

Figure 27- Design of the R9-C delivery platform

Panel A: Sequence modifications made to the PrSET-αBC construct to faciliatate linkage to the R9-C peptide

Panel B: The R9-C peptide

Panel C: A schematic of the molecule following the linkage reaction between R9-C and AAE

A. Amino Acid Sequence of the AAE Protein

```
MRGSHHHHHH GMASMTGGQQ MGRDLYDDDD KDRCGSELED IAIHHPWIRR
PFFPFHAPSR LFDQFFGEHL LESDLFSTAT SLAPFYLRPP SFLRAPEWID
TGLSEMRMEK DRFSVNLDVK HFSPEELKVK VLGDVIEVHG KHEERQDEHG
FISREFHRKY RIPADVDPLT ITSSLSSDGV LTVNGPRKQA SGPERTIPIT
REEKPAVTAA PKK
```

Key feature of the modified αBC Protein

- αB-Crystallin Sequence
- Location of AAE modifications
- Cysteine added to permit linkage to R9C
- 9 Amino acids that remain following enterokinase cleavage
- Sequence removed following enterokinase cleavage

B. Amino acid Sequence of the R9 Peptide

RRRRRRRRRC

C. Schematic of the molecule when it is ready for application

```
DRCGSELE-|  AAE Protein
        |
        S
        |
        S
        |
RRRRRRRRRC
```
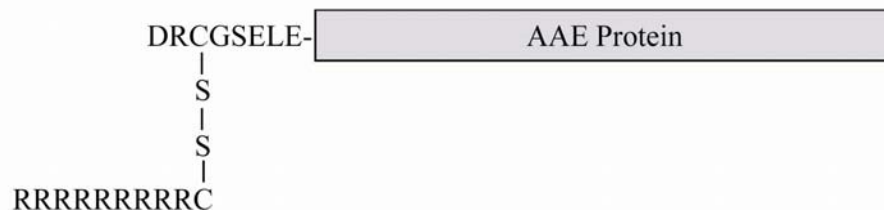
A. *in vitro* linkage of AAE and R9C
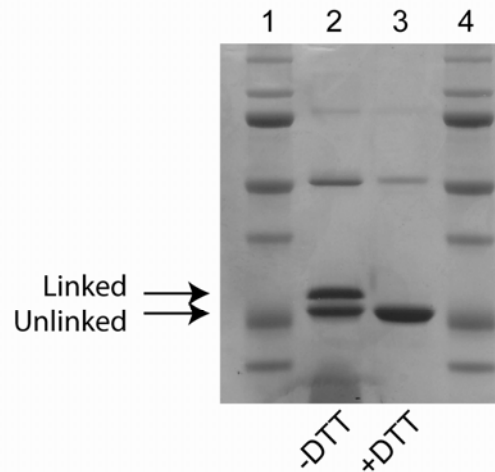
B. Delivery of AAE-R9C into HeLa Cells
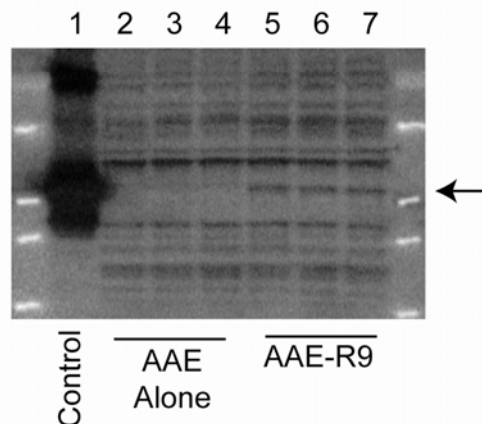
Figure 28- Linkage of R9-C and AAE and delivery of AAE into HeLa cells

Panel A: The linkage reaction is carried out *in vitro*, following the 30 minute incubation time a protion of the linkage reaction was treated with or without DTT, separated on SDS-PAGE and visualized by Coomassie stain. Apporximatley 50% of the AAE was linked with the R9 following the linkage reaction (lane 2). Addition of DTT uncouples the the peptide and its cargo (lane 3).

Panel B: HeLa cells were incubated for 30 minutes with AAE alone or AAE linked to the R9-C peptide. Cells were collected by trypsinization and analyzed by western blot for the presence of AAE in the cell extract. AAE was found in the extracts fo cells incubated with the R9-linked protein (lanes 5-7), but not in the cells incubated with aee alone (lanes 2-4)

4. Discussion

Unfortunately, due to time constraints, this project did not advance much beyond the creation of the delivery platform. There is still extensive testing to be done regarding the efficacy of this system in delivering not only αBC-AAE, but other proteins and even small molecules to cells in culture as well as organs in both *ex vivo* and *in vivo* studies. However, what has been demonstrated is that the linkage can be made between the R9-C peptide and, in this case, αBC-AAE, and that the linked protein is deliverable, at least to HeLa cells.

The most popular method for delivering proteins via CPP's has been to create fusion proteins consisting of the CPP and the protein of interest[209]. However, this method can be unreliable as the addition of the CPP sequence and other related sequences can alter protein folding leading to destabilization and loss of function in the protein of interest. The results from the studies utilizing the tat-αBC fusion proteins are a prime example. From discussions with others in the field that have experience with CPP fusion proteins, this is common and only a third of CPP fusion proteins that they have created functioned as expected.

The system created here is unique due to the placement of the cysteine in the sequence downstream from the EK site, but upstream of the multiple cloning site in the vector. The expression vector can now be used to make any protein compatible with this system. This creates a powerful tool for both

research and the development of therapeutics as it becomes simple to insert proteins into the vector, express and purify them and link them to the CPP peptide. What we have created is essentially a modular system for the transduction of proteins across cell membranes. The combination of CPP peptide and cargo proteins can be mixed and matched nearly effortlessly to find the right combination that provides the desired result. Continued development of this package could lead to several interesting research and therapeutic tools.

Chapter Five

1. Perspectives

In the course of performing scientific research, the most intriguing results aren't necessarily the answers the data provide; instead, it's the new questions that arise. Presented here are two studies that define two new roles for αBC in protecting the myocardium. The first study demonstrates that during ischemia, αBC translocates from the cytosol to the mitochondria resulting in the inhibition of cytochrome c release, possibly through interactions with VDAC and BAX. The second study establishes a role for αBC in supporting glutathione recycling in a manner that protects cells from oxidative stress. The results from both studies demonstrated that αBC inhibits apoptotic signaling. However, perhaps the more interesting convergence of these two studies is created by the questions that arise regarding compartmentalization of redox environments within distinct organelles.

The intraorganellar environments vary from organelle to organelle, for instance, the redox status of the ER is shifted toward a more oxidizing environment, presumably to assist in folding proteins by inhibiting unfavorable disulfide bond formations. However, in contrast to the ER, mitochondria are believed to be shifted towards a more reducing environment. The different environments are established by the redox machinery and the reducing equivalents present within the organelle. The same glutathione recycling

components are present within each organelle, but there are organelle-specific

differences in the protein levels and activities of the redox cycling enzymes.

Given, the findings in the two studies discussed above, the question

arises as to whether αBC, when it translocates to the mitochondria during

stress, affects the redox status of the mitochondria through its ability to

enhance GR activity. The data demonstrate that αBC may exist within the

mitochondria, which suggests that it would have the ability to interact with

intramitochondrial GR and, perhaps, increase glutathione recycling, shifting

the redox status towards a more reducing environment. Moreover, glutathione

import is stimulated by malate and pyruvate [219]. Interestingly, the MudPIT

analysis found that mitochondrial αBC co-immunoprecipitated with a malate

carrier protein.

To add yet another layer of complexity are the preliminary findings that

αBC may play a role in regulating metabolism. The MudPIT analysis of

mitochondrial αBC immunoprecipitations identified several members of the

electron transport chain as well as proteins involved in β-oxidation. The

electron transport chain proteins identified are located within complex I and V.

Dr. Ivor Benjamin's lab has reported reduced complex I activity in αBC/HSPB2

knockout mice [220]. Furthermore, when they examined skinned fibers from

the same knockout mice, they found reductions in ATP that were much larger

than would be expected for the changes observed in their mitochondrial

respiration studies. This may suggest that ATP synthase is affected by the

loss of αBC; our preliminary MudPIT results show that αBC co-immunoprecipitiates with at least two ATP synthase subunits. The effects of αBC on mitochondrial respiration definitely warrant further examination. The interactions with VDAC, beyond possible inhibiting cytochrome c release, may also play role in regulating mitochondrial metabolism.

In addition to examining these new roles for αBC, the possibility that the R9-C, CPP based, protein transduction system could be developed as a research tool, or perhaps even a therapeutic, deserves further examination. This system, or one similar to it, may prove invaluable in harnessing the protective effects of proteins, such as αBC. Since it lies downstream of several complex signaling pathways, modulation of αBC levels and /or phosphorylation status may not lend itself well to traditional small molecule drugs. Additionally, the effects of small molecule drugs can take hours or days to occur. This is well beyond the time frame of the early, damaging events of acute ischemic injury; however, CPP transduction has been shown to occur rapidly with uptake of tat-βgal into the mouse heart occurring within 15 minutes of delivery via intraperotineal injection, which is within the therapeutic window to minimize mycoyte cell death during MI [213]. The literature suggests that αBC may be one of the most powerful pro-survival factors of the heart during I/R. However, there is a large gap between being able to recognize a protective agent and being able to harness its power, hopefully, either R9-C or some other technology will one day be able to bridge that gap.

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