Mechanisms of Apoptosis in the Heart

By Kelly Marianne Regula

A thesis submitted to the Faculty of Graduate Studies in Partial Fulfillment of the Requirements for the Degree of

Doctor of Philosophy

Department of Physiology
Faculty of Medicine
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THE UNIVERSITY OF MANITOBA

FACULTY OF GRADUATE STUDIES

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ABSTRACT

There is an increasing awareness that programmed cell death or "apoptosis" may contribute to cardiac cell death and ventricular dysfunction; however, the molecular mechanisms that underlie this cell death phenomenon are poorly defined. In this thesis, I investigated the underlying mechanisms of cardiac apoptosis with specific attention to the role played by oxygen deprivation, the tumor suppressor protein p53 and BNIP3, a recently identified prodeath member of the Bcl-2 gene family.

Using post-natal ventricular myocytes, I ascertained that p53, independent of DNA binding and transactivation, was sufficient to provoke apoptosis of ventricular myocytes. I also demonstrated that a mutation that rendered p53 defective for transactivation was sufficient to provoke mitochondrial defects including permeability transition pore opening, loss of mitochondrial membrane potential and cytochrome c release. In related studies, I demonstrated that hypoxia was sufficient to trigger mitochondrial defects and apoptosis of ventricular myocytes. Moreover, I successfully cloned BNIP3 and found it to be up-regulated in ventricular myocytes during hypoxia. Importantly, I determined that a mutant form of BNIP3, defective for mitochondrial targeting, was sufficient to suppress hypoxia-mediated cell death of ventricular myocytes.

The data suggest that the mitochondrial death pathway is an important component of the apoptotic signaling cascade in ventricular myocytes. Therapeutic interventions designed to abrogate mitochondrial dysfunction may prove beneficial in averting apoptosis in patients with heart failure.

ACKNOWLEDGEMENTS

I would like to thank my parents, Gordon and Mildred Regula, for their love and patience. They taught me the value of a good education and supported my efforts through each learning curve. I'd also like to thank my brother and sister-in-law, Jason and Pam, for introducing me to a world beyond biology.

My gratitude is extended to the Manitoba Health Research Council and the Canadian Institutes of Health Research for their continued financial support of my research endeavors and graduate training.

I would like to thank the many members of the Kirshenbaum laboratory who I have had the great privilege to work with over the past 6 years: Danielle De Moissac, Hui Zheng, Shareef Mustapha, Rhonna Gurevich, Joseph Bednarczyk, Irwin Eydelnant, Christine Robinson, Karen Ens, Julie Roth, Erin Oliver-Landry, Tong Zhang and Marek Rzeszutek. Their technical assistance, scientific insight and friendship have been an integral part of my graduate training experience.

I am grateful to my advisory committee members past and present: Dr. Naranjan Dhalla, Dr. Pawan Singal, Dr. Francis Amara and Dr. Ian Dixon for their encouragement and advice throughout my graduate training.

Finally, I would like to thank my supervisor, Dr. Lorrie Kirshenbaum. I could not have asked for a better mentor. As leader in his field, he was an immeasurable resource of knowledge and life experience from I which I was able to learn. Despite his busy schedule and many responsibilities, he was always there to support and guide me. He inspired me to think outside the box and believe in myself. His philosophy was simple:

"if you think you can or you think you can't, you will." I will never forget the lessons I learned from Dr. Kirshenbaum. They will serve me well in all aspects of my life.

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LIST OF ABBREVIATIONS

$\Delta\Psi m$	Change in mitochondrial transmembrane potential
Ac-DEVD-CHO	N-acetyl-Aspartate-Glutamate-Valine-Aspartate Aldehyde
Ac-YVAD-CHO	N-acetyl-Tyrosine-Valine-Alanine-Aspartate Aldehyde
AIF	Apoptosis inducing factor
ANT	Adenine nucleotide translocase
Apaf-1	Apoptosis activating factor-1
ATP	Adenosine triphosphate
Baxluc	Bax luciferase reporter gene
BH	Bcl-2 homology
BNIP3	Bcl-2/E1B Nineteen kDa interacting protein 3
BSA	bovine serum albumin
C. elegans	*Caenorhabditis elegans*
CARD	Caspase recruitment domain
Caspase	Cysteinyl aspartate specific protease
CCCP	Carbonyl cyanide 3-chlorophenylhydrazone
Ced	Cell death abnormal
c-FLIP	Cellular flice inhibitory protein
c-IAP	Cellular inhibitor of apoptosis protein
CMV	Cytomegalovirus
CNTL	Control
CrmA	Cytokine response modifier protein A
dATP	Deoxy- adenosine triphosphate
DED	Death effector domain
Diablo	direct IAP binding protein with low pI
DISC	Death inducing signaling complex
DMEM/F12	Modified Eagle medium/Ham's nutrient mixture F12,1:1
DR	Death receptor
DTT	Dithiothreitol
EGL-1	Egg-laying defective
EMSA	Electromobility shift analysis
endo G	Endonuclease G
ER	Endoplasmic reticulum
FADD	Fas-Associated protein with Death Domain
FASL	Fas Ligand
H^+	proton
HEK293	Human embryonic kidney cells 293
HIF-1	Hypoxia inducible factor
HIV	Human immunodeficiency virus
HPYX	Hypoxia
HtrA2	High temperature requirement A2
IAP	Inhibitor of apoptosis protein
IETD	Isoleucine-Glutamic acid-Threonine-Aspartic acid
IGF-1	Insulin-like growth factor

JC-1	5',6,6'-tetrachloro-1,1',3,3' tetraethylbenzimidazolylcarbocyanine iodide
NIX	NIP3-like protein-x
NO	Nitric oxide
NFκB	Nuclear factor kappa beta
PARP	Poly-(ADP)-ribose-polymerase
PLAGL2	Pleomorphic adenoma gene-like-2
PTP	Permeability transition pore
PVDF	Polyvinylidene difluoride
ROS	Reactive oxygen species
SDS	Sodium dodecyl sulphate
Smac	second mitochondrial activator of caspases
TdT	Terminal deoxy transferase
TM	Transmembrane domain
TMRM	Tetra-methyl rhodamine methyl ester perchlorate
TNFR	Tumor necrosis factor receptor
TRAIL	TNF-related apoptosis inducing ligand
TUNEL	TdT -mediated deoxy-triphosphate nick end labeling
VDAC	Voltage dependent anion channel
v-FLIP	Viral FADD-like inhibiting protein
XIAP	x-linked IAP
zVAD-fmk	z-benzyloxycarbonyl-valine-alanine-aspartyl-methoxy fluoromethyl ketone

I. INTRODUCTION

Heart disease represents a major cause of morbidity and mortality worldwide. Risk factors including smoking, diabetes, uncontrolled hypertension, sedentary life style and viral infection have been identified as key underlying factors that contribute to loss of ventricular function and heart failure. Given the meager and limited ability of cardiac muscle for repair and/or self renewal after injury, an inordinate loss of working ventricular muscle cells has been suggested to be a predominant underlying cause of contractile failure in patients with ischemic heart disease or impaired coronary reserve([1]).

Recently, there has been considerable interest in the role of apoptosis in heart failure. Apoptosis, or programmed cell death, is a genetically regulated form of cell death that permits multicellular organisms to selectively discard damaged or superfluous cells through an intrinsic cell suicide program ([2;3]). Apoptosis has been detected in the myocardium in a number of cardiac pathologies including hypoxia ([4-11]), ischemia-reperfusion ([12-19]) and myocardial infarction ([16;20-25]). There is now considerable evidence from studies in animals as well as in humans that demonstrates increased apoptosis in the failing heart ([26;27]). Whether the loss of cells through an apoptotic process is adaptive, maladaptive or contributes to ventricular remodeling and failure is less clear. Undoubtedly, the acute loss of working ventricular myocytes in the absence of de novo myocyte regeneration can be seen as a major clinical impediment and underlying event leading to heart failure. A definitive therapeutic goal in treating patients with heart failure would therefore be to preserve the number of pre-existing myocytes through modulation of apoptosis.

The mechanisms that govern apoptosis in the heart are poorly understood; however, recent evidence suggests the involvement of a group of cysteine proteases, known as caspases (cysteinyl aspartate specific proteases)([28]). Caspases, of which 14 have been identified, are produced in cells as inactive zymogens that require proteolytic cleavage to become activated. In response to a pro-death stimulus, upstream initiator caspases such as caspases 8 or 10 cleave and activate down-stream death effector caspases (e.g. caspase 3) leading to the active destruction of the cell [reviewed in ([29;30])]. A model of this apoptotic signaling cascade has been shown to involve the mitochondria ([31]). Mitochondria react to prodeath signals by opening large mitochondrial multi-protein conductance channels known as permeability transition (PT) pores that are situated between the inner and outer mitochondrial membranes. Opening of these pores is associated with the loss of mitochondrial membrane potential($\Delta\Psi_m$) and permeabilization of the outer mitochondrial membrane ([32;33]). Subsequently, pro-death factors are released from the mitochondrial intermembrane space contributing to the activation of death effector caspases and apoptosis. Collectively, these events constitute the mitochondrial death pathway. One key factor released from mitochondria is cytochrome c. It forms a complex with Apoptosis Activating Factor-1 (Apaf-1) and dATP which serves to recruit and activate the death effector caspase, caspase 9. Caspase 9 then signals back into the caspase cascade leading to the activation of caspase 3 and cell death ([34;35]).

To test the impact of mitochondrial perturbations and caspase activation during normal and diseased conditions of the heart, I examined the impact of hypoxia, p53 and BNIP3 on ventricular myocyte cell death. Prolonged ischemia leads to cardiac cell death and ventricular dysfunction. Hypoxia, a major component of ischemia, has been shown

to trigger apoptosis of ventricular myocytes ([4;6;36;37]). Despite this important finding, the molecular regulators of hypoxia-mediated apoptosis are poorly defined. I hypothesize that caspases and the mitochondrial death pathway play an important role in hypoxia-mediated apoptosis.

The tumor suppressor protein p53 is an important transcription factor and regulator of apoptosis in cells. It is normally expressed at low levels but rapidly accumulates in response to cellular stressors such as DNA damage, aberrant growth signals and treatment with chemotherapeutic agents ([38-40]). Subsequent to its accumulation, apoptosis quickly ensues. Several studies in the heart have suggested a role for p53 in cardiac disease. Long et al. observed increased p53 expression in myocytes subjected to hypoxia ([5]). Further, Kajstura et al. found elevated levels of p53 in the border zone of infarcted hearts ([41]). Importantly, experiments from our laboratory have established that overexpression of p53 was sufficient to trigger cardiomyocyte apoptosis through a Bcl-2 regulated pathway ([42]). The mode by which p53 triggers cell death of ventricular myocytes is poorly understood but is thought to involve the transcription of death promoting genes. Whether *de novo* gene transcription is required for p53 to cause apoptosis in the heart is unknown. Moreover, it is equally unknown whether p53 impinges upon mitochondria and caspases to provoke cell death in the heart.

There is increasing awareness that the bcl-2 gene family of proteins may play a pivotal role in cardiomyocyte apoptosis ([42;43]). These proteins exert their pro- or anti-apoptotic function by impinging on key components of the "apoptotic machinery" within the cell, including the mitochondria ([32]). I have cloned a novel protein related to the bcl-2 gene family known as BNIP3. BNIP3 is a death-inducing protein found to localize to

mitochondria ([44]). Evidence from transformed cell lines shows that BNIP3 is induced by hypoxia and causes cell death when over-expressed ([45]). Whether BNIP3 is responsive to hypoxia or provokes apoptosis in the heart is unknown. Moreover, the impact of BNIP3 on the mitochondrial death pathway and caspase activation in the heart remains to be elucidated.

I. OBJECTIVE

The objective of my thesis research is to test the impact of hypoxia, p53 and BNIP3 on caspase activation and mitochondrial function for the induction of apoptosis in rat neonatal ventricular myocytes.

Specific aims of the hypoxia project:

(I) To demonstrate that hypoxia triggers apoptosis of neonatal ventricular myocytes.

(II) To investigate whether caspase activation and cytochrome c release occurs during hypoxia -mediated apoptosis of neonatal ventricular myocytes.

(III) To determine the impact of caspase inhibition on hypoxia-mediated cytochrome c release and apoptosis.

(IV) To evaluate mitochondrial membrane potential changes and the requirement of active caspases for these changes in response to hypoxia.

Specific aims of the p53 project are:

(I) To generate a replication defective adenovirus encoding a mutant form of p53 that does not bind to DNA or transactivate p53 response genes.

(II) To determine whether p53 triggers apoptosis independent of DNA binding and gene transactivation in neonatal ventricular myocytes.

(III) To evaluate the occurrence of mitochondrial defects such as changes in membrane potential and cytochrome c release in response to wildtype and mutant p53 expression in neonatal ventricular myocytes.

(IV) To determine whether p53-mediated cell death of neonatal ventricular myocytes involves caspase 3.

Specific aims of the BNIP3 project are:

(I) To demonstrate that hypoxia induces BNIP3 expression in neonatal ventricular myocytes independent of other Bcl-2 family members.

(II) To confirm that BNIP3 provokes apoptosis of neonatal ventricular myocytes in a caspase regulated fashion.

(III) To demonstrate that hypoxia is sufficient to provoke the integration of BNIP3 into mitochondrial membranes.

(IV) To evaluate the ability of BNIP3 to provoke mitochondrial defects consistent with mitochondrial targeting of the protein.

(V) To determine whether a mutant form of BNIP3, defective for mitochondrial membrane targeting, would suppress hypoxia induced cell death of myocytes.

OVERALL SIGNIFICANCE: These studies expand our understanding of the molecular mechanisms that govern apoptosis in the heart with the long term goal of developing new therapeutic interventions to prevent myocardial apoptosis, ventricular remodeling and heart failure.

II. REVIEW OF THE LITERATURE

1. The Heart

The human body is sustained through an intricate network of blood vessels linked to a four chambered pump known as the heart. Acting in a tightly regulated fashion, the right ventricle receives blood from the body via the right atrium and delivers it to the lungs for reoxygenation. To complete the circuit, oxygen-rich blood then enters the left ventricle, the largest of the four chambers, via the left atrium and is forced out to the rest of the body.

Numerous cell types make up the heart. Fibroblasts form heart connective tissue; SA nodal cells provide the impulse that generates a depolarizing wave across the heart, but it is the concerted effort of cardiac myocytes, particularly those in the ventricles that give the heart its pumping power. Through functional and electrical coupling they contract in synchrony to propel blood throughout the body. Interestingly, while cardiac myocytes represent only 25% of the total cell number within the heart, they constitute over 80% of the heart mass ([46]).

When the body's demand for nutrient-rich blood exceeds the heart's pumping capacity, heart failure ensues. The term heart failure is somewhat misleading given that the heart does not stop but rather weakens over time due to conditions that damage the heart or cause it to work too hard. These include coronary artery disease, myocardial infarction, hypertension, congenital heart disease, cardiomyopathy, abnormal heart valves or diabetes. Once the heart becomes damaged, the workload shifts to the viable portion. Increasing workload translates to cardiac hypertrophy, increased chamber volume and elevated heart rate. As the functional capacity of the heart declines, the renin-angiotensin

system becomes activated to maintain blood pressure and adequate tissue perfusion. With time these compensatory mechanisms become a liability leading to cardiac overload, dilatation and more pronounced dysfunction.

Heart failure is a chronic and progressive disease. It often goes undetected for years until the heart has lost sufficient pumping capacity that the patient becomes outwardly symptomatic. In later stages, heart failure patients typically suffer exertional dyspnea, fatigue and peripheral edema resulting from left ventricular dysfunction. In the absence of pharmacological or surgical interventions, heart failure eventually leads to multiple organ failure and death.

The specific mechanisms underlying heart failure are poorly understood. In ventricular myocytes isolated from failing hearts, defects in excitation-contraction coupling ([47]), ATP production ([48]) and intracellular calcium handling ([49]) have been identified. Further, changes in ventricular myocyte contractile protein composition and increased fibroblast collagen deposition are characteristic of the remodeling process which takes place in the heart during heart failure. A key feature of heart failure is the loss of working myocytes ([50;51]). Because cardiac muscle cells have a limited capacity for repair and regeneration, it is clear to see how progressive or acute myocyte loss could have devastating consequences on ventricular performance. Traditionally, necrosis was thought to be the primary cause of myocardial cell loss. It is now apparent that apoptosis also contributes to myocardial cell death. Furthermore, there is now strong and convincing evidence in the literature that myocardial cell death by apoptosis may contribute to the development of heart failure. This review highlights the key features of eukaryotic cell apoptosis with an emphasis on the mechanisms of apoptosis in the heart.

2. Apoptotic cell death

In order to purposefully eliminate cells, eukaryotic organisms have evolved an orderly cell suicide process known as apoptosis ([52]). Apoptosis is an active, gene-directed form of cell death triggered by the addition of a stimulus or by the removal of a suppressive agent and is carried out by death machinery found within the cell. Apoptosis has unique morphological and biochemical features ([53]). Apoptotic cells shrink in size, forming tight spheres with blebbed membranes. The nuclear membrane disintegrates and endonucleases cut the DNA into 150-200 base pair fragments. Eventually apoptotic cells are broken up into apoptotic bodies which are silently removed by adjacent cells in the absence of an immune response ([52;54]).

Before the discovery of apoptosis, the predominant mode of cell death in multicellular organisms was believed to be necrosis. Necrosis is regarded as a passive, reactive and unregulated form of cell death resulting from severe perturbations to the extracellular environment or direct injury to the cell. It is irreversible and almost always associated with tissue pathology. Necrotic cells are characterized by edema, depletion of ATP stores as well as progressive shrinkage (pyknosis) and dissolution (karyolysis) of the nucleus (Table 1). Non-specific DNA degradation and extensive plasma membrane damage also occur. As a consequence of plasma membrane rupture during necrosis, chemotactic factors are released from the cell, which trigger an inflammatory response.

Apoptosis is the predominant form of cell death during normal physiology of multicellular organisms and is integral to their existence. It is necessary for the selective removal of cells during development as exemplified by the loss of the vertebrate tail during phylogeny ([55]), the loss of mesenchymal cells between digits during mammalian

fetal development ([56]), the reduction in neuron number in the developing brain ([57]) and in immune cell maturation ([58;59]). Likewise, apoptosis is required for the targeted removal of damaged or redundant cells during normal cell maintenance and turnover ([53]). Untimely, inappropriate or inadequate apoptosis has been linked to many pathophysiological conditions. Loss of growth control in genetically unstable cells leads to cancer in the absence of sufficient apoptosis. As well, excessive apoptosis is believed to contribute to neurodegenerative disorders, immunodeficiencies and cardiac disease. Since apoptosis is a gene-based, highly ordered process, it is an exceptional target for pharmacological or genetic intervention to modulate the death response. Future therapies to treat disease may be aimed at harnessing the cellular machinery responsible for apoptosis to prevent or provoke apoptosis in the context of different disease states.

Table 1: General characteristics of apoptotic and necrotic cells.

Characteristics	Apoptosis	Necrosis
Nucleus	Nucleosomal fragmentation Chromatin condensation	Random DNA degradation Pyknosis Karyolysis
Cell Membrane	Intact Blebbed	Vacuolated Loss of integrity
Cytoplasm	Reduction in volume Organelles intact	Edematous Organelle swelling
Energy requirement	ATP dependent	ATP-independent
Removal of the cell	Disintegration into apoptotic bodies Phagocytosis by neighboring cells and/or macrophages	Lysis Inflammation

2.1. Apoptosis in the normal and diseased heart

Over the last decade, compelling evidence for the occurrence of myocardial apoptosis has emerged, which has generated much speculation as to its role in the heart. Myocyte apoptosis may play a role in cardiac development. It has been observed during embryogenesis ([60]) and post-natal growth ([61]) of myocytes. It is thought to play a role in establishing proper vascular connections between the ventricular chambers and arterial trunks ([62]) and in shaping conductance patterns at the sinus node, AV node and bundle of His ([63]). Myocyte apoptosis may be protective. By deleting cells that have suffered irreparable DNA damage, defective myocytes are prevented from disrupting normal cardiac function. Despite these beneficial effects, untimely, inappropriate or excessive cardiac apoptosis may contribute to cardiac pathologies. Evidence documenting apoptosis in the heart under pathological conditions is extensive and includes hypoxia ([4-11]), ischemia-reperfusion ([12-19]), myocardial infarction ([16;20-25]), stretch ([64]), aging ([65]), rapid ventricular pacing ([66]), congenital heart defects ([67]), arrhythmogenic right ventricular dysplasia ([68-71]), pressure overload hypertrophy ([72]), acromegaly ([73]), viral infection ([74;74;75]), oxidative stress ([76;77]) and cardiac allograft rejection ([78]). Similarly, substantial evidence has accumulated to implicate apoptosis during heart failure ([26;27;79-82]). Therefore modulation of myocardial apoptosis may have significant therapeutic value for the treatment and/or amelioration of cardiac disorders such as heart failure.

2.2. Methods of detecting apoptosis

Since Wyllie and co-workers first described apoptosis in 1972, numerous techniques have evolved to distinguish apoptotic cells among a population of living and dead cells. Electron microscopy is the gold standard for visualizing ultra-structural

features of cells and thus is one of the best methods for identifying apoptosis at the cellular level. Unfortunately, electron microscopy is extremely labor intensive. It requires arduous sample preparation and is restricted by the limited number of cells and regions within each cell that can be visualized at a given time. Because of this, the use of electron microscopy to distinguish apoptosis is limited. Instead, the vast majority of detection techniques monitor nuclear changes associated with apoptosis. The cleavage of nuclear DNA into 180-200 base pair fragments is one of the key features of apoptosis. When separated by agarose gel electrophoresis, these fragments form a distinct ladder pattern ([83]). In contrast, necrotic cells will have undergone random DNA degradation and appear as a smear following electrophoresis. DNA gel electrophoresis is a reliable method of apoptosis detection; however, it does not distinguish between different cell types in a mixed population and is difficult to quantify.

To avoid the limitations of DNA gel electrophoresis, *in situ* biochemical assays have been developed for the detection of single or double DNA strand breaks. Most of these methods monitor single DNA strand breaks. TUNEL (terminal deoxy transferase (TdT) -mediated deoxy-triphosphate nick end labeling), one of the most widely used methods, utilizes TdT to catalyze the polymerization of fluorescein labeled dUTP nucleotides to exposed 3'-OH ends of single-stranded DNA ([84;85]). Labeled nucleotides may also be added to 3'-OH ends using E. coli polymerase by *in situ* nick translation ([86]). Alternatively monoclonal antibodies specific for single strand DNA ends have been generated which when conjugated to fluoroscein provide another means of monitoring DNA damage ([87]). Other methods have been developed for the detection of double-strand DNA breaks including in situ ligation of hairpin oligonucleotide probes ([88]). Once

processed, flow cytometry may be employed to quantify the amount of label that has been incorporated into apoptotic cells. Fluorescent microscopy may also be used to visualize and quantify cells with labeled nuclei. One caveat to these methodologies is that DNA strand breaks are not specific for apoptotic cells but may also occur during necrosis. Although this is less frequent, it may result in an over-estimation of the level of apoptosis.

Nuclear dyes paired with fluorescent microscopy are also employed for detection and quantification of nuclear DNA fragmentation. Hoechst 33258 or 33342 ([89;90]), for example, allow for the visualization of nuclei in isolated cells and tissue samples which can subsequently be scored on the basis of apoptotic features. Healthy Hoechst-stained cell nuclei are round and uniform in coloration while those undergoing apoptosis are hyper-chromatic, crescent shaped or distinctly fragmented. Hoechst staining is useful in that it provides a measure of the incidence of apoptosis among a mixed population of cells identified with cell type specific antibodies. However, this method of detection is largely subjective unless performed in a blinded fashion and may underestimate the incidence of apoptosis if apoptotic cells become dislodged from the coverslip during processing.

Overall there are a wide variety of methodologies available for the visualization and quantification of apoptosis. While this is only a partial list, it is apparent that each technique has its advantages and disadvantages highlighting the necessity of using multiple detection techniques in the evaluation process.

3. Molecular Mechanisms of Apoptosis

3.1. Genetic analysis of *Caenorhabditis elegans*

Much of our understanding of apoptotic signaling in mammalian cells is the result of seminal studies conducted in the nematode *Caenorhabditis elegans (C. elegans)*. During *C. elegans* development precisely 131 cells die by apoptosis in a predictable fashion([91]). The simplicity of the organism has facilitated the identification of 4 principle genes responsible for this programmed cell death. These genes are *Ced-3, -4, -9,* (cell death abnormal) and *EGL-1* (egg-laying defective) ([91-93]). Ced-3, Ced-4 and EGL-1 trigger cell death whereas Ced-9 prevents it. Ced-3 is a cysteine protease ([94-96]). It is produced in an inactive form which becomes functional following proteolytic cleavage. Ced-4 is an adapter for Ced-3 ([97]). Its oligomerization promotes the proteolytic activation of Ced-3. Ced-4 and Ced-3 are regulated by Ced-9 and EGL-1. Ced-9 binds to Ced-4 which interferes with the interaction between Ced-4 and Ced-3, preventing Ced-3 activation ([98]). Ced-9 can also bind to EGL-1 ([99]). Once apoptosis has been induced, EGL-1 disrupts the interaction of Ced-9 with Ced-4 and promotes Ced-3 activation ([100]). This simple pathway for the programmed death of cells within *C. elegans* has provided a framework for understanding the complex array of apoptotic pathways found in mammals.

Mammalian homologues to Ced-3, -4, -9, and EGL-1 have been identified. A group of proteases known as Cysteinyl-aspartate -specific-proteases (caspases) have been found to be homologous to Ced-3 ([28;101]). Fourteen caspases have been identified in mammals (caspase 1-14). Apoptosis-activating factor-1 (Apaf-1)([34]) and Caspase Recruitment Domain 4 (CARD4)/Nod-1 are two putative mammalian homologues of

Ced-4([102;103]). Bcl-2 ([104]) and pro-apoptotic BH3-containing members of the Bcl-2 family ([105]) are homologous to Ced-9 and EGL-1, respectively.

3.2. Caspases

One of the distinct features of apoptosis is the controlled dismantling of cells from the inside-out. This is the predominant function of the caspases ([29;106;107]). Of the fourteen mammalian caspases, caspases 2, 8, 9, and 10 are thought to be initiators of apoptosis which cleave and activate the death effector caspases 3, 6 and 7. Caspases 1, 4, 5, 11, 13 and 14 have been implicated in the inflammatory response ([30;108]). Caspase 12, which localizes to the endoplasmic reticulum (ER), may play a role in the ER stress response ([109]). As cysteine proteases, caspases cleave target proteins at specific aspartic acid residues within a distinct tetrapeptide sequence. The active cysteine residue responsible for proteolytic cleavage is harbored within a highly conserved region of the enzyme identified by QACXG (where X is R, Q, or G) ([29;110]). More than 100 different caspase substrates have been identified including regulatory, structural and enzymatic proteins ([30;111;112]). Poly-ADP ribose polymerase (PARP) is one of the best characterized caspase 3 targets and is believed to function in DNA repair ([113]).

3.2.1. Caspase activation

Caspases are expressed in cells as inactive pro-enzymes, commonly referred to as procaspases (Figure 1). Procaspases contain an amino terminal pro-domain, a large subunit (~ 20kDa) containing the active cysteine residue, a small subunit (~10 kDa) and a variable length linker region which separates the large and small subunits ([114]). Aspartate cleavage sites are found between the pro-domain and the large subunit as well as within the inter-domain linker. Caspase activation occurs by aspartate-driven proteolytic

cleavage of the linker region thus allowing the large and small subunits to assemble into a functionally active heterodimer ([107;114;115]). During the activation process, the amino terminal pro-domain is discarded. While it does not contribute to the enzymatic activity of the caspase per se, the amino terminal pro-domain may identify caspase binding partners required for caspase processing and activation ([116-118]). Initiator caspases have a long prodomain which contain either a caspase recruitment domain (CARD) (caspase 2 and 9) or a death effector domain (DED) (caspase 8 and 10). Interaction of either the DED or CARD with complementary domains among initiator caspases or on adaptor proteins facilitates clustering and auto-proteolysis of initiator caspases. This is exemplified by the activation of procaspase 9 through its adaptor protein Apaf-1 (Apoptosis activating factor-1). Apaf-1 promotes autoactivation of procaspase 9 by recruiting and concentrating the enzyme within the local area ([30]). Once activated, initiator caspases act in a hierarchical manner to cleave and activate death effector caspases. Subsequently, activated effector caspases can activate remaining initiator caspases. This system of activation enables rapid caspase mobilization and cascade amplification ([107]). Another mechanism of caspase activation is known to exist and involves non-caspase proteases. The most recognized example of this is granzyme B, a serine protease which also cleaves intracellular substrates after an aspartate residue. By activating caspases 3 and 7, Granzyme B carries out the cell death directive initiated by specific immune cells ([119;120]).

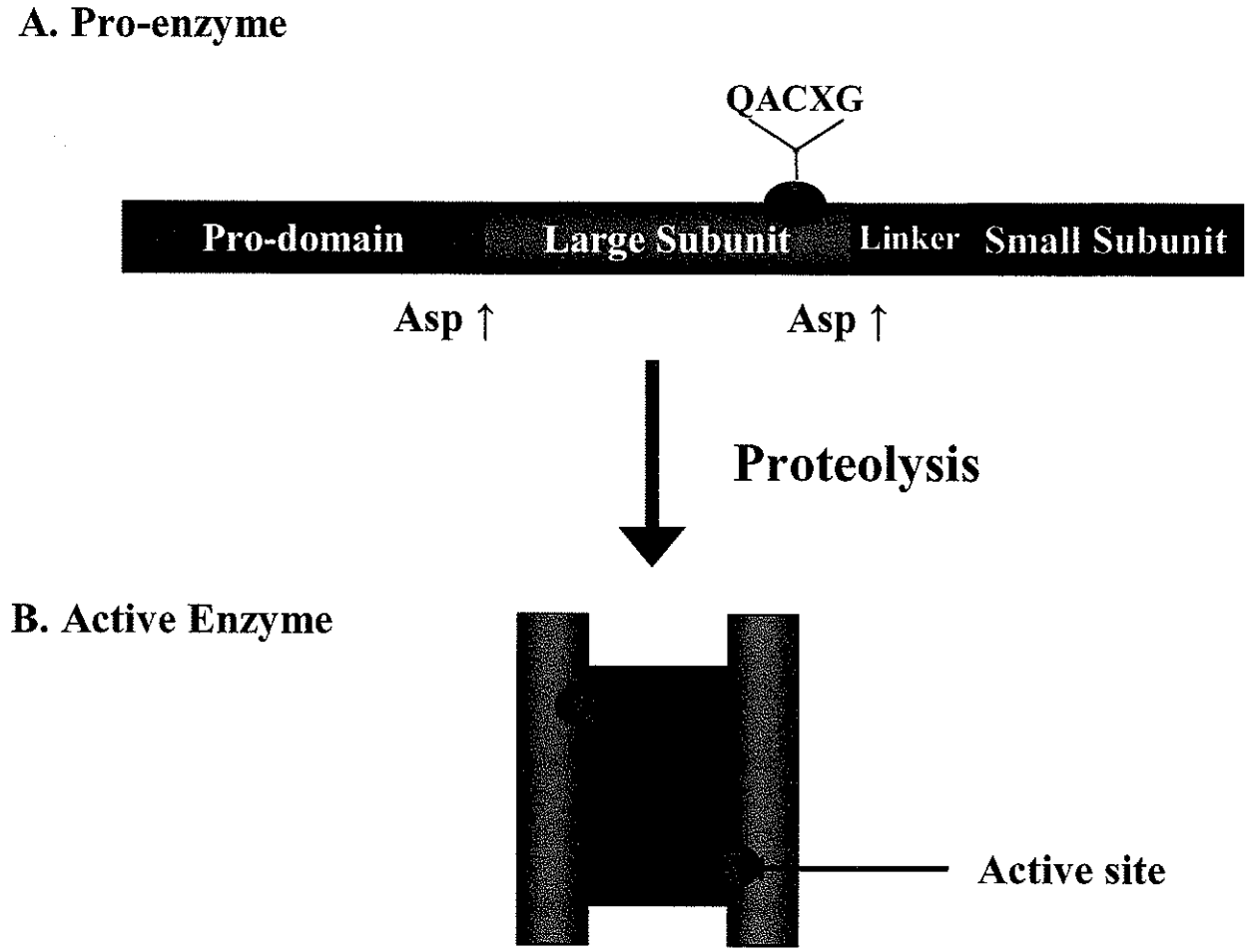

Figure 1: Schematic representation of caspase enzyme structure
Caspases are cysteine proteases. They are produced in cells as inactive precursors with common structural elements: a prodomain, large and small subunits and linker region. The active site cysteine is harbored within a conserved QACXG motif in the large subunit. Proteolysis is required to activate the pro-enzyme and occurs at aspartate residues which reside between the prodomain and the large subunit and within the linker. The active caspase is a tetramer consisting of two large/small subunit heterodimers each with an active site.

3.2.1.1 A role for caspase activation in cardiomyocytes

Caspase 3 is a key mediator of myocardial apoptosis. It is highly expressed in cardiac myocytes ([121]) and its activation is known to promote cardiomyocyte apoptosis. Evidence of active caspase 3 has been documented in human specimens of cardiomyopathy and heart failure ([36;81;122]). Experimentally, the cleavage and/or activation of caspase 3 has been detected in models of cardiomyocyte apoptosis in response to H_2O_2 ([123]), hypoxia ([7;8]), ischemia ([124]), ischemia-reperfusion ([125]), cytokines ([126]), doxorubicin ([127;128]), p53 ([129]), PKC inhibition ([130]), staurosporine ([131]) heart failure induced by rapid ventricular pacing ([132;133]) or intracoronary microembolizations ([134]) and myocardial infarction ([135;136]). Underscoring the importance of caspase 3 activity to myocardial apoptosis is substantial evidence demonstrating cardio-protection following caspase 3 inhibition, including reduced cardiomyocyte apoptosis ([7;8;124;129;131;136]), reduction of infarct size and delayed progression of heart failure ([15]).

The pathophysiological effects of caspase 3 activity are becoming increasingly apparent in models of ischemia/reperfusion. Transgenic mice which overexpress cardiac specific caspase 3, show an increase in infarct size following ischemia/reperfusion ([137]) whereas caspase 3 inhibition has been shown to protect against ischemia/reperfusion-induced cardiac myocyte apoptosis ([15;138]), reduce infarct size ([138;139]) and improve contractile recovery of the heart ([140]). The myofibrillar proteins essential myosin light chain (vMLC1) and cardiac troponin T were recently identified as novel substrates for caspase 3 ([141;142]). Further, Chandrashekhar and colleagues found that prolonged caspase inhibition prevented troponin I cleavage and preserved left ventricular function after myocardial infarction ([136]). These data suggest that caspase 3-mediated cleavage of

cardiac cytoskeletal proteins resulting in contractile dysfunction may precede cardiomyocyte cell death and contribute to the contractile dysfunction associated with cardiac disease.

Unlike caspase 3, a role for other caspases in the heart is less defined. Evidence of caspase 8 and 9 involvement in cardiac disease has been documented in studies of explanted hearts from patients with dilated cardiomyopathy or coronary artery disease ([143]). Moreover, pharmacological inhibition of these caspases has been shown to limit infarct size due to reperfusion injury ([139]). Caspase 7 and 8 activity has been detected in models of ischemia/reperfusion ([138;144]) and inhibition of caspase 8 activity has been shown to prevent hypoxia-induced apoptosis of isolated myocytes([7;8]). Emerging evidence also suggests that caspase 9 is activated in myocytes in response to hypoxia ([145]) and simulated ischemia ([124]). Likewise, caspase 2 processing has been observed in models of pacing induced heart failure in dogs ([133]), ischemia/reperfusion injury ([138]) and lovostatin-induced cardiotoxicity ([146]) while elevated levels of caspase 1 have been reported during various cardiac disease states ([135;147]). At present it is unclear what role caspase 1 plays in cardiomyocyte apoptosis since knock-out studies suggest that loss of this protease has no appreciable effects on development or apoptosis. Not unlike other cell types, caspases appear to play a significant role in cardiomyocyte apoptosis. Future therapies for the modulation of cardiac apoptosis will require a thorough understanding of the caspases that are operational in cardiomyocytes.

3.2.2 Regulation of caspase activity

Propagation and execution of apoptotic signals in cells is largely dependant upon active caspases. Not surprisingly, a subset of proteins has evolved to prevent the activation of procaspases and inhibit the activity of mature caspases in order to regulate apoptotic signaling. Many of these inhibitory proteins are produced by viruses in an attempt to maintain viability of the host cell for viral replication. The herpes virus, for example, generates v-FLIP (viral FADD-like inhibiting protein), a competitive inhibitor of the caspase 8 cofactor FADD (Fas-Associated Protein with Death Domain). In the presence of v-FLIP, caspase 8 is retained in its latent form ([148-152]). Alternatively, the cowpox virus inhibits caspase 8 activity by producing the cytokine response modifier protein A, CrmA. CrmA acts as a pseudosubstrate of caspase 8 preventing the cleavage of endogenous proteins. CrmA has also been shown to be weakly inhibitory toward caspase 2, 3, 7 and 10 ([120;153]). Our laboratory has shown that expression of CrmA in neonatal ventricular myocytes is sufficient to block hypoxia-mediated caspase 8 activation and cell death ([8]). Baculovirus is known to encode at least 2 different classes of caspase inhibitors. The first is the pseudosubstrate p35 which functions as a general caspase inhibitor ([154-156]). In vivo overexpression of p35 has been shown to block caspase 3 activation in a rabbit model of pacing-induced heart failure ([141]). Additionally, baculovirus produces IAPs (inhibitors of apoptosis proteins) which selectively inhibit caspase 3, 7 and 9. IAP proteins are characterized by the presence of one or more baculovirus IAP repeat (BIR) domains, an approximately 70 amino acid motif conserved from yeast to human that is important for caspase inhibition ([157]).

Mammalian cells have their own complement of caspase inhibitors several of which are homologous to those found in viruses. For example, c-Flip (cellular FLIP) is the mammalian orthologue to v-flip. c-Flip was originally cloned from human melanoma where it protected cells from apoptosis. Similarly, several mammalian IAPs have been characterized, including X-chromosome-linked IAP (XIAP), cellular IAP (cIAP)1, cIAP2, and survivin,. The best characterized of the IAPs is XIAP, which inhibits the activities of caspase 9, caspases 3 and 7 ([158]). Emerging evidence suggests that these endogenous caspase inhibitors are expressed in the heart although their role is mostly unknown ([143;159;160]). A study of apoptosis in patients with end-stage heart failure suggested the abundant expression of cFLIP in the heart may play an important role in the inhibition of cardiomyocyte death ([159]). Downregulation of XIAP, cIAP1, and cIAP2 in the failing myocardium has been suggested to contribute to increased myocyte apoptosis ([143]). Recently a novel caspase inhibitor known as ARC (apoptosis repressor with caspase recruitment domain) was identified, which is expressed at high levels almost exclusively in heart and skeletal muscle ([161]). ARC has been reported to selectively interact with caspases 2 and 8 and to inhibit receptor-induced apoptosis. ARC has been shown to abrogate apoptosis induced by hypoxia and hydrogen peroxide in H9c2 cells ([162;163]) and in isolated perfused hearts subjected to ischemia and reperfusion (I/R) ([164]).

4. Pathways of caspase activation in mammalian cells

Complex signaling pathways originating from the cell surface or mitochondria have evolved to activate caspases in mammalian cells. These pathways are known as the extrinsic, type I or death receptor pathway and the intrinsic, type II or mitochondrial death pathway (Figure 2). The death receptor pathway is mediated by specific death

receptors (DR) at the cell surface which activate intracellular apoptotic signaling pathways in response to unfavorable extracellular conditions. In contrast, the mitochondrial death pathway is mediated primarily by the mitochondria which sense unfavorable intracellular conditions. Initiator caspases that participate in the death receptor and mitochondrial death pathways are structurally and mechanistically distinct. Initiator caspases 8 and 10, which contain DED regions, are activated in response to DR stimulation, while caspase 9, which is recruited and concentrated through its CARD domain, participates in the mitochondrial death pathway.

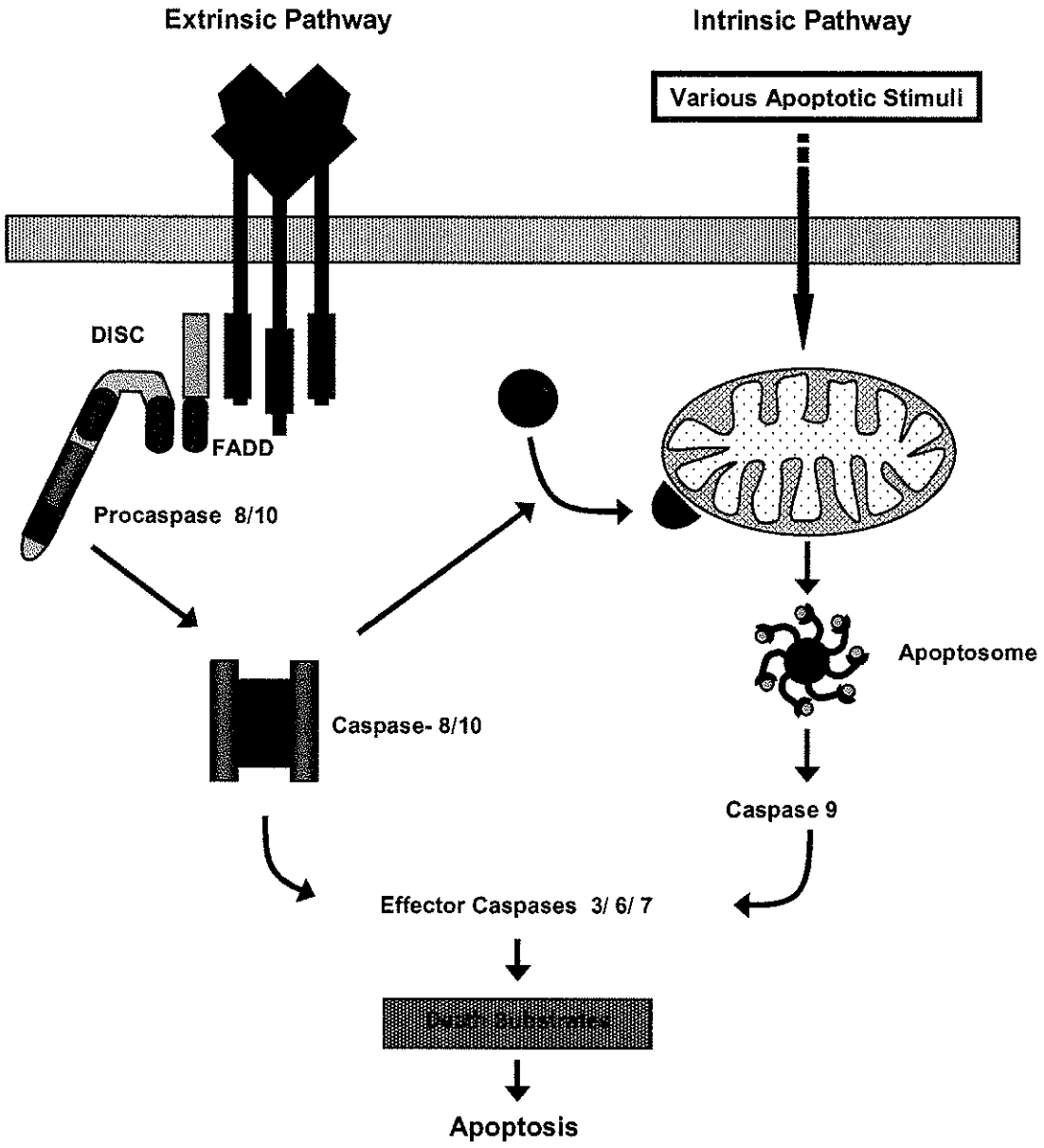

Figure 2: Pathways of caspase activation in mammalian cells. The extrinsic pathway is mediated by members of the death receptor superfamily such as TNF-α and Fas. Activation of the receptor triggers receptor oligomerization followed by the recruitment of the adaptor molecule, FADD. This results in the generation of a death inducing signaling complex (DISC) capable of activating pro-caspase 8/10. The intrinsic pathway is mediated by intracellular or extracellular stimuli. These stimuli activate cellular factors that impinge upon mitochondria causing the release of cytochrome c, apoptosome formation and subsequent caspase 9 activation. The extrinsic and intrinsic pathways converge upon the effector caspases, resulting in the cleavage of death substrates and apoptosis.

4.1 Death receptor signaling

The vast majority of death receptors belong to the tumor necrosis factor receptor (TNFR) superfamily which includes Fas, TNFR1 and DR3 to DR6. These receptors are single pass transmembrane proteins with cysteine rich ligand binding domains at the extracellular face. In general, ligand binding to its cognate death receptor triggers the recruitment of death domain (DD) and DED containing proteins to the intracellular surface of the receptor which assemble into a death-inducing signaling complex (DISC) ([165-167]). Formation of the DISC facilitates recruitment and activation of caspase 8/10 which initiates the proteolytic cascade (reviewed in ([168])).

The TNFR1 and Fas receptor pathways are well characterized. TNFR1 is activated by TNF-α or lymphotoxin α, soluble cytokines found in the general circulation. TNF-α binding to the TNFR1 induces receptor trimerization and DISC formation. The adaptor proteins TNFR1-associated death domain protein (TRADD) and FADD (Fas-associated protein with death domain) are recruited to the receptor through homotypic DD interactions between proteins. FADD is a key molecule required for the activation of pro-caspase 8/10. Protein-protein interactions between requisite DED motifs of pro-caspase 8/10 and FADD, recruit pro-caspase 8 to the DISC where it undergoes autoprocessing and activation ([169;170]). A similar process is observed during Fas receptor activation. Fas ligand (FasL) binding consolidates activated Fas receptors into a homotrimeric complex. FADD is then recruited to the receptor complex to form the DISC leading to pro-caspase 8/10 activation and induction of the caspase cascade. Caspase 8/10 also cleaves the prodeath Bcl-2 family member Bid, which translocates to the mitochondria to initiate the mitochondrial death pathway ([168]).

4.1.1 Death receptors in the heart

Death receptor signaling appears to play an important role in cardiac development and disease. FADD knockout mice are embryonic lethal and die with severe cardiac abnormalities ([171]). A similar scenario is observed in caspase $8^{-/-}$ knockout mice ([172]). Interestingly, Fas receptor null mice are viable ([173]) which highlights a functional redundancy in the receptors which trigger caspase activation.

Elevated levels of TNF-α are observed in failing hearts ([174-176]) and during hypertrophic cardiomyopathy ([177]). Overexpression of TNF-α leads to increased cardiac myocyte apoptosis in isolated adult ventricular myocytes ([178]). Furthermore, TNF-α transgenic mice are susceptible to dilated cardiomyopathy and heart failure ([179;180]). Interestingly, TNF-α alone does not necessarily induce apoptosis in neonatal myocytes unless paired with the protein synthesis inhibitor cycloheximide. This suggests that *de novo* protein synthesis is required to prevent TNF-α-induced cell death ([181]). Our laboratory has shown that the cytoprotective effects of TNF-α are mediated through the transcription factor, nuclear factor kappa beta (NFκB), potentially by increasing the expression of cytoprotective genes, since inhibition of the NFκB pathway increases cardiac myocyte apoptosis in response to TNF-α ([181]). The difference in cytotoxicity mediated by TNF-α in adult myocytes versus neonatal myocytes cultures is difficult to explain but may reflect an age dependent disparity in the activities of downstream signaling pathways. Components of the TNF death receptor pathway, including caspase 8, are expressed in cardiac myocytes but there are also significant levels of antagonist proteins such as cFLIP (cellular FADD-like inhibiting protein) ([182]). Whether this or

other regulatory proteins are differentially regulated in the neonatal and adult heart is currently unknown.

4.2 The mitochondrial death pathway

Mitochondria are the major energy producing organelles of the cell. Housing the constituents of the citric acid cycle and the electron transport chain, mitochondria have the formidable task of making enough ATP to sustain cellular functions. Recently, mitochondria have been found to play a novel role in the cell, acting as intracellular sensors for transduction of apoptotic signals. The apoptotic signaling pathways involving mitochondria constitute the mitochondrial death pathway and the predominant mechanism of caspase 9 activation.

The mitochondrion is organized into two distinct compartments separated by an inner mitochondrial membrane. The innermost compartment, the matrix, houses the enzymes of the citric acid cycle, β oxidation and as well as the mitochondrial DNA. The inner mitochondrial membrane is highly convoluted with numerous cristae projecting into the mitochondrial matrix. Cristae contain the constituents of the electron transport chain and the F_0F_1 ATPase ([183]). The restricted permeability of the inner mitochondrial membrane to protons (H^+) creates a mitochondrial transmembrane potential ($\Delta\Psi m$) and facilitates the production of ATP through the F_0F_1 ATPase ([184]). The outermost compartment, the intermembrane space, is situated between the inner and outer mitochondrial membranes and within the luminal space of the cristae. The intermembrane space contains highly reactive proteins which when released into the cytosol, trigger an apoptotic response ([34;35]) (Figure 3).

In most systems, release of cytochrome c is an important mediator of the mitochondrial death pathway. Cytochrome c is found loosely tethered to the inner mitochondrial membrane where it shuttles electrons between complex III and complex IV of the electron transport chain. In response to a prodeath stimulus, cytochrome c is released into the cytosol where it can form a complex with the Ced-4 homologue, Apaf-1, in the presence of dATP/ATP (deoxy adenosine triphosphate) (Figure 4). The interaction between cytochrome c and Apaf-1 induces a conformational change in Apaf-1 which promotes the formation of a wheel-like structure comprised of 7 molecules each of Apaf-1, dATP, and Cytochrome c. Pro-caspase 9 is recruited to the core of this complex of proteins which is known collectively as the apoptosome ([185;186]). The apoptosome facilitates processing and activation of pro-caspase 9 within the cytosol leading to caspase 3 activation and DNA fragmentation ([185;187;188]). Despite elucidation of apoptosome structure, the precise mechanism of pro-caspase 9 processing and activation is unknown. Two models have been proposed. One model suggests that pro-caspase 9 harbored within the apoptosome recruits and activates cytosolic pro-caspase 9. Alternatively, interaction between two apoptosomes may bring pro-caspase 9 into sufficient proximity to promote activation of the enzyme found within the complex itself.

Apoptosis is an energy-dependent process that requires sufficient levels of ATP to proceed. Cytochrome c, a component of the electron transport chain, plays an important role in ATP production. The fact that reduced mitochondrial cytochrome c levels do not disrupt ATP levels sufficient to block apoptotic mechanisms is surprising. Frey et al. suggest that mitochondria contain releasable pools of cytochrome c constituting approximately 10-15% of the total complement of cytochrome c ([189]). Alternatively,

there may be two pools of mitochondria, one of which maintains the normal complement of cytochrome c sufficient to sustain ATP production ([190]).

Formation of the apoptosome mediated by cytochrome c is not always sufficient to provoke the caspase cascade due to the presence of endogenous inhibitors like the IAPs ([191]). Murine Smac (second mitochondrial activator of caspases) and its human ortholog DIABLO (direct IAP binding protein with low pI) are released to the cytosol in response to an apoptotic trigger where they bind to and neutralize XIAP, c-IAP1 and c-IAP2, baculoviral Op-IAP, and surviving ([192-194]). Smac/DIABLO competes with caspase 9 for binding to the IAPs which cascade.

The mammalian serine protease Omi, also known as HtrA2 (high-temperature requirement A2), is a serine protease that is released from the mitochondria to the cytosol during apoptosis ([195;196]). Similar to Smac/DIABLO, Omi/Htra2 has the ability to bind and antagonize IAPs although it does not interact with survivin ([197]). Omi/Htra2 is released from mitochondria to the cytoplasm during apoptosis where it contributes both to caspase-dependent and caspase-independent apoptosis ([197-200]). In the cytoplasm, Omi/HtrA2 interacts with cytosolic IAP proteins similar to Smac/DIABLO ([197;199]). However, in contrast to Smac/DIABLO HtrA2 promotes the catalytic cleavage of IAPs leading to their irreversible inactivation and progression of apoptosis ([201;202]). The significance of Smac/Diablo and/or Omi/HtrA2 in cardiac myocyte apoptosis remains to be explored, although Smac/Diablo is expressed in cardiac myocytes (K Regula and L. Kirshenbaum, unpublished data).

Many of the apoptotic mitochondrial proteins described thus far, function in caspase dependent apoptosis. Notwithstanding, Kroemer and associates identified a

novel mitochondrial flavoprotein protein, termed AIF for apoptosis inducing factor, which could trigger apoptosis in the absence of caspase activation ([203]). AIF is produced as a 67-kDa-precursor protein consisting of an N-terminal mitochondrial localization sequence ([203]). Once imported into the mitochondrion, AIF is processed into its mature 57-kDa form. The unique feature of AIF stems from its ability to translocate to the nucleus following an apoptotic signal where it promotes high-molecular weight DNA fragmentation in the absence of caspase activation. That AIF can trigger DNA fragmentation independent of caspase activation is supported by the fact that neither Bcl-2 overexpression nor caspase inhibition blocked its ability to cleave DNA ([203]). Furthermore, AIF can trigger DNA fragmentation in either Apaf-1 or caspase 9 deficient cells ([204]). Endonuclease G (Endo G) is also released from the mitochondria during apoptosis. Itself a nuclease, Endo G can directly induce high molecular weight DNA fragmentation ([205-208]). Evidence of Endo G-mediated DNA fragmentation has been reported during UV-or TNF-α induced apoptosis ([209]). Both Endonuclease G and AIF are expressed in the heart ([210;211]) but the significance of these proteins in cardiac myocyte apoptosis is undetermined.

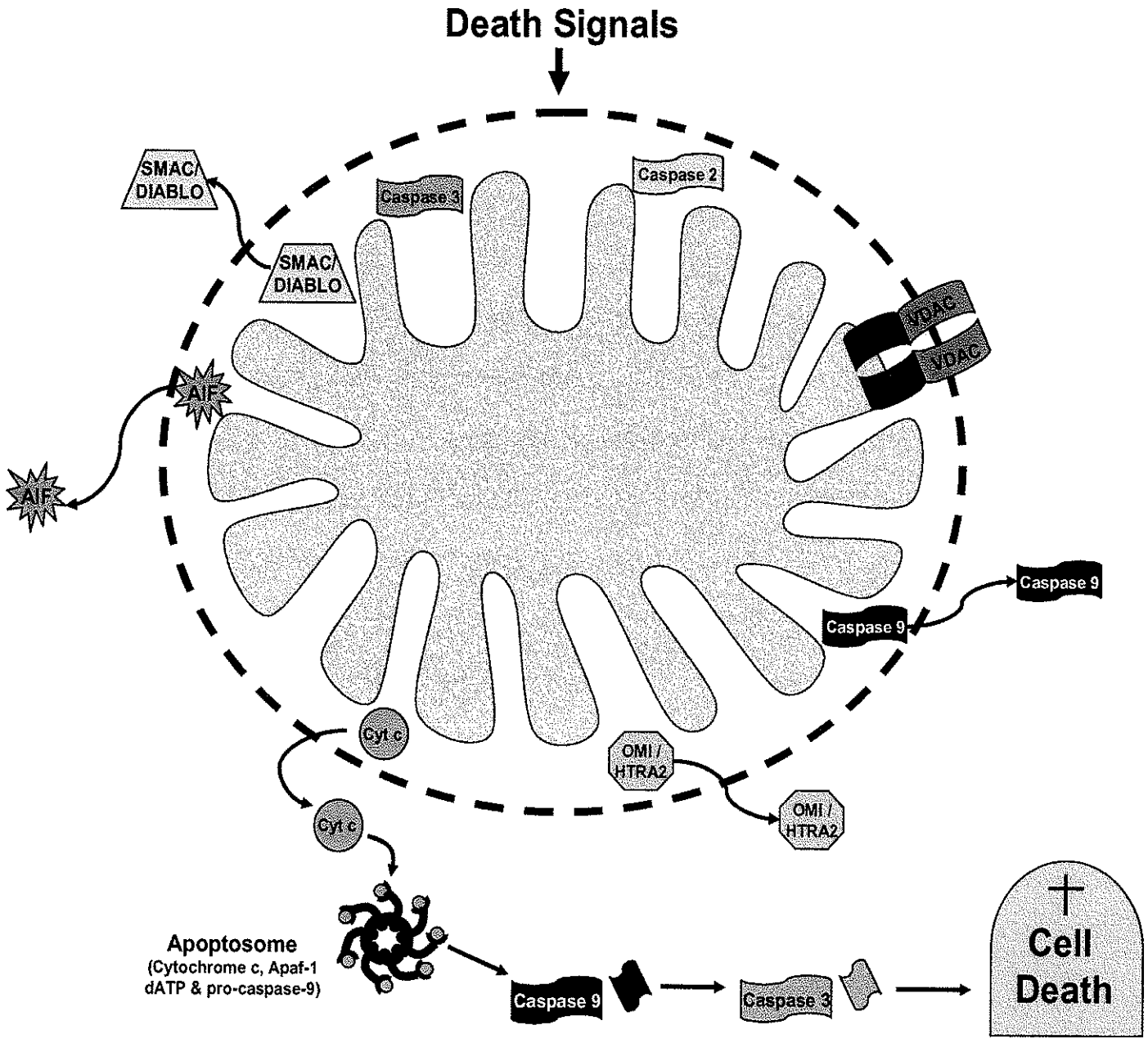

Figure 3: The intrinsic/mitochondrial death pathway. The mitochondrial death pathway is mediated by intracellular and extracellular death signals that impinge upon mitochondria leading to the disruption of normal mitochondrial physiology. Perturbations to the mitochondria trigger opening of the permeability transition pore which is believed to contribute to the release of prodeath factors from the mitochondrial intermembrane space and ultimately, cell death. These factors include AIF, Smac/Diablo, Omi/Htra2 and cytochrome c.

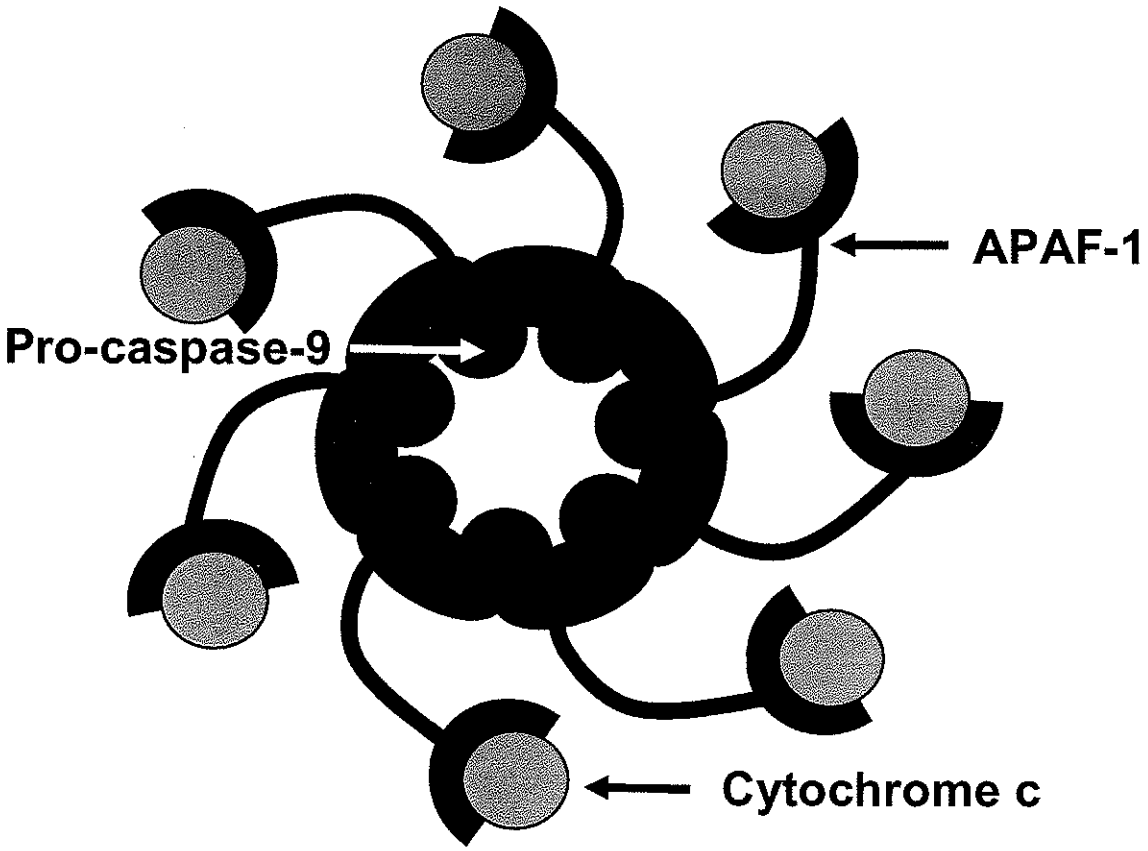

Figure 4: The apoptosome. The apoptosome is a cytosolic structure that facilitates caspase 9 activation. Formation of the apoptosome begins when cytochrome c is released from the mitochondria. In the cytosol, cytochrome c binds to Apaf-1 causing it to undergo a conformational change that facilitates interactions with other Apaf-1 molecules. Stabilized by dATP, molecules of Apaf-1 unite to form a wheel-like structure which recruits procaspase 9 dimers to its core. The entire assembly consists of 7 molecules each of dATP, Apaf-1, and cytochrome c as wells as 7 dimers of pro-caspase 9 [reviewed in ([212])].

4.2.1 Permeability transition pore (PTP)

For the past several years the mitochondrial permeability transition pore (PTP) has been at the center of considerable interest and debate for its potential involvement in mitochondrial membrane permeabilization and regulation of cell death. The PTP is a nonspecific ion channel comprised of the voltage dependent anion channel (VDAC), the adenine nucleotide translocase (ANT) and cyclophilin D as well as other proteins ([213-216]). It spans the inner and mitochondrial membranes at regions where they come into close apposition allowing the passage of molecules up to 1500 Da between the mitochondrial and cytoplasmic compartments. When the pore is open, H^+ ions enter the mitochondrial matrix resulting in loss of mitochondrial membrane potential ($\Delta\psi_m$) and expansion of the mitochondrial matrix ([215]). Secondary to mitochondrial uncoupling, ROS (reactive oxygen species) are produced and ATP levels fall. Because the surface area of the inner mitochondrial membrane is substantially larger than that of the outer membrane, matrix swelling is believed to cause rupture of the outer membrane and leakage of apoptogenic factors into the cytoplasm ([217;218]) (Figure 5).

The PTP exists in either an open or closed state and is regulated by environmental cues. Elevated calcium, nitric oxide, reactive oxygen species, and caspases open the pore (reviewed in ([216])). Conversely, the PTP favors a closed conformation in the presence of ADP, low matrix pH and Mg^{2+}. Experimentally, cyclosporin A (a ligand of cyclophilin D) and Bongkrekic acid (BA; a ligand of ANT) are used to pharmacologically inhibit the pore ([219-221]). Members of the Bcl-2 gene family are also reported to regulate the PTP ([183]). Importantly, factors such as ROS and elevated calcium which contribute to initial PTP opening are also secondary consequences of permeability transition. This begs the

question which comes first: PTP opening or disruption of mitochondrial homeostasis? The answer is currently unknown.

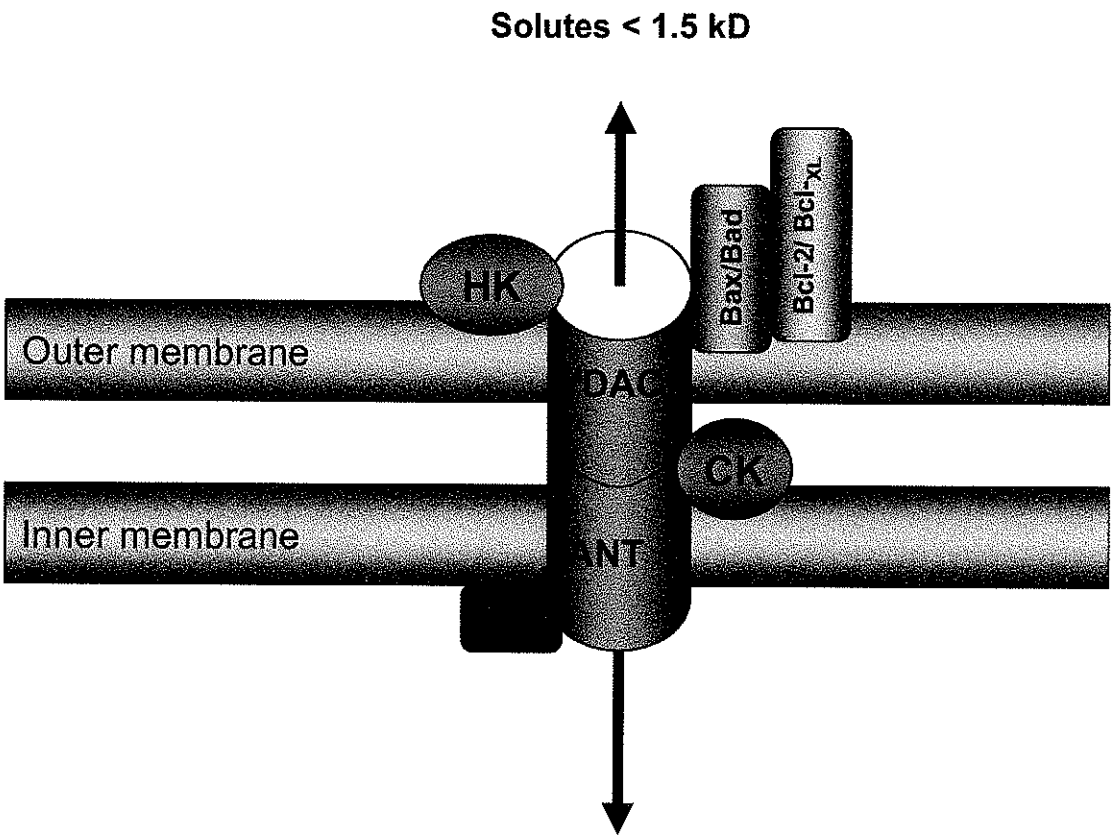

Figure 5: Permeability Transition Pore (PTP). The PTP is a nonspecific pore comprised of the voltage dependent anion channel (VDAC), the adenine nucleotide translocase (ANT) and cyclophilin D as well as other proteins. The PTP permits the movement of small molecules (<1500 Da) between the cytosol and the mitochondrial matrix.

4.2.2 Mitochondrial death pathway in the heart

Cardiomyocyte contraction is an energy dependant process that demands a constant supply of ATP. To satisfy the need for energy, cardiomyocytes contain a large proportion of mitochondria, constituting approximately 30% of the mammalian cell's volume ([222]). Given the preponderance of mitochondria in cardiomyocytes, it is not surprising that they play a role in cardiomyocyte apoptosis. In fact, an increasing number of studies are now reporting mitochondrial defects that are consistent with activation of the mitochondrial death pathway.

Cytochrome c, as a key player involved in caspase 9 activation and apoptosis, has been the focus of much scrutiny in the heart. Hypoxia induces cytochrome c release in isolated cardiomyocytes, resulting in caspase 3 activation and nuclear DNA fragmentation ([7;9]). Similar findings have been reported in response to H_2O_2 ([123]), hypoxia-reoxygenation ([223]), ischemia ([224;225]), serum and glucose deprivation ([124]) as well as ischemia-reperfusion ([226]). Cytochrome c release is also observed in hearts subjected to ischemia/reperfusion ex vivo ([224]) and in response to rapid ventricular pacing ([227]). Moreover, cytochrome c release has been observed in human cardiomyopathic samples, suggesting the operation of a mitochondrial death pathway in human cardiac disease ([81]).

Recently, loss of $\Delta\Psi_m$ and PTP opening have been observed during cardiomyocyte apoptosis ([8;9;228]). We have demonstrated that hypoxia-mediated apoptosis of ventricular myocytes involves a reduction in $\Delta\Psi_m$ and PTP opening ([8;9]). Bongkrekic acid, an inhibitor of the PTP is sufficient to block hypoxia-mediated PTP opening, loss of $\Delta\Psi_m$, and apoptosis, suggesting that mitochondrial defects contribute to

myocyte apoptosis in response to low oxygen tension ([8]). Opening of the PTP has also been implicated in reperfusion injury.

5. Bcl-2 Gene Family

The Bcl-2 family is comprised of a large group of related proteins with pro- or anti-apoptotic capabilities. The prototypic member of this family is Bcl-2, which shares sequence and functional homology with the *C. elegans* gene *Ced-9* and the adenovirus E1B genes ([229;230]). Bcl-2 was first identified in human B-cell lymphomas following a translocation breakpoint mutation t(14:18) that shifted the Bcl-2 gene to the immunoglobulin locus. Cells derived from these lymphomas constitutively expressed Bcl-2 and were resistant to apoptosis. Interestingly, Bcl-2 is not a proto-oncogene but rather favors cell survival in a sub-optimal conditions as in UV radiation, heat shock, growth factor withdrawal and oxidation stress ([231]).

To date, a total of 24 Bcl-2 family members have been identified, each of which contains one to four Bcl-2 homology (BH) domains (BH1-4) and often, a transmembrane domain ([232])(Table 2) (Figure 6). While the functional significance of the BH domains is poorly understood, they are believed to be key mediators of apoptosis. Most pro-survival members contain all four BH domains. The BH4 domain participates in heterodimerization ([233]) and has previously been shown to be essential for the prevention of apoptotic cell death ([234]). Pro-apoptotic members of the Bcl-2 family contain at least a BH3 domain and are further subdivided into a Bax subfamily and a BH3-only subfamily [reviewed in ([235])]. Members of the Bax subfamily contain the BH1, BH2 and BH3 domains. The BH1 and BH2 domains of Bax are required for homodimerization with Bcl-2/Bcl-xl. The BH3-only subfamily is unique in that it possesses only the short 9 to

16 residue BH3 domain. It has been speculated that members of the BH3 subfamily may represent the physiological antagonists of the pro-survival Bcl-2 proteins. This is based upon evidence that members of this subfamily are pro-apoptotic and that programmed cell death in *C. elegans* is contingent upon EGL-1, the sole non-mammalian BH3 family member [reviewed in ([236])]. The BH3 domain of Bad, Bik or Bid interacts with other Bcl-2 proteins to initiate apoptosis ([237-239]). As mentioned, most Bcl-2 homologues contain a hydrophobic C-terminal transmembrane (TM) domain. The TM domain facilitates binding to intracellular membranes including membranes of the endoplasmic reticulum, mitochondria and nuclear envelope ([240-242]).

Anti-apoptotic Bcl-2 proteins	BH4	BH3	BH1	BH2	TM
Bcl-2	X	X	X	X	X
Bcl-X_L	X	X	X	X	X
Mcl-1	X	X	X	X	X
A1/Bfl-1	X	X	X	X	X
Bcl-w	X	X	X	X	X
Boo/Diva/Bcl-B	X	X	X	X	X
Bcl2-L12				X	
Pro-apoptotic Bcl-2 proteins Multi-domain					
Bcl-Rambo	X	X	X	X	X
Bax		X	X	X	X
Bak		X	X	X	X
Mtd/Bok		X	X	X	X
Bcl- X_S	X	X			X
Pro-apoptotic Bcl-2 proteins BH3 only					
Bik/Nbk		X			X
Bim/Bod		X			X
Blk		X			X
Hrk		X			X
Noxa		X			
Bad		X			
Bid		X			
Bfk		X		X	
Bcl-G_L		X		X	
Hrk/Dp5		X			X
Puma					
Bmf		X			
Pro-apoptotic Bcl-2 proteins BH3-like					
Bnip3		X			X
Nix		X			X
MAP-1		X			
p193		X			

Table 2: Classification of Bcl-2 family members. Summary of the pro- and anti-apoptotic Bcl-2 family members identified to date and their structural elements (Adapted from Scorrano and Korsmeyer, 2003([243]) and Kelekar and Thompson, 1998([231])).

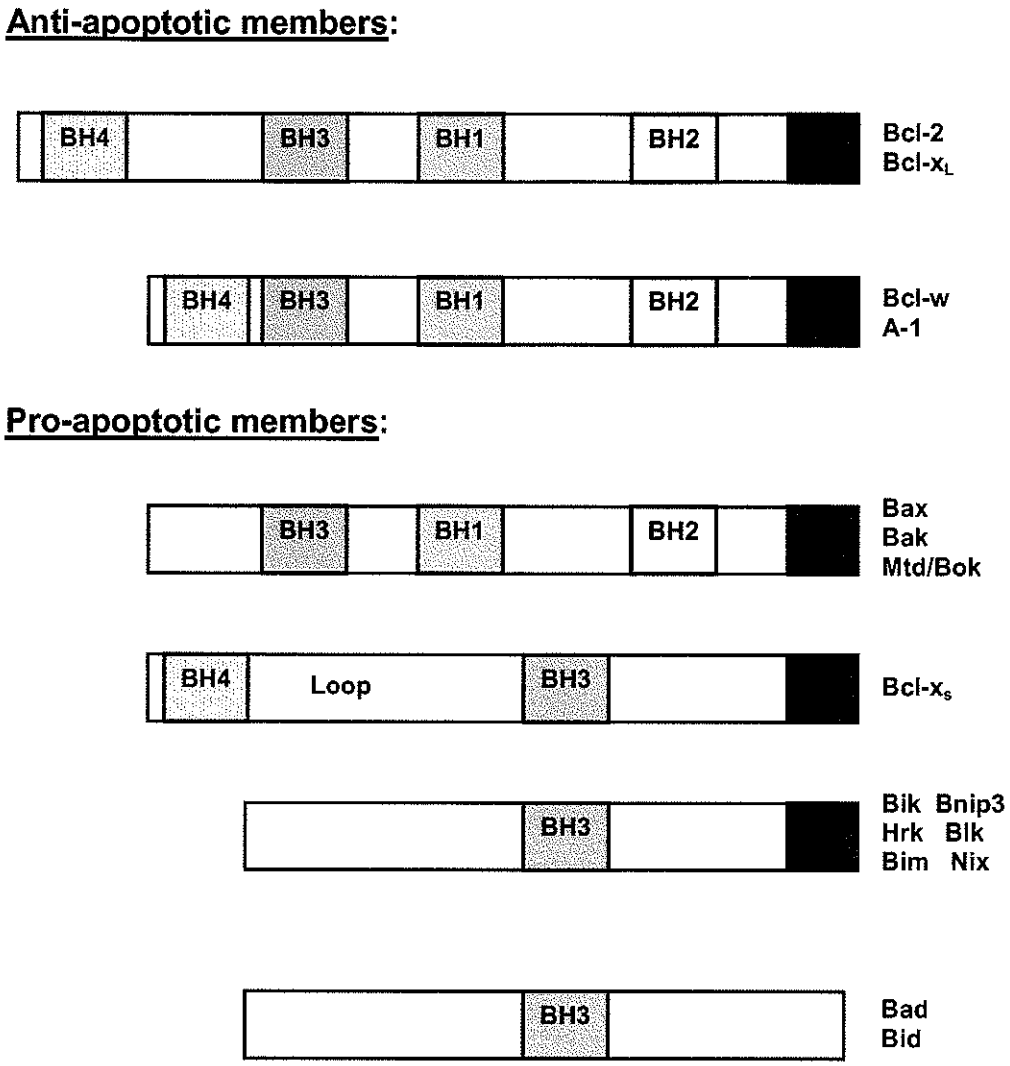

Figure 6: Structural domains of Bcl-2 family proteins. Pro- and anti-apoptotic family members consist of one to four Bcl-2 homology (BH) domains and in most cases, a transmembrane (TM) domain. A subset of Bcl-2 family members are indicated (Adapted from Kelekar and Thompson ([231])).

5.1 Mitochondria and the Bcl-2 Gene Family

Early models of apoptosis suggested that protein-protein interactions between pro- and anti-apoptotic Bcl-2 family members were responsible for regulating apoptosis. However, Bcl-2 proteins have been shown to prevent apoptosis independent of heterodimerization ([244]). Since these early models were reported, it has become increasingly apparent that Bcl-2 family members control apoptosis by regulating mitochondrial membrane permeability. Mitochondria from cells that overexpress Bcl-2/Bcl-$_{XL}$ are resistant to loss of mitochondrial membrane potential and increased mitochondrial outer membrane permeability ([245-248]), functions contingent upon an intact BH4 domain ([249]). Forced dimerization of Bax or Bak diminish mitochondrial membrane potential, increase ROS production and trigger cytochrome c release ([250-254]). It is currently unknown how Bcl-2 family members mediate these effects but the physical association with the outer mitochondrial membrane is required. In 1993, Krajewski et al. reported that Bcl-2 family members target to the outer mitochondrial membrane through a hydrophobic domain in the c-terminus known as the transmembrane domain ([241]). When this domain is removed, both localization and function of the protein are disturbed. Bcl-2 lacking the transmembrane domain is primarily cytosolic and unable to rescue apoptosis. The same mutation in Bax renders it cytosolic and defective for induction of apoptosis. In either circumstance, substitution with the same or a heterologous TM domain restores mitochondrial membrane targeting and function ([255-257]).

One way that Bcl-2 family members are believed to influence mitochondrial permeability is by altering the status of the permeability transition pore. Mitochondrial

defects associated with Bax are abrogated by cyclosporin A or Bongkrekic acid suggesting that the PTP is involved ([258]). Direct interactions between Bax and VDAC as well as Bax and ANT have been observed ([220;259]). This may account for Bax induced PTP opening following an apoptotic signal ([220]). The death inducing factors Bim, Bad and Bid also translocate to mitochondria where they presumably provoke PTP opening. Notably, defects associated with opening of the PTP are blocked by Bcl-2. It has been proposed that Bcl-2 may directly or indirectly promote PTP closure by inhibiting VDAC or by disrupting the interaction of Bax or Bak with VDAC, ANT or both ([260]). Importantly, regulation of PTP conformation be it open or closed, is contingent upon the ability of the Bcl-2 family member to localize to mitochondria.

Regulation of the PTP has been suggested to be an important property of Bcl-2 family members to control the release of proteins concealed beneath the outer mitochondrial membrane. However, given that Bax and Bax-like proteins share structural similarities with pore-forming proteins, it is conceivable that changes in mitochondrial membrane permeability may be brought about by the oligeromization and insertion of Bax into the outer mitochondrial membrane independent of an effect on the PTP ([257;261]). This would provide an explanation for the cytochrome c release observed in the absence of matrix swelling and membrane potential changes in some models ([258;262]) ([252;263;264]). The story is further complicated by new evidence which suggests that Bax and VDAC together form a pore that is larger than that of Bax or VDAC alone and capable of redistributing cytochrome c ([265]).

5.2 BNIP3

BNIP3 (Bcl-2/E1B Nineteen kDa interacting protein 3), formerly known as NIP3, is mitochondrial prodeath protein belonging to the BH3-only subfamily. BNIP3 was originally identified from a yeast two hybrid screen using the adenoviral pro-survival protein E1B 19K as bait ([266]). Subsequently, BNIP3 was found to interact with Bcl-2 through a region of the Bcl-2 protein that shares significant homology with EIB 19K ([266]). Several proteins share homology with BNIP3. These include BNIPL, BNIP3h, Nip3-like protein-x (NIX; also referred to as BNIP3L/BNIP3α/B5), as well as the C. elegans orthologue ceBNIP3 ([267-273]).

BNIP3 is comprised of three main structural elements: an N-terminal PEST sequence, a putative BH3 domain and a C-terminal transmembrane domain (Figure 7). As the acronym suggests, PEST sequences consist of numerous proline, glutamic acid, serine, threonine and aspartic acid residues which are bordered by charged amino acids. PEST sequences are typically found in proteins with high rates of turnover due to degradation by the proteasome ([274]). Chen et al., 1999 reported that cells transfected with BNIP3 demonstrate a time dependent reduction in BNIP3 protein levels as would be expected of a PEST containing protein ([269]). Further, this trend could be reversed protein levels in presence of the proteosome inhibitor, lactacystin. The significance of proteosome-mediated degradation of BNIP3 is unknown but likely serves to protect against inappropriate activation of BNIP3 specific death pathways.

The BH3 domain, common among pro-apoptotic Bcl-2 relatives, is comprised of eight amino acids with a leucine residue at position 1 and an aspartate residue at position 6. These residues are believed to be integral for heterodimerization between pro-and

anti-apoptotic Bcl-2 family members and apoptotic function ([231]). Based on the limited sequence homology between residues 110 to 118 of BNIP3 and other BH3-only proteins, BNIP3 is considered to have a putative BH3 domain (also known as a BH3-like domain) ([44;275]). It is commonly accepted that the BH3 domain plays a key role in the toxicity of prodeath Bcl-2 family members. Interestingly, deletion of the BH3 domain has no effect on the ability of BNIP3 to cause cell death ([275]). Amino acids 110 and 115 of BNIP3 are leucine and aspartate residues, respectively. The region demarcated by these key amino acids was earlier shown to be important for heterodimerization with Bcl-X_L or E1B19K ([44]). However, using yeast two hybrid analysis, coimmunoprecipitation and in vitro binding assay, Ray et al. demonstrated that the BH3 domain of BNIP3 is in fact dispensable for protein interactions with itself, Bcl-2, Bcl-X_L and Ced-9 ([275]). Instead, the transmembrane domain of BNIP3 is required for heterodimerization with Bcl-2 or Bcl-X_L and homodimerization. Similarly, neither NIX nor ceBNIP3 require the BH3 domain for homo- or heterodimerization ([44;270;271;273]).

The carboxyl-terminal transmembrane domain of BNIP3 is located at residues 164 to 184. As mentioned above, it is required for homo and heterodimerization with itself, Bcl-2 or Bcl-X_L. While homodimerization has been suggested to be a requirement for killing by some members of the Bcl-2 protein family, Ray et al. generated a series of BNIP3 mutants which illustrated that homodimerization was not a requirement of BNIP3 to induce cell death 9 ([275]). However, heterodimerization with Bcl-2 or Bcl- X_L has been shown to inhibit BNIP3 induced cell death. It remains to be determined whether these anti-apoptotic proteins protect against BNIP3 induced cell death through a direct mechanism or by inhibiting downstream defects triggered by BNIP3.

Perhaps the most important function of the transmembrane domain is to position BNIP3 within the outer mitochondrial membrane ([44;271;276]). Under basal conditions, BNIP3 is loosely associated with the outer mitochondrial membrane but becomes fully integrated via the TM domain when overexpressed ([277]). In the absence of residues 164 to 184 (BNIP3Δ164-184), BNIP3 maintains a cytosolic distribution and importantly, cannot trigger cell death ([269;272;278]). A major function of BNIP3 is to regulate PTP status suggesting that BNIP3 may promote cell death by impinging upon normal mitochondrial physiology.

Recent investigations have identified both positive and negative regulators of BNIP3 expression and function. Hypoxia is a potent inducer of BNIP3 protein expression ([9;279;280]). Hypoxia is associated with both tumor formation and ischemic injury. BNIP3 proteins levels are elevated in a variety of human tumors and in the heart in response to hypoxia ([281]). HIF-1 (Hypoxia inducible factor 1) is a transcription factor commonly elevated in cells following hypoxic insult. Notwithstanding, HIF-1 responsive elements have been identified within the BNIP3 promoter and HIF-1 reportedly induces BNIP3 expression. Activation of BNIP3 has largely been attributed to intracellular signaling events. This notion is expanding, however, with the observation that CD47, a cell surface receptor implicated in lymphocyte apoptosis, is capable of inducing BNIP3 translocation to the mitochondria and apoptosis ([282]). Rapid degradation of BNIP3 by the proteasome and undefined mechanisms which prevent mitochondrial integration serve to inhibit BNIP3 function. Importantly, Zamora et al. have identified nitric oxide (NO) to be a potent inhibitor of BNIP3 expression in mouse hepatocytes ([283]). This finding

should be viewed with caution; however, since the stimulatory or inhibitory contribution of NO to apoptotic cell death may fluctuate depending on the cellular context ([284]).

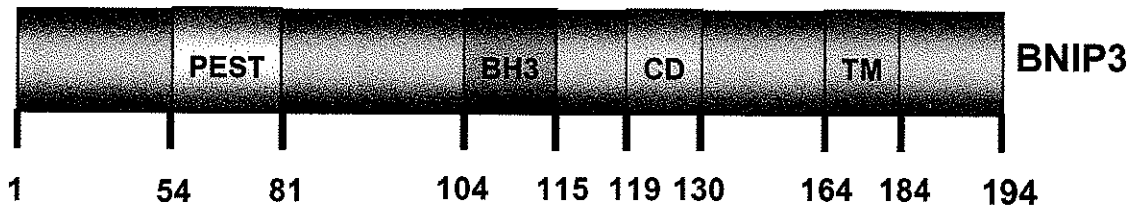

Figure 7: Schematic representation of BNIP3. The major domains of BNIP3 are depicted. Presence of a PEST sequence (PEST) suggests that BNIP3 has a high rate of turnover. A BH3-like domain (BH3) is located centrally within the protein and is believed to be important for its pro-apoptotic function. The carboxyl-terminal transmembrane domain (TM) is required for mitochondrial membrane targeting. The function of the conserved domain (CD) located between residues 119 to 130 is currently undetermined.

5.3 Bcl-2 family proteins in the heart

Many Bcl-2 family proteins are expressed in the heart. Bcl-2, Bcl-xL and BNIP3 are detected in the mitochondrial fraction of the cell, whereas Bad, Bax, and Bid shuttle between the soluble fraction of the cell and the mitochondria ([9;123;126;285;286]). Other proteins, including the Bcl-2 homologue Bcl-rambo ([287]) and BH3-only proteins Bim ([288]) and Blk ([289]) are expressed in the heart but the significance for cardiac myocyte apoptosis is unknown. Further studies are necessary to determine the total complement of Bcl-2 family members that are expressed in the heart.

Bcl-2 family proteins modulate cardiac myocyte apoptosis. Our laboratory demonstrated that overexpression of Bcl-2 is cytoprotective against p53-induced apoptosis of neonatal ventricular myocytes ([42]). Further, overexpression of Bcl-2 has been shown to abrogate apoptosis of ventricular myocytes in culture ([145]) and in vivo([286]). Prodeath Bcl-2 family members promote cardiac myocyte apoptosis through the mitochondrial death pathway. Bid is cleaved and inserts into myocyte mitochondria during ischemia/reperfusion, an event which is mediated by the calcium activated protease calpain ([286]). H_2O_2-induced myocyte apoptosis is associated with translocation of Bad ([123]) and Bax ([77]) to the mitochondria and mitochondrial defects. Recently, BNIP3 and related BH3-only family member NIX (NIP-like Protein-X) have been shown to be important mediators of cardiac cell apoptosis. Elevated levels of BNIP3 are detected in cardiomyocyte cell cultures subjected to hypoxia and whole heart following myocardial infarction ([9;280]). Overexpression of BNIP3 is sufficient to provoke apoptosis of ventricular myocytes in a caspase dependent manner. Importantly, deletion of the carboxyl-terminal transmembrane domain of BNIP3 which is crucial for insertion of

BNIP3 into mitochondrial membranes, renders BNIP3 defective for provoking mitochondrial defects and apoptosis, suggesting that this function of BNIP3 is necessary for mitochondrial dysfunction and cell death ([9]). Similar to BNIP3, elevated NIX levels are associated with cardiac dysfunction and apoptosis ([269;290]). NIX reportedly contributes to cardiac hypertrophy that transitions into overt heart failure in a Gαq-transgenic mouse model ([290]). Overexpression of NIX provokes apoptosis in a caspase dependent manner. Notably, a naturally occurring splice variant of NIX lacking the carboxyl-terminal transmembrane rescued the Gq-induced phenotype and heart failure, a phenomenon reminiscent of BNIP3([290]). BNIP3 and NIX may represent a new class of death proteins that regulate mitochondrial function and apoptosis in cardiac pathologies ([291]).

6. Tumor Suppressor Protein p53

p53 is one of the body's most important defense mechanisms against cancer having the capacity to regulate the cell cycle, apoptosis or both in a variety cell types ([292;293]). Early characterization of p53 suggested it to be an oncogene. Unfortunately, these preliminary studies were based upon a mutant form of p53 that when overexpressed was sufficient to cause oncogenic transformation of cells ([294;295]). It was not until the next decade with the identification of wild-type p53 that the true nature of p53 as an anti-oncogene was realized. Introduction of the wild-type p53 gene was found to inhibit growth of cells, a feature typical of a tumor suppressor gene ([296-298]). DNA screening found an unusually high number of p53 mutations in tumorigenic tissue taken from colon cancer patients. Indeed, it was found that the majority of human cancers contained one or more loss of function mutations in the p53 gene ([299-302]). Similarly, individuals who

suffered from Li-Fraumeni syndrome, a condition associated with excessive tumor formation, were shown to carry a germline defect in one of the p53 alleles ([303]). Importantly, p53 null mice, although developmentally normal, were highly susceptible to spontaneous tumor formation ([304]).

Tumor suppression by p53 has been largely attributed to its cell cycle control and apoptosis functions. Fibroblasts derived from p53 deficient tumors demonstrate unlimited growth potential and resistance to apoptosis. Reintroduction of functional p53 to cells restores cell cycle arrest and apoptosis ([302;304;305]). Moreover, overexpression of wildtype but not mutant p53 is sufficient to induce apoptosis and cell cycle arrest in cells ([305;306]). We and others have demonstrated that p53 causes apoptosis in ventricular myocytes ([5;42;307]).

An important property of p53 may be to protect the genome from accumulating mutations in response to genotoxic stress. p53 is typically present at low levels in normal cells with a half-life of less than 30 minutes but accumulates in response to DNA damaging agents such as ionizing radiation, UV radiation or cytotoxic compounds ([208;308-310]). Cells exposed to these agents undergo apoptosis and cell cycle arrest in a p53 dependent manner ([208;309]). Lowe et al. demonstrated that mouse thymocytes deficient for one or both p53 alleles were resistant to radiation-induced apoptosis in a manner consistent with the level of p53 expression ([293]). Moreover, hematopoietic cells that either lacked p53 gene expression or overexpressed a mutant form of the p53 gene did not exhibit cell cycle inhibition after gamma-irradiation ([308]). By arresting the cell cycle and permitting time for DNA repair or by permanently removing the cell by apoptosis, the cell cannot transmit harmful DNA mutations to daughter cells ([311]). More recently,

this notion of p53 as a guardian within the cell has been extended beyond genotoxic stress. Other pathological stimuli including hypoxia ([312]), alterations in redox balance ([313]), heat shock ([314]) and nucleotide depletion ([315]) have been shown to induce p53 (Figure 8).

6.1. The structure of the p53 protein

P53 is a tetrameric, sequence specific DNA binding protein responsible for the transcriptional activation of numerous target genes ([316-319]). Human p53 protein consists of 393 amino acid residues, which has been characterized on the basis of 4 major domains. The N-terminus of p53 (residues 1-42) constitutes the acidic transcriptional activation domain required for the activation of p53 dependent genes ([320]). Centrally within the protein lies the core domain (residues 102-292) which enables sequence specific DNA-binding of p53 to responsive genes ([321-323]). Oligomerization (residues 300-355) and nuclear localization (residues 363-393) motifs are located at the C-terminus (Figure 9).

Numerous genes involved in apoptosis or cell cycle control are transcriptionally activated by p53. These include Bax ([324]), Puma ([325]), p21 ([326]), insulin-like growth factor 1 (IGF-1) receptor, IGF-BP3 ([327]) and MDM2 ([328]). Not surprisingly, the majority of human tumors contain mutations within the DNA binding domain of p53 ([302]). Several oncogenic DNA viruses have evolved mechanisms to block the *trans*-activation potential of p53. The adenovirus E1B 55-kD protein ([329]), SV40 large T antigen ([330]), and the E6 protein of oncogenic forms of human papillomavirus (HPV E6) ([331]) produce proteins that associate with and inhibit the *trans*-activation function of p53. Endogenously, *trans*-activation by p53 can be abrogated through interactions with MDM2. MDM2 directs the

ubiquitination and degradation of p53. Because the MDM2 gene is also activated by p53, this creates a negative feedback loop which physiologically disengages p53 whereby it can no longer function as an activator of transcription.

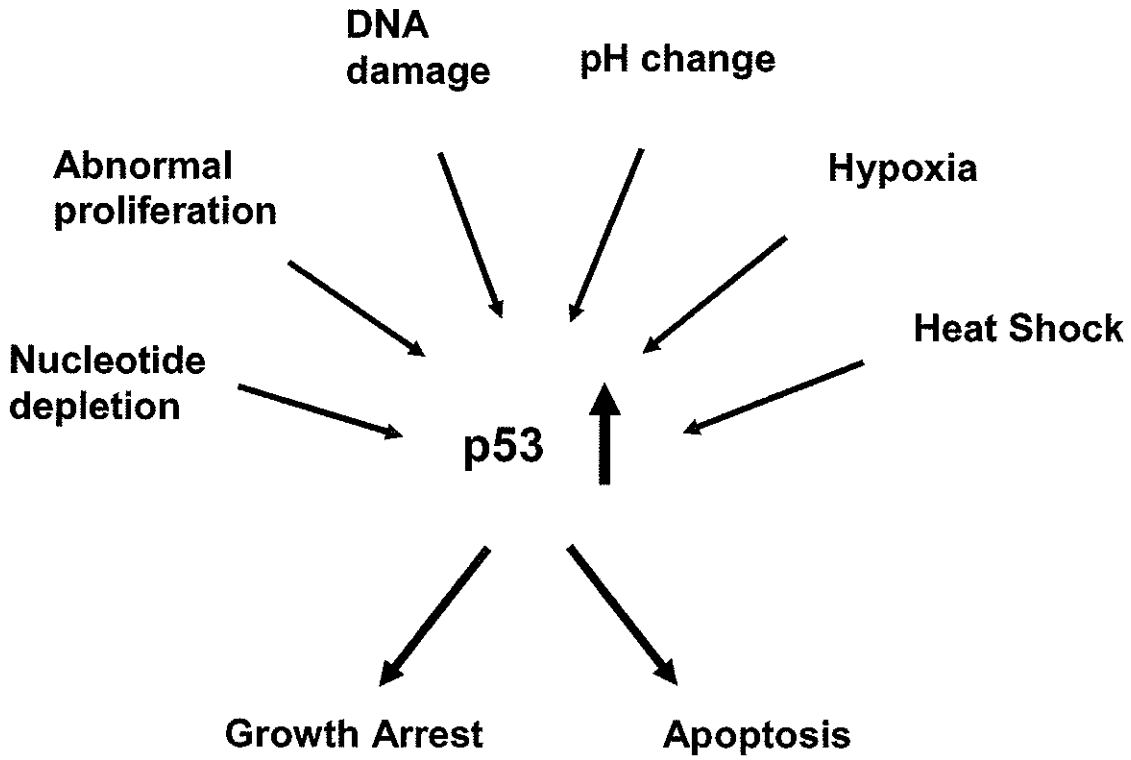

Figure 8: Induction of p53. Numerous pathological stimuli trigger the accumulation and activation of p53 protein in cells resulting in growth arrest and apoptosis.

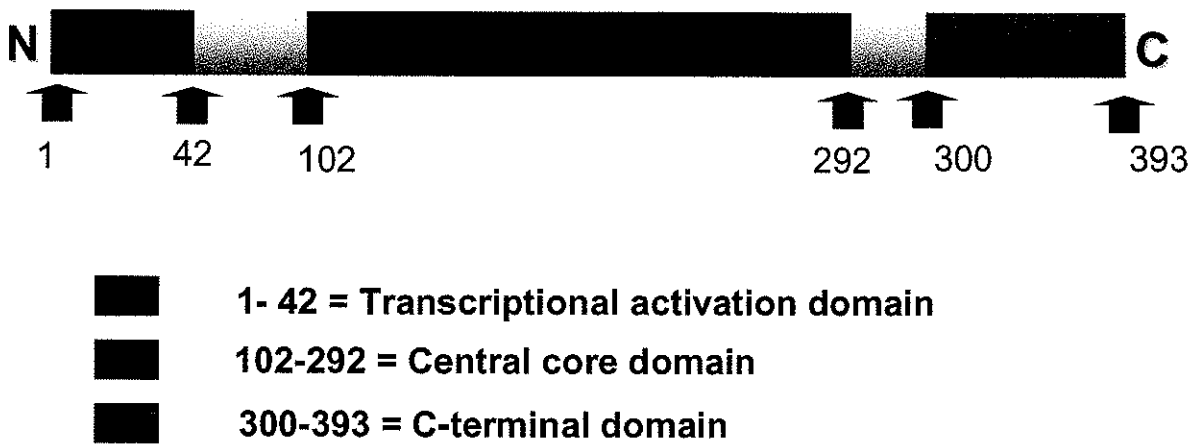

Figure 9: Schematic representation of p53.

6.2 Homologues of p53

Recently 2 homologues of p53 have been identified known as p63 and p73. Two isoforms of p63 have described which are known as p63α-γ and ΔNp63α-γ. Conversely, nine different isoforms of p53 have been identified. Additionally, a second class of p73 variants has recently been identified which lacks the N terminal transactivation domain. p63 and p73 share considerable homology with the activation, DNA binding, and oligomerization domains of p53; however, these proteins appear to be differentially regulated by pro-apoptotic signals and have different transcriptional targets ([332;333]). For example, while full length p73 variants are not induced by DNA damage, their overexpression causes growth arrest and apoptosis ([334]). It has been postulated that full length p73 may functionally substitute for p53 for cell cycle arrest. Interestingly, the truncated isoforms of p73 inhibit the pro-apoptotic function of p53 and full length p73, suggesting a complex relationship between homologues and variants (reviewed in ([335])).

6.3. The role of p53 in the heart

Although the molecular mechanisms by which apoptotic cell death is activated in the heart are unknown, there is evidence to suggest a role for p53. Elevated levels of p53 associated with increased apoptosis have been detected in the heart following myocardial hypoxia ([5]), receptor-mediated Angiotensin II activation ([307]), hyperglycemia ([336]), ischemia ([80]), dog hearts subjected to rapid ventricular pacing ([337]) and in the border zone of infarcted hearts ([338;339]). A substantial reduction in apoptosis is observed in heart allografts obtained from p53 null mice ([340]). Furthermore, over-expression of p53 is sufficient to trigger apoptosis of cardiomyocytes, a finding that supports the involvement of p53 in the apoptotic cell death observed during cardiac disease. A few reports suggest

that p53 is not required for cardiomyocyte apoptosis ([6;11]). Importantly, these studies used acute models of injury where p53 levels would not have had sufficient time to accumulate. Prolonged exposure to the toxic stimulus would likely demonstrate an involvement of p53.

The ability of p53 to cause apoptosis has largely been attributed to transcriptional activation of the death promoting gene Bax. In Bax knock-out mice, granulosa cells, certain neurons, and immature germ cells were found to be defective for apoptosis in the absence of Bax ([341]). Despite this, Bax null lymphocytes exhibit normal sensitivity to gamma irradiation suggesting that Bax may not be essential for p53-dependent apoptosis in all cases ([342]). In the heart, elevated levels of p53 often couple with increased Bax expression ([343-345]). However, we have recently shown that a mutant p53, deficient for DNA binding and gene transactivation, is sufficient to trigger cardiac cell apoptosis with activation of the mitochondrial death pathway. Recent reports of a mitochondrial pool of p53 suggest that p53 may directly activate the mitochondrial death pathway. Alternatively, p53 may influence existing pools of Bax protein to trigger cell death. Mutational analysis of p53 may be required to fully understand its role at the level of the mitochondria.

III. MATERIALS AND METHODS

1. Primary cell culture

Ventricular myocytes were isolated from 1 to 2 day old Sprague-Dawley rats ([346-348]). Rats were sacrificed by cervical dislocation and submerged in 70 % ethanol. Hearts were removed and minced in ice-cold phosphate buffered saline containing 10 g/L of glucose (PBS^{-2}). Connective tissue and exposed DNA were digested with collagenase CLSII, trypsin and DNase. Myocytes were then purified in a two-step manner consisting of Percoll gradient centrifugation followed by 45 minutes of pre-plating onto a 150mm polystyrene tissue culture dish (Sarstedt, Inc, Montreal, QC). Following purification, myocytes were submitted to primary culture in Dulbecco's Modified Eagle medium/Ham's nutrient mixture F12,1:1 (DMEM/F-12; Gibco, Burlington, ON) with 2mM L-glutamine, 15mM Hepes (Roche, Laval, QC), 3mM $NaHCO_3$ (Sigma-Aldrich Canada, Oakville, ON),10 ug/ml gentamycin (Gibco, Burlington, ON) and 10% fetal bovine serum (Gibco, Burlington, ON). Cells were maintained in a tissue culture incubator at 37°C under 5% CO_2 on 6 well Primaria tissue culture dishes (VWR, Mississauga, ON) or on collagen coated coverslips (Fisher Scientific, Whitby ON). Culture media was replaced 24 hours later with serum free DMEM/F-12 containing 15mM Hepes (Roche, Laval, QC), 3mM $NaHCO_3$, (Sigma-Aldrich Canada, Oakville, ON), 1nM Na_2SeO_4 (Sigma-Aldrich Canada, Oakville, ON), 5 µg/ml transferrin (Sigma-Aldrich Canada, Oakville, ON), 1nM LiCl (Sigma-Aldrich Canada, Oakville, ON), 1 µg/ml insulin (Sigma-Aldrich Canada, Oakville, ON), 25 µg/ml ascorbic acid (Sigma-Aldrich Canada, Oakville, ON) and 10 µg/ml gentamycin (Gibco, Burlington, ON).

2. Cell Transfection

For transfection of neonatal myocytes, cells were incubated for 40 minutes with DMEM containing DEAE-Dextran, 2.5 μg of cytomegalovirus (CMV) promoter driven β galactosidase plasmid, 5 μg of a Bax luciferase reporter gene and 2.5 % newborn calf serum ([346-348]). The Bax luciferase reporter gene, designated Baxluc, consisted of nucleotides –318 to –688 of the human Bax promoter which contains tandem p53 consensus binding sites 5'-GAGACAAGCCTGGGCGTGGGGCT-3' ([42]). Immediately thereafter, cells were stimulated for 60 seconds with 10% dimethyl sulfoxide in DMEM. Cells were maintained in serum free DMEM/F-12 until harvest 24 hours later. At harvest, cells were washed once in PBS^{-2} and lysed in 200μl of 25mM Tris, 2mM DTT, 2mM 1,2-diaminocyclohexane-N,N,N',N'-tetraacetic acid, 10% glycerol and 0.1% Triton-X-100. Following a 10 minute incubation at 4°C, 20 μl of cell lysate was mixed with 100μl of 20mM tricine pH 7.8, 1.07 mM $MgCO_3 \cdot 4\ Mg(OH)_2 \cdot 5\ H_2O$, 2.67 mM $MgSO_4$, 0.1 mM EDTA, 3.33 DTT, 270 μM coenzyme A (Sigma-Aldrich Canada, Oakville, ON), 470 μM D-luciferin (Roche, Laval, QC) and 530 μM ATP. *Luciferase activity:* Samples were assayed in triplicate for the oxidation of luciferin using a Lumat LB9501 luminometer ([349]). *β-galactosidase activity:* 80 μl of the aforementioned cell lysate was added to 4.85 mg/ml chlorophenol red-β-$_D$ galactopyranoside SQ, 51.1 mM Na_2HPO_4, 11.22 mM NaH_2PO_4, 1mM $MgCl_2$, 45mM β-mercaptoethanol (Eustice DC e al, 1991). β-galactosidase activity was then determined colorometrically as absorbance at 575 nm by spectrophotometry. To account for potential differences in transfection efficiency between conditions, luciferase activity was normalized to β-galactosidase activity and expressed as relative light units.

3. Recombinant adenoviruses

Adenoviruses encoding CrmA, wildtype p53 (p53WT) and a mutant form of p53 with an Alanine to Valine substitution at position 135 (p53MT) were generated. First, the cDNA was subcloned into an adenoviral shuttle vector. The 1.4 kb CrmA cDNA (a gift from Dr. David Pickup, Duke University) ([153]) was subcloned into PCA3 while the 1.4 kb cDNA fragments of p53WT and p53MT were subcloned into PCA4. Next, the shuttle vector containing the cDNA insert was rescued into PJM17 adenoviral particles by homologous recombination using HEK 293 cells as the vehicle ([350]). Adenoviruses encoding CrmA, wildtype p53 and mutant p53 were designated AdCrmA, Adp53WT and Adp53MT, respectively. A control virus lacking the cDNA insert was also generated and designated AdCMV. All adenoviruses were driven by the cytomegalovirus promoter (CMV). Replication defective adenoviruses were propagated in Human embryonic kidney (HEK) 293 cells and harvested by freeze-thawing. Adenoviral titers were determined by plaque assay in HEK cells.

4. Adenovirus infections

Myocyte cultures were infected with recombinant adenovirus diluted to $2x10^8$ plaque forming units (pfu) per mL in serum free DMEM/F12 for 4 hours. This equates to a multiplicity of infection of between 10-200 viral particles per cell and achieves gene delivery to ≥95% of neonatal ventricular myocytes ([348]). Subsequently, fresh medium was applied in the absence of virus and cells were incubated for an additional 44 hours.

5. Hypoxia

Serum free DMEM/F-12 was made hypoxic by pre-gassing for 1 hour with 95% nitrogen and 5% CO_2. Hypoxic media was then applied to primary cultured neonatal ventricular myocytes and continuously gassed in an airtight chamber. Control myocytes were maintained under standard conditions with 95% O_2 and 5% CO_2.

6. Assays of apoptosis

A. Analysis of cellular viability: Myocytes were assessed for viability by staining with the vital dyes Calcein-AM (acetoxymethyl ester; 2μM) and ethidium homodimer-1 (2μM) for 30 minutes (Molecular Probes, Eugene, OR, USA). Cells were washed three times with PBS^{-2}, inverted on glass slides and visualized by immunofluorescence microscopy using an Olympus AX70 Research microscope (Carsen Scientific Imaging Group, Markham, ON) with the capacity to visualize both living (green) and dead (red) cells, simultaneously.

B. Hoechst 22358 staining: To visualize the nuclear morphology of cardiac myocytes, cells cultured on glass coverslips were fixed for 15 minutes in 70% ethanol and myocytes identified by indirect immunocytochemistry using MF20 hybridoma supernatant (D. Bader, 1:5 dilution) against sarcomeric heavy chain and 10 μg/ml rhodamine-conjugated goat $F(ab)'_2$ anti-mouse IgG (Molecular Probes; Eugene, OR, USA). Myocytes were stained for 3 minutes at room temperature with Hoechst 33258 dye (1 μg/ml; Molecular Probes; Eugene, OR, USA). Following staining, myocytes were washed three times with PBS^{-2}, inverted and mounted on glass slides with antifade FluorSave reagent (Calbiochem; La Jolla, CA) ([42]). Cell analysis was conducted on an Olympus AX70

Research microscope (Carsen Scientific Imaging Group, Markham, ON) equipped with excitation and emission filters enabling the detection of Hoechst 33258.

C. TUNEL Assay: To visualize apoptotic nuclei in situ, cardiac myocytes were fixed in 3.7% paraformaldehyde (pH 7.4) for 1 hour at room temperature and subjected to terminal transferase-mediated dUTP-biotin nick end-labeling (TUNEL) assay ([84]). Myocytes were incubated with terminal deoxynucleotide transferase (TdT) buffer containing 140 mM sodium cocodylate, 1mM cobalt chloride, 30 mM Tris-HCl, pH7.2, 1nmol of fluoroscein-conjugated dUTP and 50 units of terminal deoxynucleotide transferase (Roche, Laval, QC) for 1 hr at 37°C. Following nuclear labeling, myocytes were washed three times with PBS^{-2}, inverted and mounted on glass slides with antifade FluorSave reagent (Calbiochem; La Jolla, CA). Cell analysis was performed using the Olympus AX70 Research microscope (Carsen Scientific Imaging Group, Markham, ON) equipped with appropriate fluorescein isothiocyanate filters.

D. DNA laddering: Genomic DNA was isolated from neonatal ventricular myocytes. Genomic DNA was resolved by DNA gel electrophoresis and visualized for DNA fragmentation ([348]).

7. Caspase Inhibition

Synthetic peptide caspase inhibitors were utilized to block caspase activation under different experimental conditions (Biomol, Plymouth Meeting, PA, USA)([351]). Myocytes were pre-incubated with 100-150 μM of the caspase 3-like inhibitor Ac-DEVD-CHO (N-acetyl-Asp-Glu-Val-Asp-aldehyde), Ac-YVAD-CHO (N-acetyl-Tyr-Val-Ala-Asp aldehyde) an inhibitor of large prodomain caspases related to caspase 1, 8 and 10 or the

pan-caspase inhibitor, zVAD-fmk (benzyloxycarbonyl-valine-alanine-aspartyl methoxy fluoromethyl ketone). Alternatively, to circumvent the potential limitations of peptide inhibitors including short half-life and limited ability to permeate membranes, adenoviral mediated delivery of CrmA was employed to inhibit caspase 8 activation.

8. Detection of caspase activity

A. Caspase 3-like activity The activation of caspase 3-like protease activity was determined using the ApoAlert Fluorescent Assay Kit (BD Biosciences Canada, Mississauga, ON) as instructed by the manufacturer. In brief, 40μg of cardiac cell lysate was incubated with the caspase 3 substrate Asp-Glu-Val-Asp (DEVD) conjugated to 7-amino-4-trifluoromethyl coumarin (AFC). The fluorescence emission of AFC was monitored by fluorometry (Photon Technology International, Lawrenceville, New Jersey, USA) at an excitation setting of 400 nm and an emission setting of 505nm. A shift in fluorescence emission reflected increased caspase 8 activity. Data was obtained for n=3 independent myocyte cultures.

B. Caspase 8-like activity The activation of caspase 8-like protease activity was determined using the ApoAlert Fluorescent Assay Kit (BD Biosciences Canada, Mississauga, ON) as instructed by the manufacturer. In brief, 40μg of cardiac cell lysate was incubated with the caspase 9 substrate Iso-Glu-Thr-Asp (IETD) conjugated to 7-amino-4-trifluoromethyl coumarin (AFC). The fluorescence emission of AFC was monitored by fluorometry (Photon Technology International, Lawrenceville, New Jersey, USA) at an excitation setting of 400 nm and an emission setting of 505nm. A shift in

fluorescence emission reflected increased caspase 8 activity. Data was obtained for n=3 independent myocyte cultures.

9. Immunofluorescence microscopy

Cardiac myocytes were identified by indirect immunocytochemistry using MF20 hybridoma supernatant (D. Bader, 1:5 dilution) against sarcomeric heavy chain and 10 μg/ml rhodamine-conjugated goat $F(ab)'_2$ anti-mouse IgG (Molecular Probes; Eugene, OR, USA).

10. Confocal Microscopic detection of Cytochrome c

Neonatal Ventricular myocytes plated on glass coverslips (Fisher Scientific, Whitby ON) were incubated with 0.1 μM MitoTracker Red CMX-Ros (chloromethyl X-rosamine; Molecular Probes; Eugene, OR, USA) for 30 minutes at 37°C to label respiring mitochondria. Subsequently, myocytes were fixed in 3.7% paraformaldehyde (pH 7.4) for 1 hour at room temperature, washed three times with PBS^{-2} and permeabilized at room temperature for 10 minutes with 0.1% Triton-X-100. Myocytes were incubated overnight with a mouse monoclonal antibody directed toward mitochondrial cytochrome c (PharMingen, Mississauga, ON) followed by 0.5 μg/ml Alexa 488-conjugated donkey anti-mouse IgG (Molecular Probes, Eugene Oregon, USA). Myocytes were visualized using an Olympus IX 70 Research microscope equipped with an Olympus Fluoview laser-scanning module (Carsen Scientific Imaging Group, Markham, ON).

11. Isolation of mitochondrial and cytoplasmic fractions

Myocytes were fractionated into mitochondrial and cytosolic S100 compartments. Myocytes were rinsed once in ice cold PBS^{-2} and harvested in 200µl of isotonic buffer A (250mM sucrose, 20 mM HEPES, 10 mM KCl, 1.5 mM $MgCl_2$, 1mM EDTA, 1mM EGTA, 1mM DTT, 1mM PMSF, 8 µg/ml aprotinin, 2 µg/ml leupeptin, 5 µg/ml pepstatin, pH 7.4). A 15-minute incubation on ice followed before cells were disrupted on ice with 15 strokes of a dounce homogenizer. The resulting homogenate was centrifuged twice at 750xg for 10 minutes at 4°C. Thereafter, the resulting supernatant was removed and centrifuged to isolate the cytoplasmic and mitochondrial fractions. Finally, an ultracentrifugation was performed to ensure a pure cytoplasmic fraction devoid of mitochondrial contamination.

12. Western blot analysis

For immunodetection of proteins, myocytes were harvested in RIPA lysis buffer containing 0.5% sodium dodecyl sulfate (SDS), 150 mM NaCl and 50 mM Tris·HCl supplemented with a protease inhibitor cocktail. Cell lysate concentrations were determined by comparison against known BSA (bovine serum albumin) concentration standards. Cell lysates (50-100µg) were resolved on an 8-15% SDS-polyacrylamide gel at 140 V for 4 hours and electrophoretically transferred to polyvinylidene difluoride (PVDF) membrane. During protein detection, membranes were first incubated with 1X Blocking Reagent (Roche, Laval, QC) for 1 hour at room temperature to reduce nonspecific binding of the primary antibody. Subsequently, all antibodies were diluted in 0.5X Blocking Reagent for membrane incubation. Membranes were incubated with

primary antibodies overnight at 4° C and then washed 3 times with Tris Buffered Saline with Tween-20 (TBS-Tween; 150 mM NaCl, 50 mM Tris –HCl pH 7.4, 0.3% Tween-20, 0.1% BSA). Subsequently, membranes were incubated with secondary antibodies for 1 hour at 4° C followed by three washes with TBS-Tween. Bound secondary antibody was detected by chemiluminescence using ECL reagents (Amersham Biosciences, Baie d'Urfe, QC).

13. Antibodies

Mouse monoclonal antibody specific for amino acids 112-124 of human BNIP3 (clone Ana40) was a gift from Dr. Arnold Greenberg (University of Manitoba, Winnipeg, MB, Canada). Mouse monoclonal antibodies directed toward mitochondrial cytochrome c and cytochrome c oxidase were obtained from PharMingen (Mississauga, ON) and Molecular Probes (Eugene, Oregon, USA), respectively. Mouse monoclonal antibody specific for amino acid residues 371-380 of human p53 was purchased from Oncogene Resaerch Products (Boston MA, USA). Mouse monoclonal antibodies directed toward MDM2, Bak, and Bax and Bcl-2 were purchased from PharMingen (Mississauga, ON). Mouse monoclonal antibody directed toward Bad was purchased from Transduction laboratories (Lexington, KY, USA). Rabbit polyclonal antibody for detection of the active p17 large fragment of caspase 3 (clone R280) was a gift from Dr. Donald Nicholson (Merck Frosst laboratories, Montreal, QC, Canada). Rabbit polyclonal antibody directed toward CrmA was a gift from Dr. David Pickup, Duke University. Anti-human mouse monoclonal antibody directed toward the 40kD parent and 23kD cleavage product of Caspase 8 was obtained from PharMingen. Antibody for the detection of α-sarcomeric actin was

purchased from Sigma-Aldrich Canada (Oakville, ON). Primary antibodies were detected by goat anti-mouse or goat anti-rabbit antibody conjugated to horseradish peroxidase (Caltag Laboratories).

14. Electromobility shift assay (EMSA)

Isolation of nuclear extract: Nuclear extracts prepared from neonatal cardiac myocytes. $3X10^6$ cells were pelleted and resuspended in 200µl of 10 mM HEPES, pH 7.9, 60 mM KCl, 1.0 mM EDTA, 1.0 mM dithiothreitol (DTT), and 0.3 % Nonidet P-40 and protease inhibitors. Cells were allowed to lyse on ice for 15 minutes and then centrifuged at 1000g at 4°C to pellet nuclei. The supernatant was discarded and the cell pellet was resuspended in 50µl of 200 mM HEPES, pH 7.9, 0.4 M NaCl, 1.0 mM EDTA, 1.0 mM EGTA, 1mM DTT and 1mM phenylmethylsulfonyl fluoride at 4°C for 15 minutes. The nuclear extract was centrifuged for 5 minutes at 10000 g and was frozen at –80°C.

Electromobility shift analysis: DNA binding reactions were carried out using a ^{32}P radiolabeled probe containing p53 consensus binding sites 5'-GAACATGTCTAAGCATGCTG-3'. DNA binding reactions (20µl) were carried out on ice and contained 5 µg nuclear extract, 2 ug double-stranded probe, poly(dI-dC) (Amersham Biosciences, Baie d'Urfe, QC), 10 µg BSA in 20 mM HEPES, pH7.9, 5% gycerol, 1 mM EDTA and 5mM DTT. Nuclear-protein complexes were resolved on a 5% polyacrylamide gel in 1X tris-borate-EDTA (pH 8.0) and were detected by autoradiography.

15. Mitochondrial membrane potential ($\Delta\Psi_m$)

Mitochondrial membrane potential ($\Delta\Psi_m$) was monitored in isolated cardiac myocytes using the potentiometric dyes: JC-1 (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolyl-carbocyanine iodide; Molecular Probes; Eugene, Oregon, USA) and TMRM (tetramethyl rhodamine methyl ester perchlorate; Molecular Probes, Eugene, Oregon, USA). JC-1 is a dual emission potential sensitive probe which fluoresces green at low membrane potential and red at high membrane potentials. Alternatively, TMRM is a single emission dye which exhibits a red shift upon membrane potential driven mitochondrial uptake. Myocytes plated on glass coverslips were incubated for 30 minutes with either 1 μM JC-1 or 50 nM TMRM ([184;352]). To confirm the accuracy of the dyes, cells were pretreated with 50 μM cyanide 3-chlorophenylhydrazone (CCCP; Sigma-Aldrich Canada, Oakville, ON) for 10 minutes ([353]). CCCP, a protonophore, uncouples mitochondria resulting in complete dissipation of inner mitochondrial transmembrane potential. Fluorescent images were captured using a high-speed Sensys digital camera (Photometrics, Inc, Waterloo, Ontario, Canada) and integrated optical densities as an index of $\Delta\Psi_m$ were determined using Image Pro-plus software (Media Cybernetics Inc. Silver Spring, MD).

To confirm that red fluorescence accurately reflects polarized mitochondrial membranes, we treated cells with the protonophore carbonyl cyanide 3-chlorophenylhydrazone (CCCP) to dissipate the H^+ gradient generated by the inner mitochondrial membrane electron transport chain and monitored the reduction in red fluorescence. In contrast to control cells, cell treated with CCCP displayed a discernable

reduction in red fluorescence confirming the utility of JC-1 for monitoring membrane potential (Figure 15B & 5D).

16. Mitochondrial permeability transition (PT)

To monitor permeability transition pore (PTP) opening, ventricular myocytes plated on glass coverslips were loaded with 5 μM calcein-acetoxymethylester (calcein-AM, Molecular Probes, Eugene, Oregon, USA) in the presence of 5 mM cobalt chloride to quench the cytoplasmic signal ([184;354]). Following a 30 minute incubation period at 37°C, cells were gently washed in PBS^{-2} and visualized by fluorescence microscopy.

17. Statistical analysis

Data was obtained from at least n=3 independent cell cultures with three replicates of each condition. Within each condition, at least 200 cells were analyzed. Results were compared by unpaired two-tailed Student's t-test using a level of significance of $P \leq 0.05$.

IV. RESULTS

1. Mechanisms of hypoxia-mediated cell death of ventricular myocytes

1.1 Hypoxia-mediated apoptosis of neonatal ventricular myocytes

Neonatal ventricular myocytes were subjected to 24 hours of hypoxia (95% N_2-5% CO_2, pO_2 33mmHg) and assayed for the occurrence of apoptosis. Nuclei were stained *in situ* with Hoechst 33258 and visualized by immunofluorescence microscopy (Figure 10A). While normoxic myocytes displayed smooth intact nuclear demarcations, hypoxic myocytes displayed characteristic features of apoptosis including nuclear condensation, hyperchromatic fluorescence and nuclear fragmentation. The percentage of nuclei displaying apoptotic morphology was determined (Figure 10B). In contrast to normoxic control cells, hypoxia invoked a 9-fold increase ($p<0.05$) in apoptotic nuclei. One of the key features of apoptosis is the nucleosomal fragmentation of DNA into 180-200 base-pair fragments also referred to as DNA laddering. Hypoxia-mediated apoptosis was verified by gel electrophoresis of nuclear DNA (Figure 10C). In contrast to normoxic control cells, a significant increase in nucleosomal DNA laddering was observed in hypoxic cells.

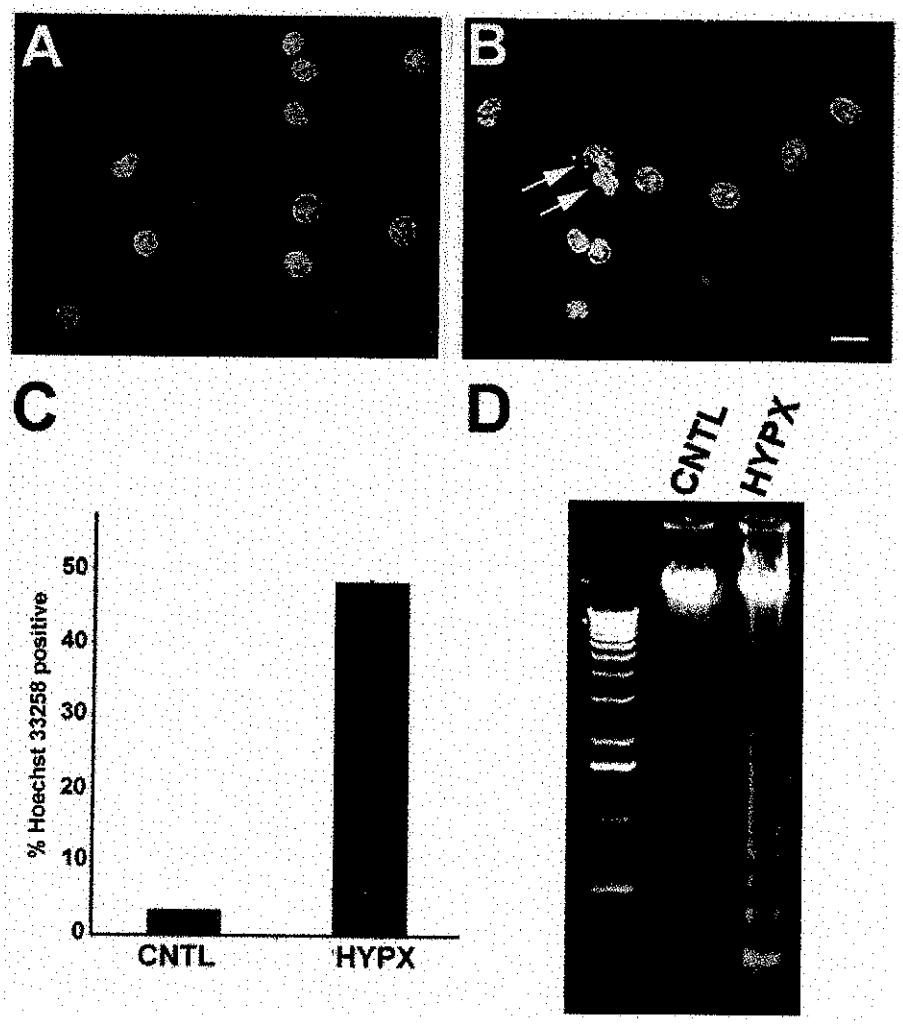

Figure 10: Hypoxia induces DNA fragmentation of neonatal ventricular myocytes Panels A&B) Fluorescent microscopic images of neonatal ventricular myocytes doubled stained for sarcomeric myosin (red) and nuclear morphology with Hoechst 33258 (blue). Panel A) Normoxic cells. Panel B) Hypoxic cells. Panel C) Histogram illustrating the percentage of apoptotic cells as determined by Hoechst 33258 nuclear staining. CNTL (normoxic cells); HYPX (hypoxic cells). Data represents mean ±SE ($P<0.001$) obtained from n=3 independent myocyte isolations with 2 replicates for each condition and >200 cells counted per condition. Arrows depict apoptotic nuclei. Bar = 10 μm. Panel D) Gel electrophoresis of nuclear DNA extracted from normoxic and hypoxic neonatal ventricular myocytes.

1.2 Activation of Caspase 8

Evidence in the literature has suggested that caspase 8 is an apical, initiator caspase important for activation of the mitochondrial death pathway, distal caspase activation and apoptosis. To determine whether procaspase 8 was processed during hypoxia, we performed western blot analysis of myocytes subjected to 24 hours of hypoxia (Figure 11A). In contrast to normoxic cells, hypoxia resulted in a significant increase in the processing of procaspase 8 to its 40 kDa and 23 kDa cleavage fragments. Ponceau-s staining confirmed even loading of proteins. To confirm that processing of caspase 8 was accompanied by increased caspase 8 activity, we performed a fluorogenic assay to monitor protease activity (Figure 11B). Cell lysate was incubated with the substrate for caspase 8 (IETD) conjugated to AFC. Active caspase 8 results in the cleavage of IETD-AFC which can be measured by fluorometry at 505 nm. A 1.5 fold induction ($p<0.01$) in caspase 8-like activity was observed in hypoxic myocytes compared to normoxic cells suggesting that hypoxia provokes caspase 8-like activity.

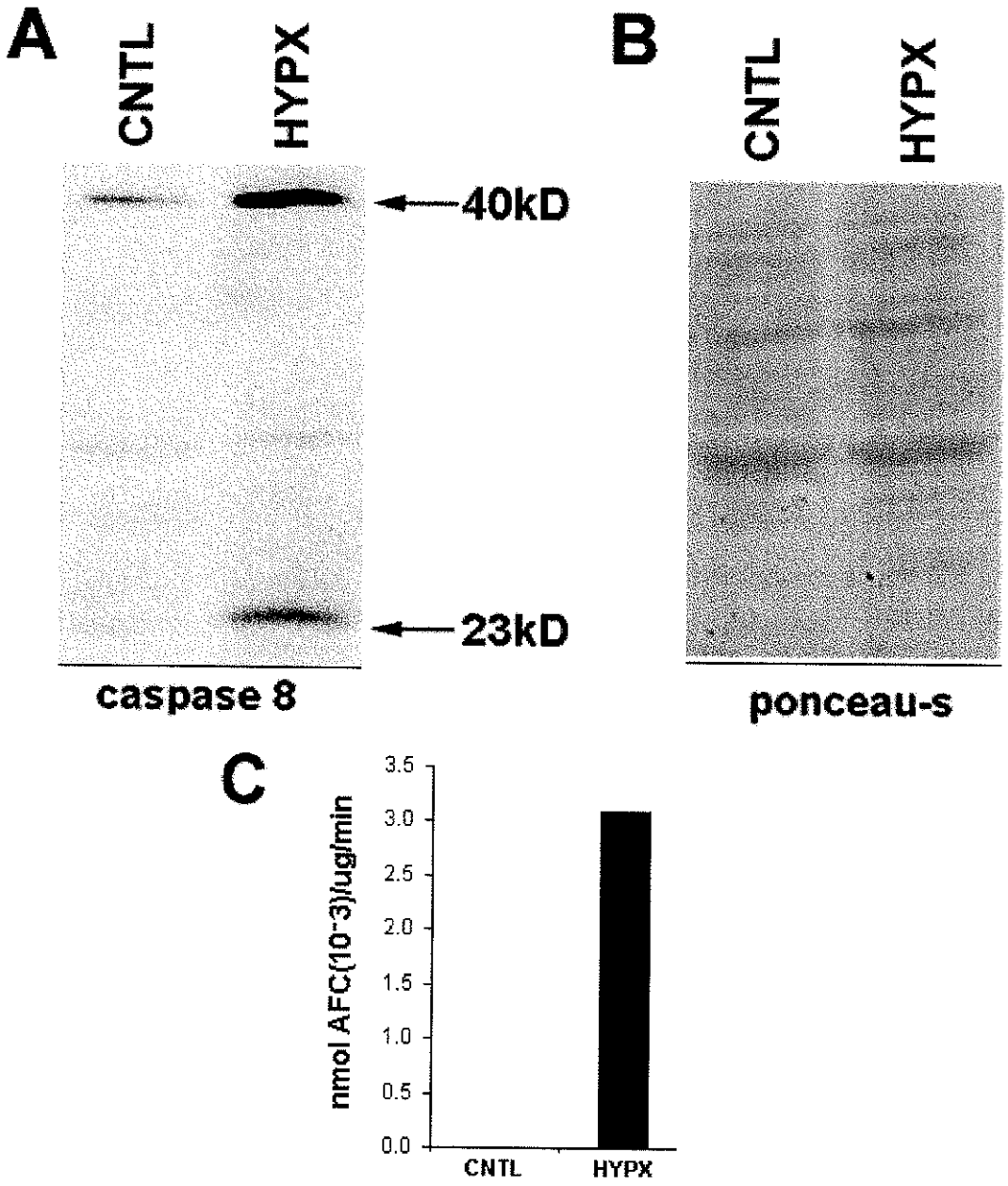

Figure 11: Hypoxia triggers proteolytic cleavage and enzymatic activation of caspase 8

Panel A) Western blot analysis of cardiac cell lysate derived from normoxic and hypoxic neonatal ventricular myocytes. Proteolytic cleavage products of caspase 8 are apparent in cells subjected to hypoxia. CNTL (normoxic cells); HYPX (hypoxic cells). Arrows depict 40 kDa and 23 kDa cleavage fragments of caspase 8. Panel B) Ponceau-s staining to demonstrate equivalent protein loading. Panel C) Fluorometric analysis of caspase 8-like activation during hypoxia. Hypoxia results in a 1.5 fold increase ($P<0.01$) in caspase 8 like activity compared to normoxic cells. Data are expressed as mean nmol AFC/µg per minute released from the IETD-AFC substrate. Data were obtained from at least n=3 independent myocyte isolations.

1.3 Caspase 8 inhibition by CrmA

To determine the role of caspase 8 during hypoxia-mediated apoptosis, we generated a replication defective adenovirus encoding CrmA, a serpin protein originating from the cow pox virus which is known to inhibit caspase 8 activity ([355]). Neonatal ventricular myocytes were infected with AdCrmA or incubated in serum-free medium for 24 hours (Figure 12A). Subsequently, cells were subjected to 24 hours of hypoxia. Western blot analysis confirmed the efficient production of the 38kDa CrmA protein in neonatal ventricular myocytes infected with AdCrmA under normoxic or hypoxic conditions. CrmA was not detected in cells infected with a control virus lacking the cDNA insert (data not shown). Of significance, cells expressing CrmA showed reduced caspase 8-like activity when subjected to hypoxia, verifying that the CrmA protein was functionally active and sufficient to inhibit caspase 8-like activity (Figure 12B).

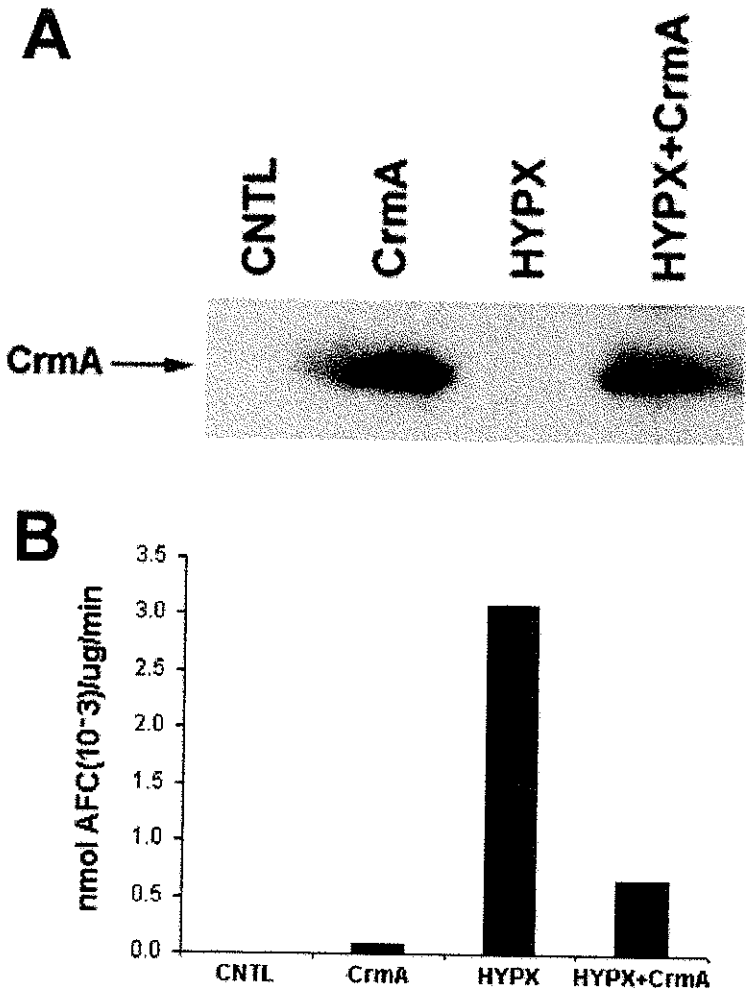

Figure 12: Crma inhibits hypoxia-mediated caspase 8-like activity.
Panel A) Western blot analysis of CrmA protein. CrmA protein is efficiently expressed in control and hypoxic cells (24hr) infected with AdCrmA but not in cells infected with a control virus. CNTL (cells infected with the empty virus AdCMV); CrmA (cells infected with an AdCrmA); HYPX (hypoxic cells); HYPX+CrmA (cells expressing CrmA and subjected to hypoxia). Panel B) Fluorometric analysis of caspase 8-like activation in cells expressing CrmA. CrmA abrogates hypoxia-mediated caspase 8 like activity. Data are expressed as mean nmol AFC/µg per minute released from the IETD-AFC substrate. Data were obtained from at least n=3 independent myocyte isolations.

1.4 CrmA suppresses hypoxia-mediated apoptosis in neonatal ventricular myocytes

To determine whether caspase 8 activity is required for hypoxia-mediated apoptosis, we overexpressed CrmA to suppress caspase 8 activity and quantified the level of hypoxia-mediated apoptosis. Myocyte nuclei were stained with Hoechst 33258 and visualized by immunofluorescence microscopy. Representative immunofluorescence images are shown in Figure 13A. The percentage of nuclei displaying apoptotic morphology was determined (Figure 13B). Myocytes subjected to hypoxia that expressed the CrmA protein showed only 8% apoptotic nuclei compared to the 48% observed with hypoxic myocytes not expressing CrmA ($p<0.05$). The effect of CrmA on hypoxia-mediated apoptosis was confirmed by a reduction in nucleosomal DNA laddering during hypoxia (Figure 13C). The data establish that AdCrmA is functionally active and confirm the requirement of caspase 8 activation for hypoxia-mediated cell death. Moreover, there was no significant difference in apoptosis between control and CrmA infected cells confirming that neither adenoviral infection nor CrmA was toxic to myocytes.

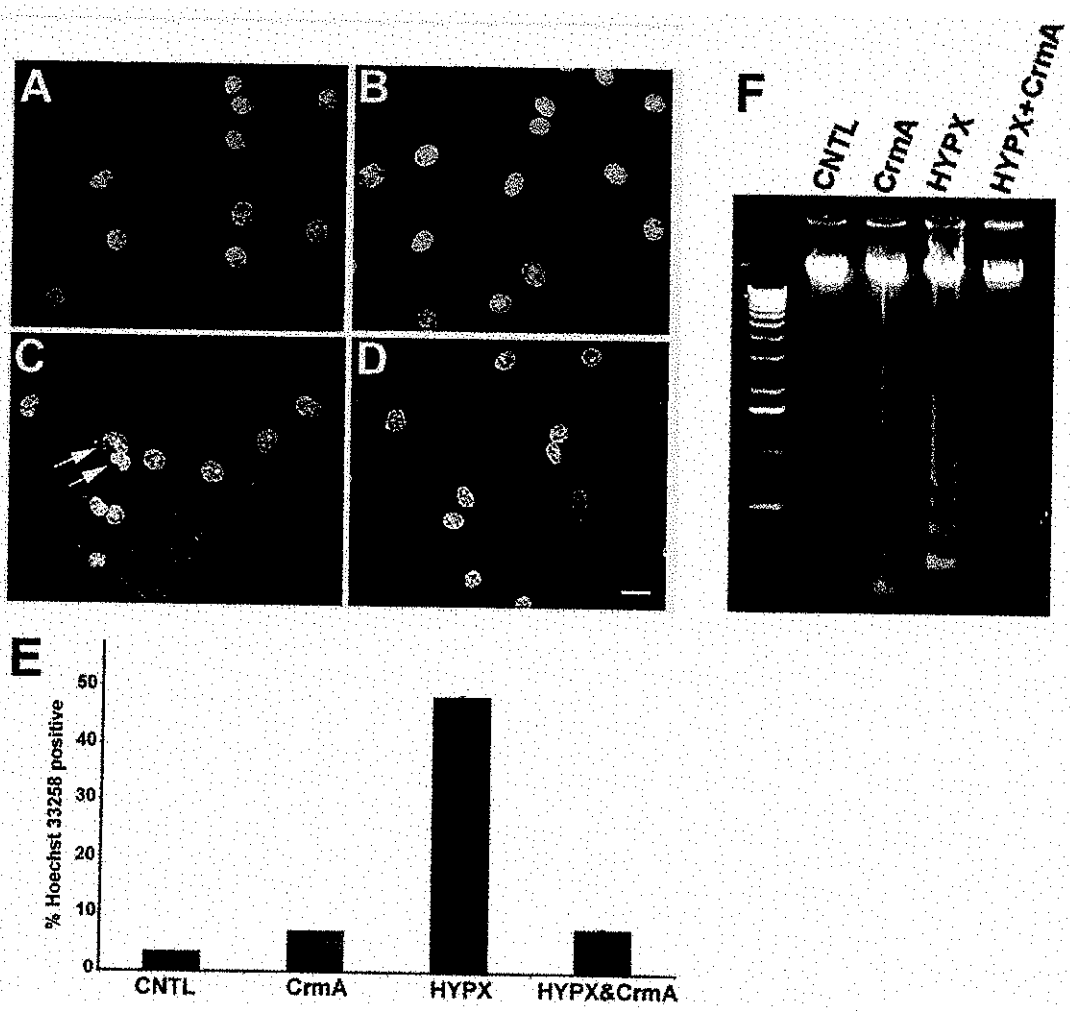

Figure 13: CrmA suppresses hypoxia-mediated DNA fragmentation of neonatal ventricular myocytes

Panels A-D) Fluorescent microscopic images of neonatal ventricular myocytes doubled stained for sarcomeric myosin (red) and nuclear morphology with Hoechst 33258 (blue). Panel A) Normoxic cells. Panel B) Myocytes expressing CrmA. Panel C) Hypoxic cells. Panel D) Myocytes expressing CrmA and subjected to hypoxia. Panel E) Histogram illustrating the percentage of apoptotic cells as determined by Hoechst 33258 nuclear staining. CNTL (normoxic cells); CrmA (Myocytes expressing CrmA); HYPX (hypoxic cells); HYPX + CrmA (Myocytes expressing CrmA and subjected to hypoxia). Data represents mean ±SE ($P<0.001$) obtained from n=3 independent myocyte isolations with 2 replicates for each condition and >200 cells counted per condition. Arrows depict apoptotic nuclei. Bar = 10 µm. Panel F) Gel electrophoresis of nuclear DNA extracted from normoxic and hypoxic neonatal ventricular myocytes in the presence and absence of CrmA.

1.5 Mitochondrial cytochrome c release

The release of cytochrome c from mitochondria is well documented as an important step in the apoptotic signaling pathway ([7;35;124;248]). To assess whether hypoxia provokes cytochrome c release from mitochondria, we monitored the subcellular localization of cytochrome c *in situ* by antibody labeling (green) followed by confocal microscopy. Myocyte mitochondria were selectively stained with Mitotraker red CMXRos (red). As shown in Figure 14A, control cells showed punctuate green staining. By overlaying the red and green signals, cytochrome c appeared to colocalize with mitochondria (Figure 14C). In contrast, myocytes subjected to hypoxia showed diffuse green staining which extended beyond the confines of the mitochondria to align with the sarcomeres. This evidence suggests that hypoxia promotes cytochrome c leakage from the mitochondria (Figure 14D&F). Surprisingly, CrmA did not prevent mitochondrial cytochrome c release due to hypoxia (Figure 14G&I). Western blot analysis of the S-100 cytoplasmic myocyte fraction confirmed the confocal observations (Figure 14J). Cytochrome c was absent in S100 fractions of normoxic and CrmA expressing myocytes but readily detectable in those cells subjected to hypoxia regardless of CrmA expression.

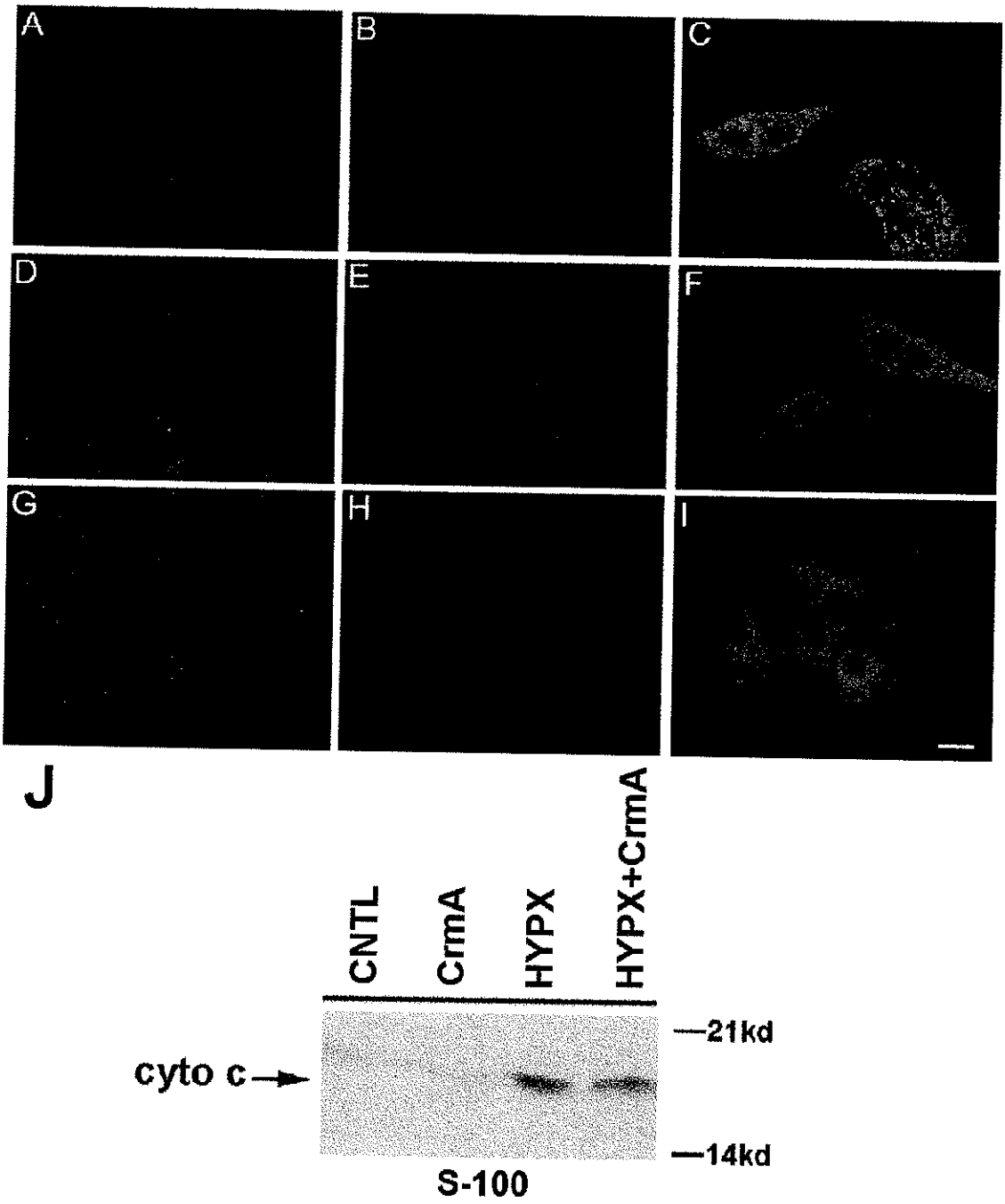

Figure 14: Cytochrome c release during hypoxia is independent of CrmA-inhibitable caspases.
Panels A-I) Confocal images of ventricular myocytes double stained for cytochrome c (green) and Mitotracker (red) for respiring mitochondria. Panels A-C) Normoxic cells; Panels D-F) Hypoxic cells; Panels G-I) cells infected with AdCrmA and subjected to hypoxia; Panel J) Western blot analysis for cytochrome c of normoxic and hypoxic myocyte S100 fractions in the presence and absence of CrmA. CNTL (normoxic cells); CrmA (Myocytes expressing CrmA); HYPX (hypoxic cells); HYPX + CrmA (Myocytes expressing CrmA and subjected to hypoxia).

1.6 Mitochondrial transmembrane potential

In recent years, a link between mitochondrial function and apoptosis has been established. For example, it has been suggested that loss of inner mitochondrial membrane potential ($\Delta\Psi_m$) is associated with transduction of apoptotic signals. In order to determine whether hypoxia reduces $\Delta\Psi_m$, we employed the dual emission potential sensitive probe 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolyl-carbocyanine iodide (JC-1; Molecular Probes, Eugene, Oregon, USA) to follow $\Delta\Psi_m$ *in situ*. At low mitochondrial membrane potential, JC-1 exists as a monomer and fluoresces green. However, at high membrane potentials, JC-1 forms aggregates which fluoresce red. As shown in Figure 15A, mitochondria of normoxic cells showed marked red fluorescence indicating a high membrane potential and a polarized state. To confirm that red fluorescence accurately reflects polarized mitochondrial membranes, we treated cells with the protonophore carbonyl cyanide 3-chlorophenylhydrazone (CCCP) to dissipate the H^+ gradient generated by the inner mitochondrial membrane electron transport chain and monitored the reduction in red fluorescence. In contrast to control cells, cell treated with CCCP displayed a discernable reduction in red fluorescence confirming the utility of JC-1 for monitoring membrane potential (Figure 15B). Further, we evaluated the effect of hypoxia on mitochondrial membrane potential. We found that in contrast to control cells, myocytes subjected to hypoxia displayed a reduction in red fluorescence suggestive of a reduction in $\Delta\Psi_m$ (Figure 15D) . Importantly, hypoxia-induced loss of red fluorescence by JC-1 was abrogated by overexpression of CrmA suggesting a role for CrmA in maintaining mitochondrial function in response to hypoxia (Figure 15D). The intensity of JC-1 red fluorescence relative to control cells was measured using ImagePro

Plus Software and plotted graphically (Figure 15E). The observed changes in $\Delta\Psi_m$ as measured by JC-1 were independently confirmed using the cationic red fluorescing dye tetramethyl rodamine methyl ester (TMRM). The accumulation of TMRM in mitochondria has been shown to be driven by the inner mitochondrial membrane potential; therefore more intense TMRM red fluorescence is suggestive of a stronger mitochondrial membrane potential. As with JC-1, hypoxia induced a loss of $\Delta\Psi_m$ as evidenced by diminished TMRM red fluorescence relative to normoxic cells (Figure 16 A&B). As expected, expression of CrmA protected against hypoxia-induced loss of $\Delta\Psi_m$ (Figure 16 C). Fluorescent intensities are summarized in Figure 16 E. The above data support the concept that hypoxia induces a reduction in $\Delta\Psi_m$ that is blocked by CrmA.

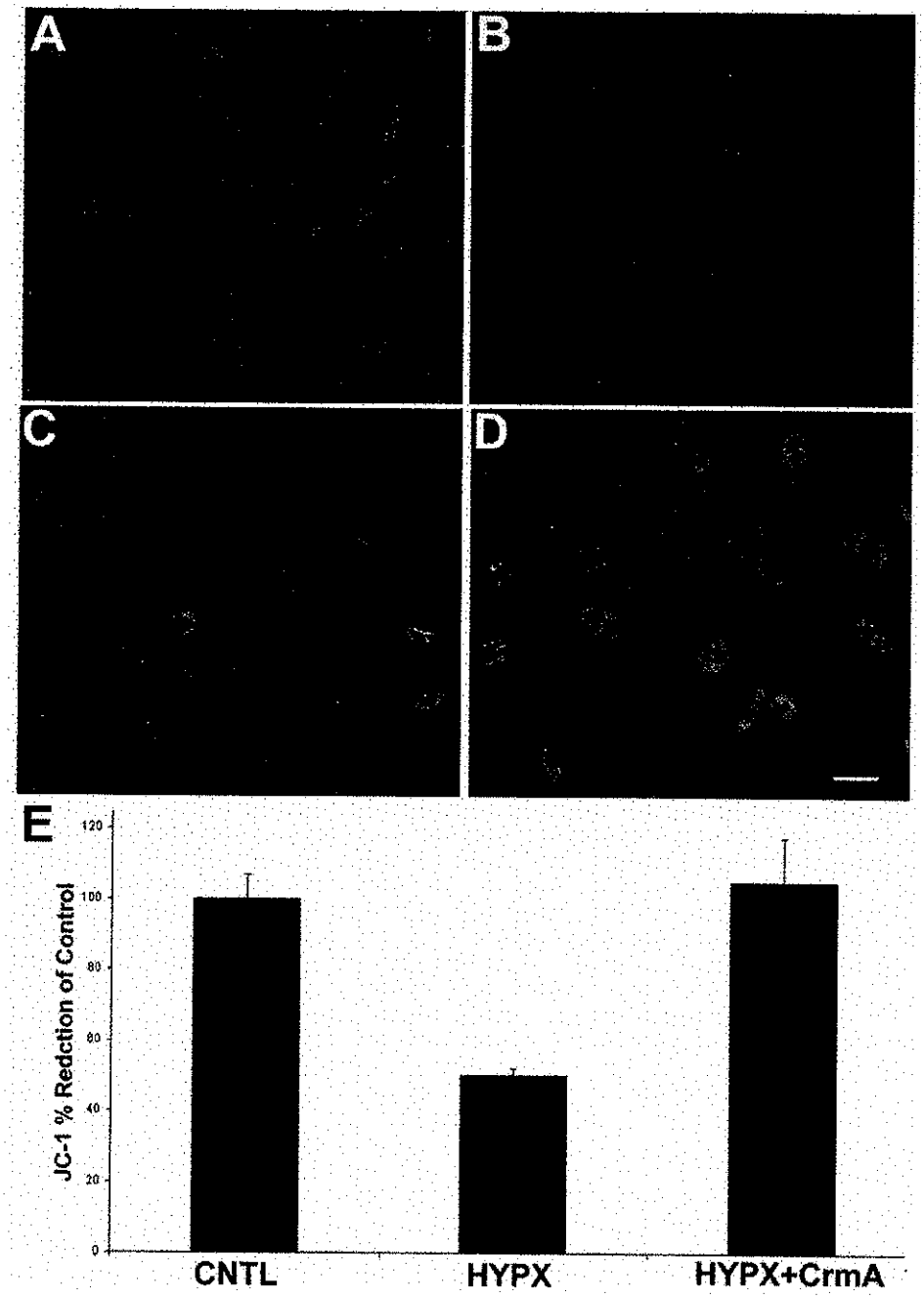

Figure 15: Hypoxia provokes a reduction in $\Delta\Psi m$ as determined by JC-1 staining. Panels A-D) Fluorescent microscopic images of myocyte mitochondria stained with JC-1. JC-1 forms red fluorescing aggregates in polarized mitochondria. A reduction of red fluorescence is indicates a loss of $\Delta\Psi m$. Panel A) Normoxic cells. Panel B) the mitochondrial uncoupler, CCCP; Panel C) Hypoxic cells. Panel D) Myocytes expressing CrmA and subjected to hypoxia. Bar = 15µm. Panel E) Histogram illustrating the percent reduction in $\Delta\Psi m$ compared to normoxic cells. CNTL (normoxic cells); HYPX (hypoxic cells); HYPX + CrmA (Myocytes expressing CrmA and subjected to hypoxia). Data are expressed as mean ±S.E. with replicates of 2 from at least n=32 independent myocyte isolations.

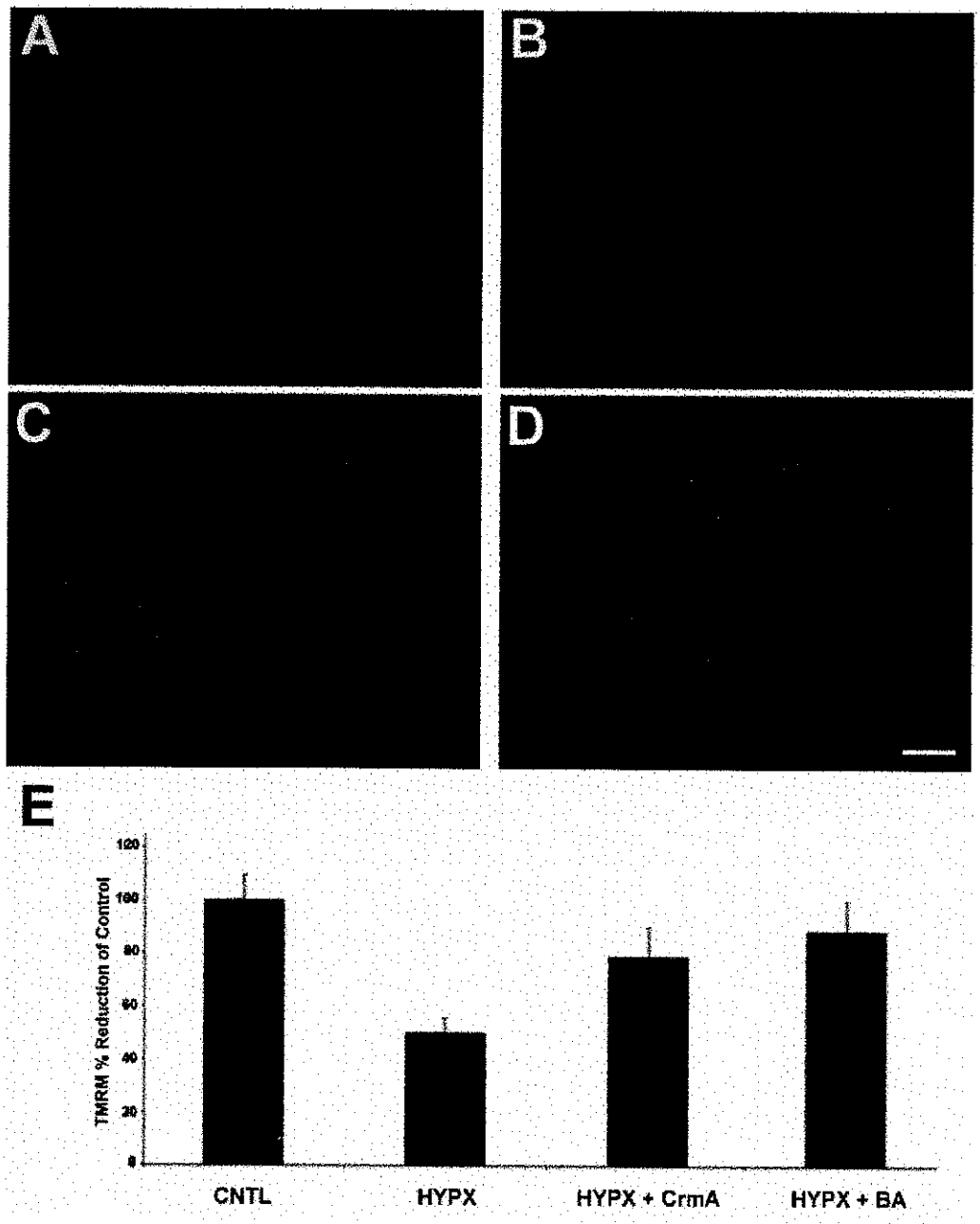

Figure 16: Hypoxia provokes a reduction in ΔΨm as determined by TMRM. Panels A-D) Fluorescent microscopic images of myocyte mitochondria stained with TMRM. TMRM exhibits a red shift when mitochondrial are polarized. Diminished fluorescence indicates loss of ΔΨm. Panel A) Normoxic cells. Panel B) Hypoxic cells. Panel C) Myocytes expressing CrmA and subjected to hypoxia. Panel D) Hypoxic cells pretreated with Bonkrekic Acid (BA). Bar = 15μm. Panel E) Histogram illustrating the percent reduction in ΔΨm compared to normoxic cells. CNTL (normoxic cells); HYPX (hypoxic cells); HYPX + CrmA (Myocytes expressing CrmA and subjected to hypoxia). HYPX + BA (Myocytes pretreated with BA and subjected to hypoxia). Data are expressed as mean ±S.E. with replicates of 2 from at least n=3 independent myocyte isolations.

1.7 Mitochondrial permeability transition during hypoxia

Changes in $\Delta\Psi_m$ are often associated with mitochondrial permeability transition. Therefore, we next determined whether the mitochondrial permeability transition pore opens during hypoxia. To do so, myocytes were loaded with the membrane permeant dye, calcein acetoxymethyl ester (calcein AM) in the presence of cobalt chloride ([184;354]). Calcein AM diffuses throughout cell including the mitochondria. Upon entry into a living cell, calcein AM becomes deesterified and fluorescent. Its localization within the cell can then be monitored by immunofluorescence microscopy of the green signal. Cobalt chloride is confined to the cytoplasm of the cell where it quenches the cytoplasmic calcein AM signal. The resultant image of a control cell is that of green fluorescing mitochondria devoid of a cytoplasmic signal. However, opening of the PTP allows the calcein AM to leach from the mitochondria and become quenched within the cytosol leaving ghost-like cell images. As shown in Figure 17A, normoxic control cells displayed punctate green staining mitochondria, which is typical of intact mitochondrial membranes. However, hypoxic myocytes displayed an obvious reduction in green fluorescing mitochondria consistent with opening of the PTP (Figure 17B). Importantly, myocytes expressing CrmA were protected from hypoxia-mediated PTP opening suggestive of a role for CrmA inhibitable caspases in modulation of the PTP in response to hypoxia (Figure 17C). In order to confirm that loss of green fluorescence was a measure of PTP opening, we treated cells with Bongkrekic acid (BA; 50 μM) which is known to inhibit opening of the pore and followed this by hypoxia ([184;356;357]) (Figure 17D). In contrast to cells subjected to hypoxia, those made hypoxic in the presence of BA were indistinguishable by fluorescence from normoxic control cells confirming the

validity of this methodology for measuring PTP opening. Interestingly, BA pretreatment of myocytes prior to hypoxia was sufficient to maintain mitochondrial membrane potential as measured by TMRM fluorescence to a level that was comparable to normoxic control cells (Figure 17D). From this observation it appears that PT and $\Delta\Psi_m$ are intimately linked. Thus the next logical step was to determine whether BA could rescue hypoxia-mediated apoptosis of ventricular myocytes. As illustrated by Hoechst 33258 nuclear staining in Figure 18, BA was sufficient to suppress hypoxia-mediated apoptosis of ventricular myocytes to control levels. This important observation suggests that hypoxia causes myocyte apoptosis through a mechanism that opens the permeability transition pore.

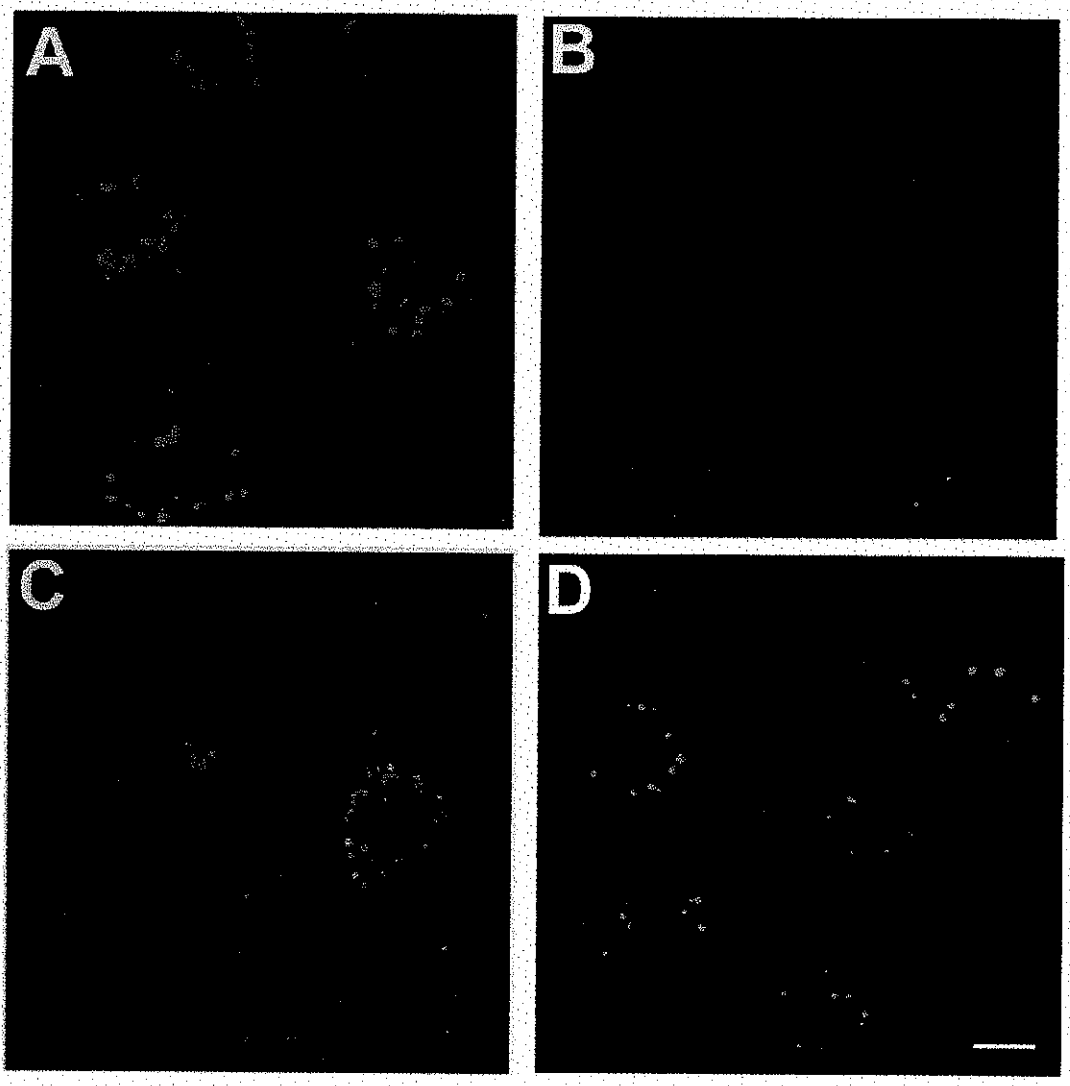

Figure 17: Hypoxia provokes permeability transition pore opening.
The state of the mitochondrial permeability transition pore was monitored in ventricular myocytes using the dye calcein-AM in the presence of cobalt chloride. Opening of the pore is marked by a loss of green fluorescence from mitochondria ([354]). Panel A) normoxic cells; Panel B) hypoxic cells; Panel C) Myocytes expressing CrmA and subjected to hypoxia; Panel D) Myocytes pretreated with BA and subjected to hypoxia. Bar = 15μm.

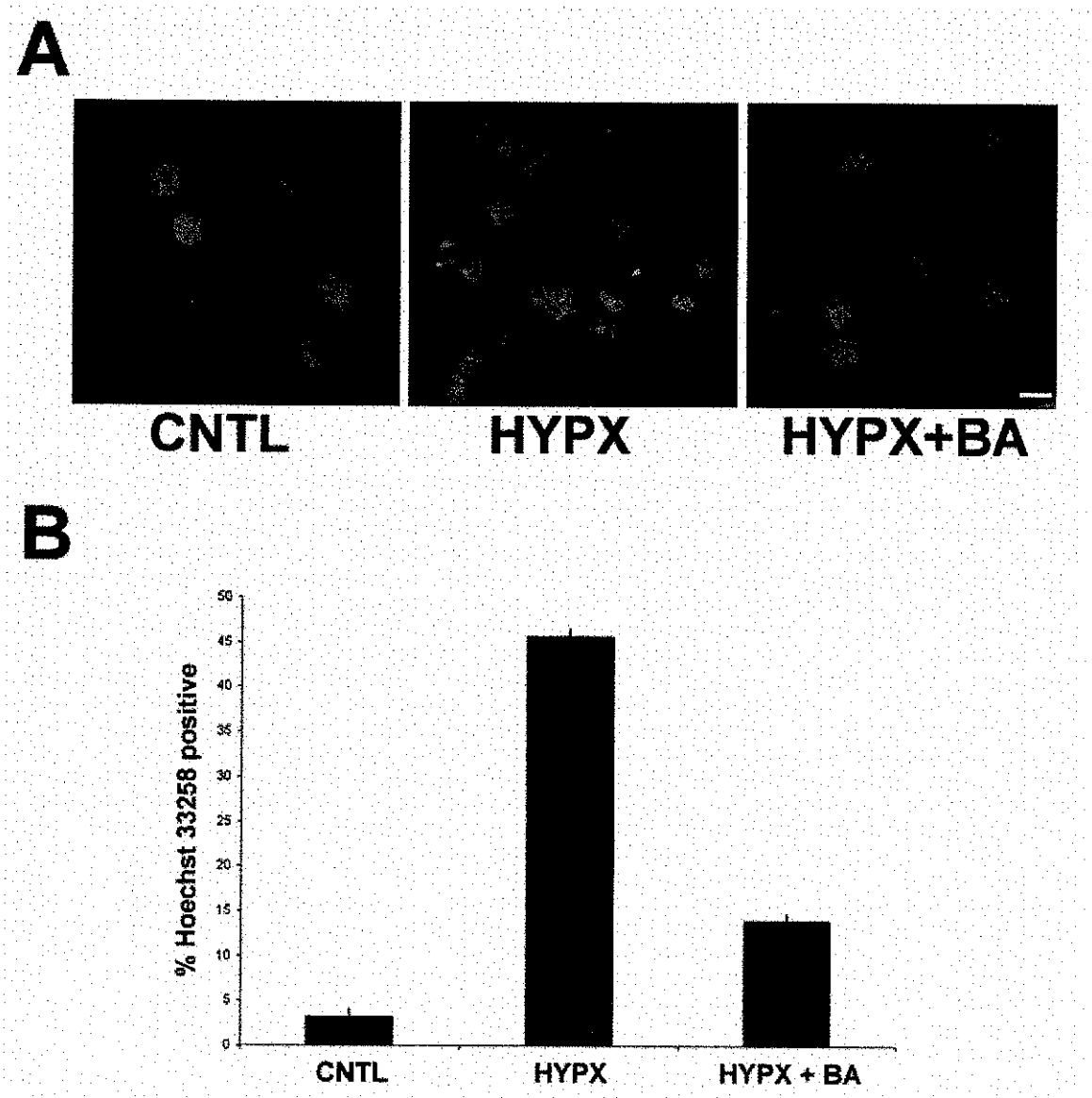

Figure 18: Hypoxia-induced apoptosis is suppressed by the permeability transition pore inhibitor Bongkrekic Acid.
Panel A) Fluorescent microscopic images of neonatal ventricular myocytes stained for nuclear morphology with Hoechst 33258 (blue). Normoxic cells (CNTL); Hypoxic cells (HYPX). Panel C) Histogram illustrating the percentage of apoptotic cells as determined by Hoechst 33258 nuclear staining. CNTL (normoxic cells); HYPX (hypoxic cells); Myocytes pretreated with BA (50μM) and subjected to hypoxia. (HYPX+BA). Panel B) Histogram illustrating the percentage of apoptotic cells as determined by Hoechst 33258 nuclear staining. Data are expressed as mean from n=3 independent myocyte isolations with 2 replicates for each condition and >200 cells counted per condition. Arrows depict apoptotic nuclei. Bar = 10 μm.

2. Mechanisms of p53-mediated cell death of ventricular myocytes

2.1 Expression of p53 proteins in ventricular myocytes

The tumor suppressor protein p53 is induced in a variety of tissues following pathological stress ([5;312;358;359]). In the heart, elevated levels of p53 have been observed in response to ischemia- reperfusion ([359;360]), hyperglycemia ([336]), hypoxia ([5]); mechanical stretch ([361;362]); ventricular pacing ([337]) Importantly, previous work from our laboratory has shown that forced expression of p53 triggers widespread apoptosis of ventricular myocytes ([42]), however, the molecular mechanisms of p53-mediated apoptosis in the heart are poorly understood. To gain a better understanding of its mode of action, we probed the importance of p53 DNA binding and gene transactivation to cardiac cell apoptosis. First we undertook a series of experiments to establish our experimental model. We generated a replication defective adenovirus encoding a mutant p53 (p53MT) that harbors a single amino acid substitution of an alanine to a valine at position 135. The functional consequence of this mutation is that it renders p53 defective for DNA binding and gene transactivation. As shown in figure 19, adenoviral-mediated gene transfer of wild-type (p53WT) or p53MT to ventricular myocytes resulted in equivalent p53 protein expression. Importantly, this western blot data confirms that both proteins were being delivered to myocytes uniformly and efficiently.

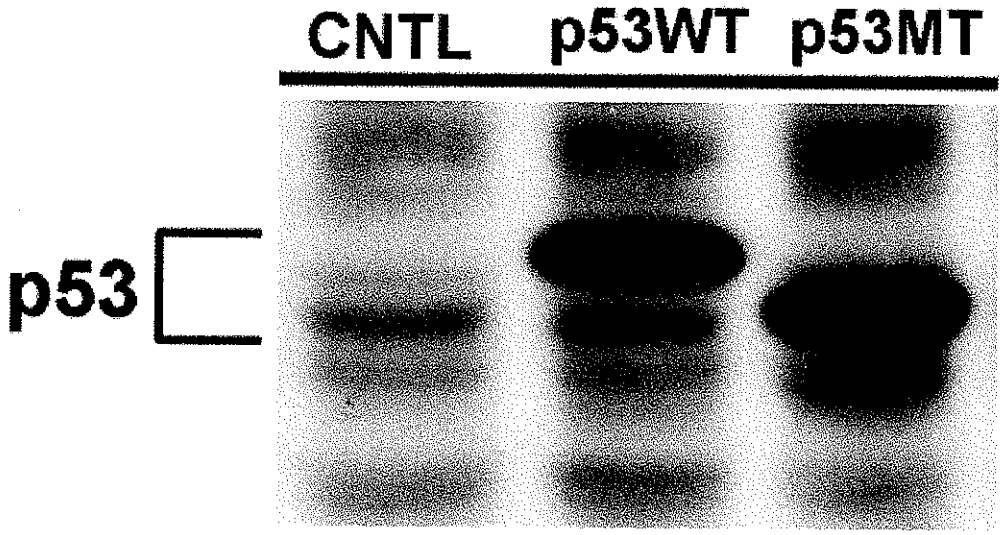

Figure 19: Expression of p53 protein in neonatal ventricular myocytes.
Western blot analysis performed on of adenovirus infected neonatal myocyte cell lysates. Wildtype (p53WT) and mutant p53 (p53MT) proteins are expressed to comparable levels. CNTL (cells infected with the empty virus AdCMV).

2.2 *Mutant p53 is defective for DNA binding*

To verify the phenotype of the mutant protein, we isolated nuclear extract from ventricular myocytes expressing p53WT or p53MT and analyzed it for p53 DNA binding activity by electromobility shift analysis (EMSA). As shown in figure 20, a p53 protein-DNA shift was observed in cells expressing p53WT but not in cells expressing p53MT, confirming that p53MT was defective for DNA binding. Cells infected with control virus lacking the cDNA insert (Adcmv) were similar to uninfected control cells, verifying that viral infection alone had no effect on endogenous p53 DNA binding. Additionally, the identity of migrating protein/DNA complex was confirmed by supershift analysis with a p53 specific antibody as well as competition binding assay with 1000x cold probe.

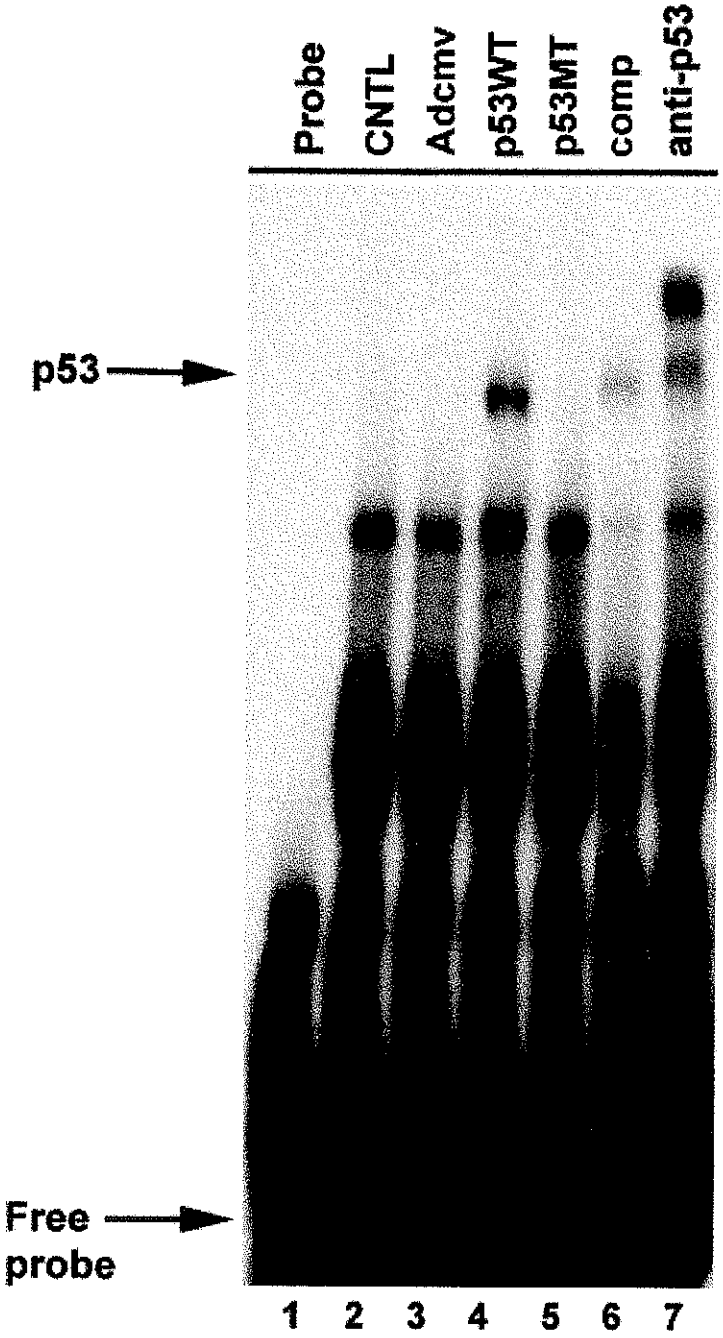

Figure 20: Electromobility shift analysis of ventricular myocytes.
Equivalent quantities of myocyte nuclear extract were prepared and analyzed for p53 DNA binding activity. p53WT, but not p53MT, is competent for DNA binding. Lane 1 (probe) probe in the absence of nuclear extract; lane 2 (CNTL) control cells; lane 3 (Adcmv) cells infected with empty virus; lanes 4&5 (p53WT and p53MT) analysis of DNA/protein complexes for wildtype p53 (p53WT) and mutant p53(p53MT); lane 6 (comp) competitive binding analysis of p53WT protein/DNA complexes with 1000 fold excess cold oligonucleotide probe; lane 7 (anti-p53) supershift analysis with a murine antibody directed toward p53. Arrow indicates band corresponding to p53.

2.3 Mutant p53 is defective for gene transcription

Although we verified p53MT to be defective for DNA binding, it was equally important to confirm that it was unable to transcribe genes. To do so, we expressed p53WT and p53MT in ventricular myocytes followed this by transfection with the human Bax promoter linked to a luciferase reporter gene; 48 hours later, cells expressing p53WT showed a 3 fold induction ($p<0.01$) in Bax gene transcription compared to control cells (figure 21A). Cells infected with the control virus Adcmv did not display Bax promoter activation confirming that viral infection alone was innocuous. Importantly, cells expressing p53MT were indistinguishable from control cells with respect to Bax gene transcription.

To further confirm the defect in p53MT gene activation we performed western blot analysis of endogenous Bax and MDM2 protein (figure 21B). In contrast to control cells, levels of endogenous Bax protein were substantially increased in myocytes expressing p53WT but not in cells expressing p53MT. A similar pattern of expression was evident for the known p53 response gene MDM2 suggesting that lack gene activation by p53MT was universal and not restricted to the Bax gene or Bax promoter (figure 21C).

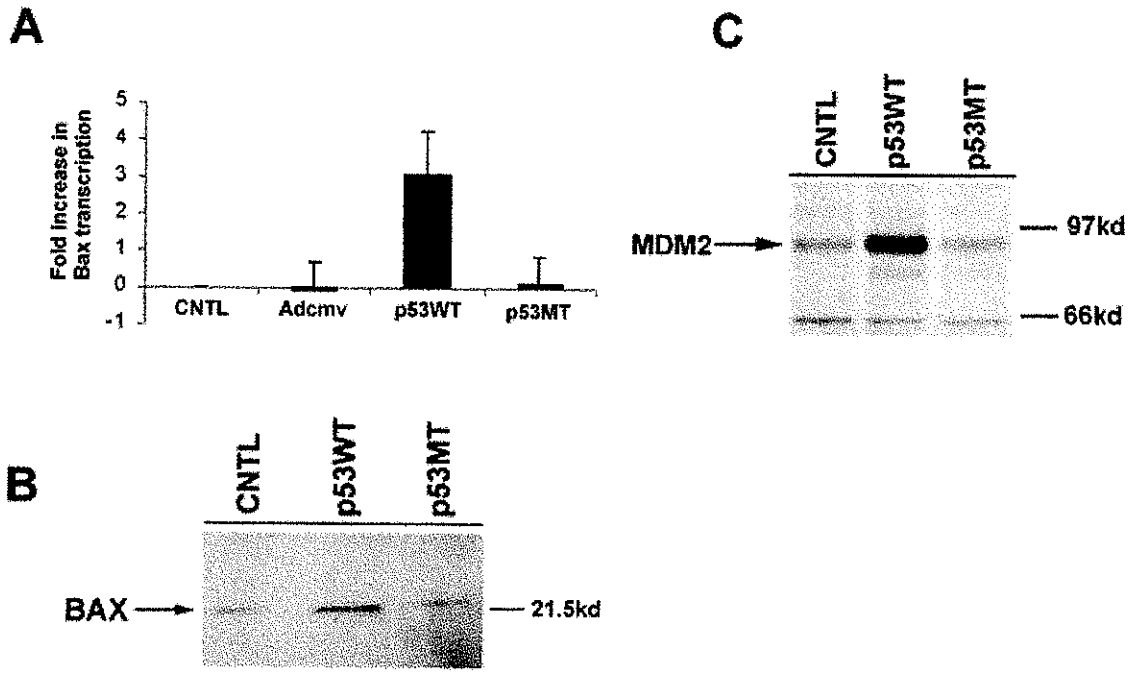

Figure 21: p53 dependent transcription of Bax and MDM2 in ventricular myocytes. Panel A) Transcription of the human Bax gene promoter is activated by p53WT but not by p53MT. CNTL (control cells); (Adcmv) cells infected with empty virus; p53WT (cells expressing wildtype p53); p53MT (cells expressing mutant p53). Data are expressed as mean ±S.E. with replicates of 3 from at least n=3 independent myocyte isolations. Panels B & C) Western blot analysis for the detection of endogenous Bax and MDM2 proteins in cardiac cell lysate expressing p53WT and p53MT.

2.4 p53 provokes apoptosis of ventricular myocytes independent of gene transcription

p53 is a potent inducer of apoptosis in ventricular myocytes; however, the requirement of *de novo* gene activation for p53-mediated apoptosis is unknown in this cellular context. Therefore, we expressed p53WT and p53MT in ventricular myocytes and analyzed the genomic DNA for evidence of fragmentation by DNA gel electrophoresis (figure 22). Cells expressing p53WT displayed a prominent DNA laddering pattern consistent with apoptotic cell death, which was absent in control cells. Interestingly, DNA laddering was apparent in cells expressing p53MT. To corroborate the DNA gel electrophoresis data, we performed immunofluorescence microscopy to investigate nuclear DNA fragmentation *in vivo* ($p<0.001$). After 24 hour, cells expressing p53WT displayed a 46% increase in apoptosis compared to uninfected control cells or those infected with a control virus ($p<0.001$). Furthermore, a 22% increase in apoptosis was observed in cells expressing p53MT ($p<0.001$). Collectively, the data establish that p53 can provoke apoptosis of ventricular myocytes independent of DNA binding and gene transcription.

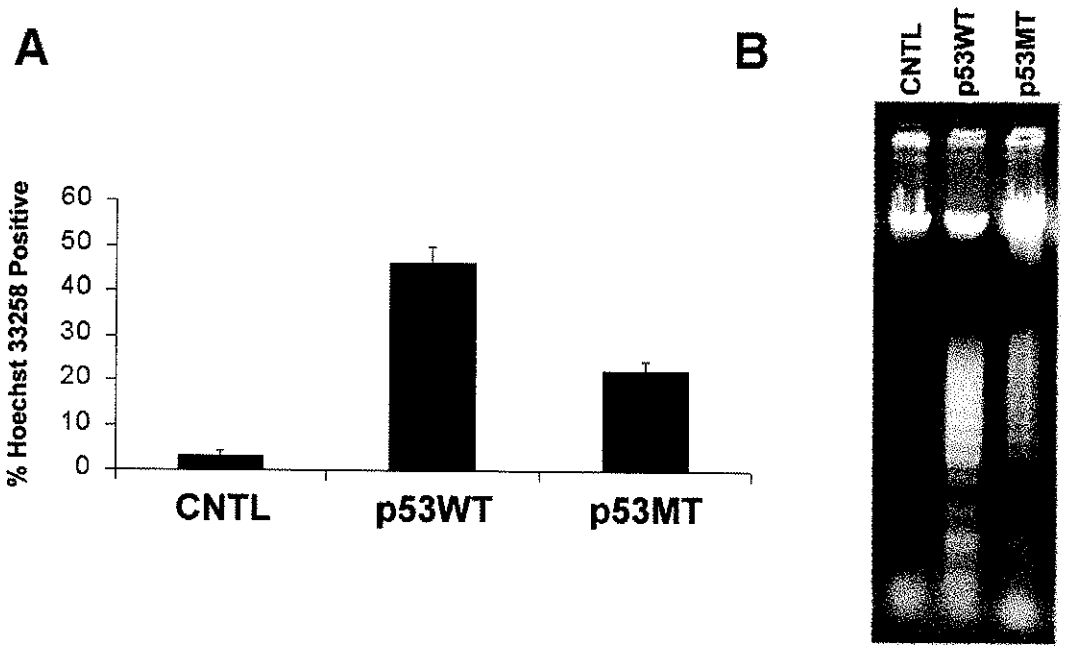

Figure 22: p53 provokes apoptosis of ventricular myocytes independent of DNA binding and gene activation.
Panel A) Histogram illustrating the percentage of apoptotic myocytes as determined by Hoechst 33258 nuclear staining and counterstaining for sarcomeric myosin to distinguish myocytes. Data were obtained from n=3 independent experiments counting >200 cells per condition and expressed as ± S.E. Panel B) Nucleosomal DNA fragmentation of ventricular myocytes is observed in ventricular myocytes expressing p53WT and p53MT proteins.

2.5 p53 proteins provoke perturbations to mitochondria including permeability transition pore opening and changes in mitochondrial membrane potential

As perturbations to mitochondria involving a reduction in membrane potential appear to provoke apoptosis, we ascertained whether p53 would influence $\Delta\Psi_m$ in ventricular myocytes (Figure 23). In the absence of p53 proteins, myocyte mitochondria treated with TMRM maintained bright red fluorescence, an indication of polarized membranes. In contrast, a 49% and 24% reduction in $\Delta\Psi_m$ from control was observed in cells expressing p53WT and p53MT, respectively. Since empty virus treated cells were indistinguishable from control cells, we could rule out the possibility that TMRM staining was affected by viral infection.

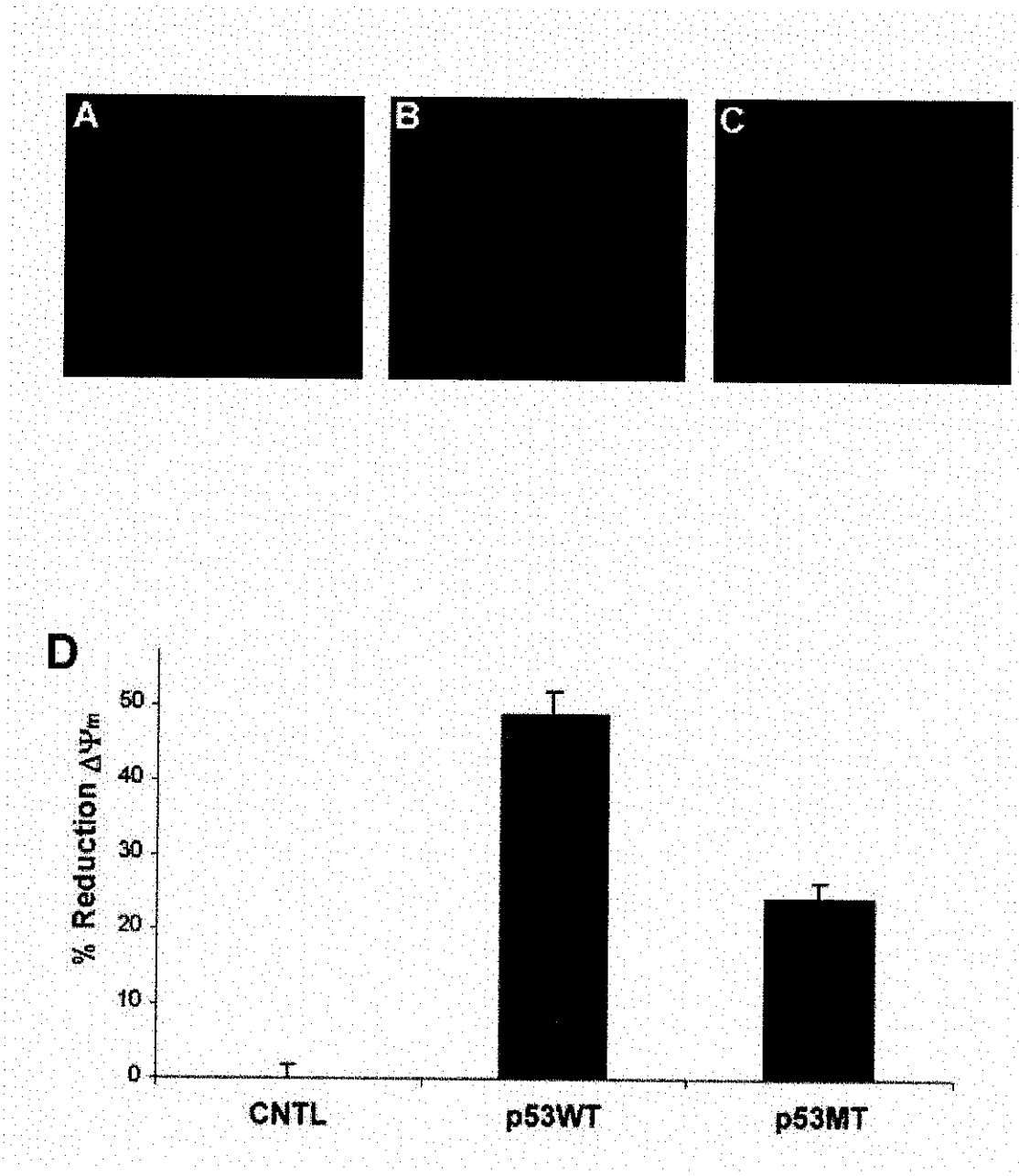

Figure 23: p53 causes a reduction in mitochondrial membrane potential ΔΨm. Panels A-C) TMRM staining mitochondria in the presence and absence of p53 proteins. Loss of red fluorescence indicates a reduction in ΔΨm. Panel A) control cells; Panel B) p53WT; Panel C) p53MT. Panel D) Histogram illustrating the percent reduction in ΔΨm of cells expressing p53 proteins compared to control cells. CNTL (control treated cells); p53WT (Myocytes expressing wildtype p53); p53MT (Myocytes expressing mutant p53). Data are expressed as mean ±S.E. with replicates of 2 from at least n=3 independent myocyte isolations.

2.6 p53 proteins provoke mitochondrial cytochrome c release

Mitochondrial cytochrome c release appears to be intimately linked with the apoptotic signaling cascade; to determine whether mitochondrial perturbations associated with p53 lead to cytochrome c release in ventricular myocytes, we evaluated the subcellular distribution of cytochrome c *in situ* by confocal microscopy and western blot analysis. As shown in figure 24A, control treated cells showed punctate cytochrome c staining (green) which was exclusively localized to mitochondria. Cells expressing p53WT or p53MT, in contrast, showed reduced mitochondrial cytochrome c staining with an apparent redistribution of the protein to the cytoplasm. Similar evidence of mitochondrial cytochrome c release was obtained through western blot analysis of the S-100 cytoplasmic fraction of p53WT and p53MT expressing cells (Figure 24B). Here, a marked increase in cytochrome c was detected in the cytoplasmic fraction of cells expressing p53WT or p53MT proteins compared to control cells. The Ponceau-S staining of the western blot demonstrated equivalent loading of proteins, adding credibility to the observed differences in cytochrome c immunostaining. Thus, as substantiated by both confocal and western blot data, myocytes expressing wild-type or mutant p53 proteins trigger mitochondrial cytochrome c release.

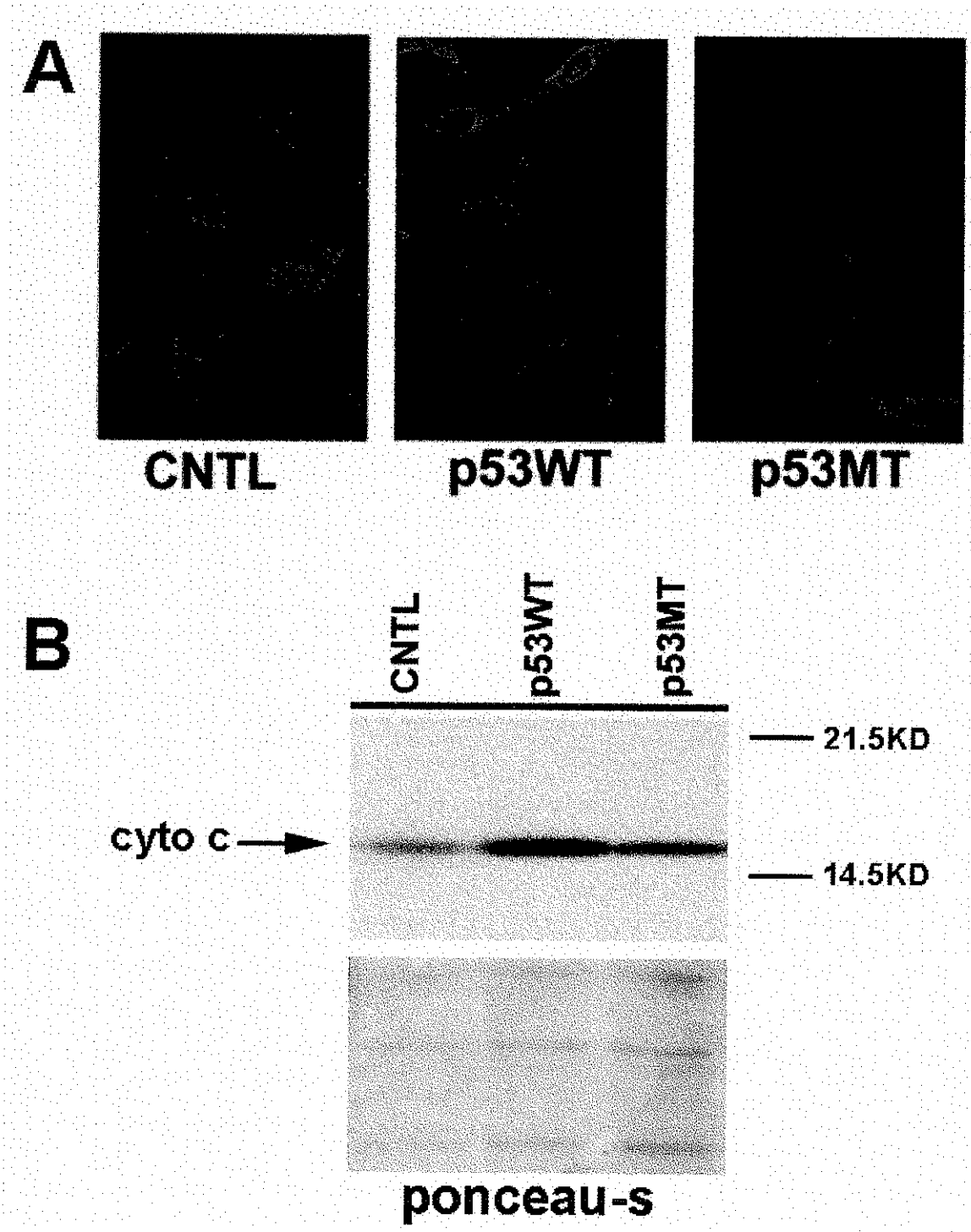

Figure 24: p53 provokes cytochrome c release from mitochondria.
Panel A) Confocal microscopy of neonatal ventricular myocytes stained for cytochrome c (green fluorescence). CNTL (control cells); p53WT (Myocytes expressing wildtype p53); p53MT (myocytes expressing mutant p53). Panel B) Western blot analysis for cytochrome c of myocyte S100 fractions in the presence and absence of p53 proteins. Ponceau-s staining of Western blot filter to verify equivalent protein loading.

2.7 p53 proteins provoke caspase activation

Given that mitochondrial defects and apoptosis triggered by p53 were not contingent upon its gene activating properties, we explored whether caspase 3 was activated in response to p53. Cell lysate was prepared from myocytes expressing p53WT, p53MT or control virus and analyzed for caspase 3-like activity (Figure 25A). p53WT resulted in a 4-fold increase in the proteolytic activation of caspase 3-like activity compared to control treated cells ($p<0.05$). Cells expressing p53MT showed a 1.4-fold induction in caspase 3-like activity ($p<0.05$). Notably, the observed increase in caspase 3-like activity and apoptosis in the presence of either p53 protein was abrogated by the peptide caspase 3 inhibitor, Ac-DVED-CHO, confirming the activation of caspase 3-like caspases and their contribution to the apoptotic signaling pathway (Figure 25B).

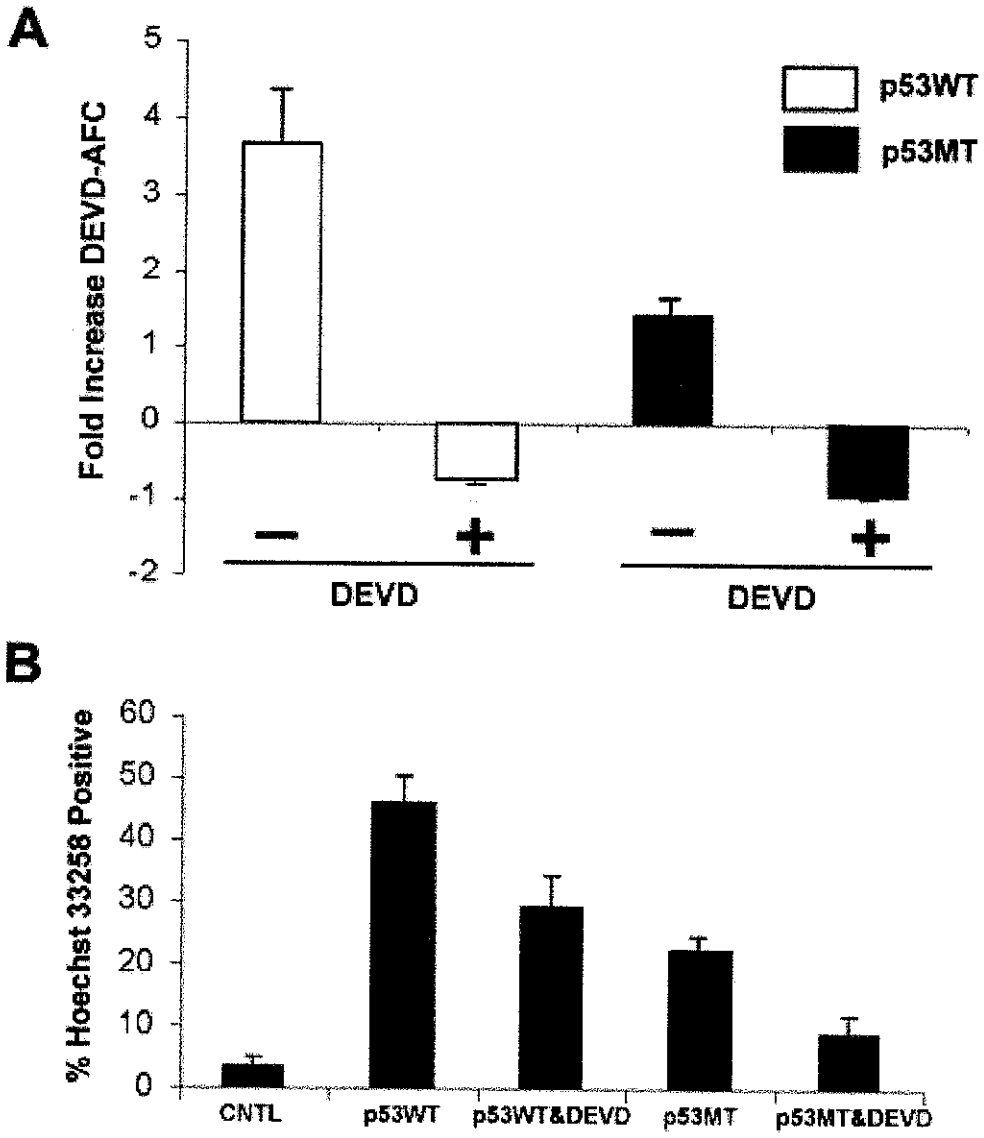

Figure 25: p53 triggers caspase 3-like activity in ventricular myocytes.
Panel A) Caspase 3-like activity is increased in ventricular myocytes expressing p53 proteins. Treatment of myocytes with 100μM of the caspase 3 inhibitor Ac-DEVD-CHO (DEVD) suppressed p53-mediated caspase 3 activation. Panel B) Histogram illustrating the percentage of apoptotic myocytes as determined by Hoechst 33258 nuclear staining and counterstaining for sarcomeric myosin to distinguish myocytes. p53-mediated apoptosis is suppressed by the inhibition of caspase 3. Data are expressed as mean from n=3 independent myocyte isolations with 2 replicates for each condition and >200 cells counted per condition.

3. The role of BNIP3 in the heart

3.1 BNIP3 protein levels increase in response to hypoxia in ventricular myocytes

Although the field of cardiac apoptosis is expanding, few regulators have been identified and described in the context of hypoxia-mediated cardiac cell apoptosis. Recently, increased transcript levels of BNIP3 have been observed in immortalized cell lines during hypoxia ([45;291]). Furthermore, a yeast two hybrid screen of a human heart cDNA library yielded BNIP3 protein, suggesting that BNIP3 is expressed in heart tissue (K.M. Regula and L.A. Kirshenbaum, unpublished data). To determine whether BNIP3 protein levels become elevated in response to hypoxia in ventricular myocytes, we performed western blot analysis. As shown in Figure 26, there was a 19.5 fold increase ($p<0.05$) in BNIP3 protein in cells subjected to 24 hours of chronic hypoxia. Interestingly, no apparent change in BNIP3 protein levels was detected during shorter durations of hypoxia. The induction of BNIP3 at 24 hours, therefore, correlates well with the occurrence of hypoxia-mediated mitochondrial defects and apoptosis of neonatal ventricular myocytes described earlier.

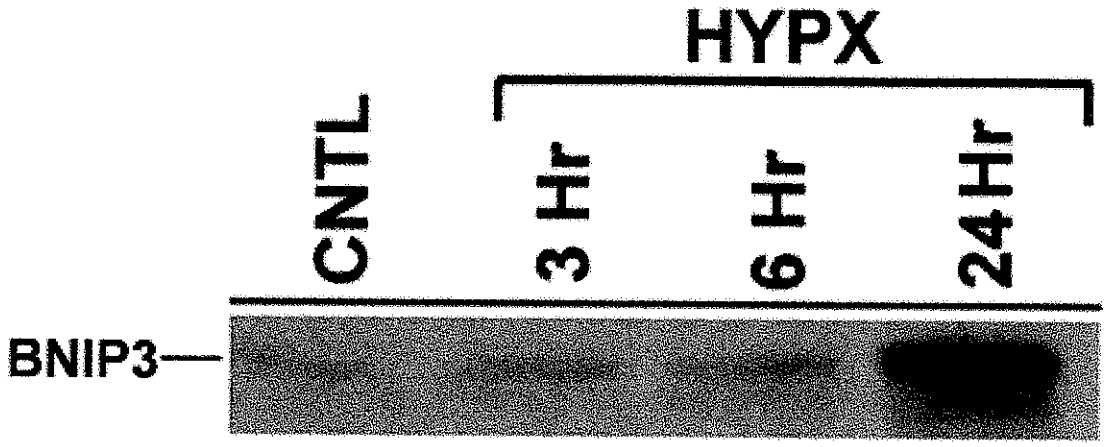

Figure 26: Hypoxia induces BNIP3 protein accumulation in neonatal ventricular myocytes. Western blot analysis of cell lysate derived from normoxic and hypoxic ventricular myocyte cultures at different time points. Densitometric scanning indicated a 19.5 fold increase ($p<0.05$) in BNIP3 protein levels following 24 hours of hypoxic compared to normoxic control cells. Data were obtained from n=3 independent myocyte isolations. CNTL (normoxic cells); HYPX (hypoxic cells).

3.2 Other Bcl2 family member protein levels are unchanged during hypoxia

To determine whether hypoxia-mediated effects on myocyte apoptosis were coincident with modified expression of Bcl-2 family members other than BNIP3, we performed western blot analysis of Bad, Bak, Bax and Bcl-2 (Figure 27). There was no perceptible change in the levels of the pro-apoptotic proteins Bad, Bak or Bax in cardiac cell lysate after 24 hours of hypoxia relative to normoxic cells. Similarly, the level of Bcl-2 protein was consistent between normoxic and hypoxic samples. This western blot data suggests that the Bcl-2 family member, BNIP3, is uniquely stimulated in response to hypoxia in cardiac myocytes.

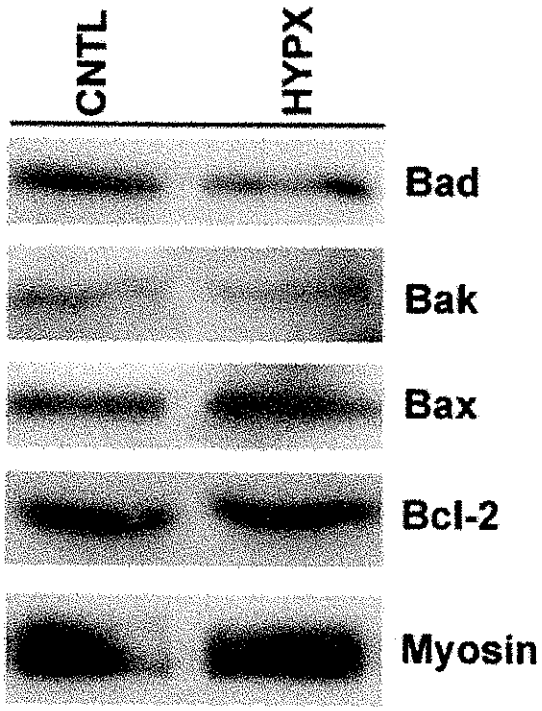

Figure 27: Protein levels of Bcl-2 family members in cells subjected to hypoxia. Western blot analysis of cell lysate derived from normoxic and hypoxic (24hr) ventricular myocyte cultures. The filter was probed with antibodies specific for representative Bcl-2 family members and sarcomeric myosin to confirm equal protein loading. Data were obtained from n=3 independent myocyte isolations. CNTL (normoxic cells); HYPX (hypoxic cells).

3.3 BNIP3 provokes cell death of ventricular myocytes

Given the observed increase in BNIP3 protein in ventricular myocytes at 24 hours of hypoxia and the independent observation of apoptosis at this time point, we sought to determine whether BNIP3 may contribute to hypoxia-mediated cardiac cell death. For these experiments, we generated a replication-defective adenovirus encoding full-length BNIP3 and expressed it in neonatal ventricular myocytes. Myocytes were then stained with Calcein AM to detect living cells (green) and ethidium homodimer-1, to simultaneously identify dead cells (red) ([181]). As illustrated in Figure 28A, myocytes expressing BNIP3 showed an 8.3 fold increase ($p<0.05$) in cell death relative to cells infected with a control virus lacking the BNIP3 cDNA insert. When myocytes were stained for nuclear morphology with Hoechst 33258, the cell death due to BNIP3 overexpression appeared to be apoptotic (Figure 28B). Importantly, the pan caspase inhibitor z-VAD-fmk (25 to 100 μM) prevented BNIP3-induced cell death of ventricular myocytes in a dose dependent manner (Figure 28C). These findings suggest that BNIP3 induces cell death of ventricular myocytes with features of apoptosis in a caspase dependent manner.

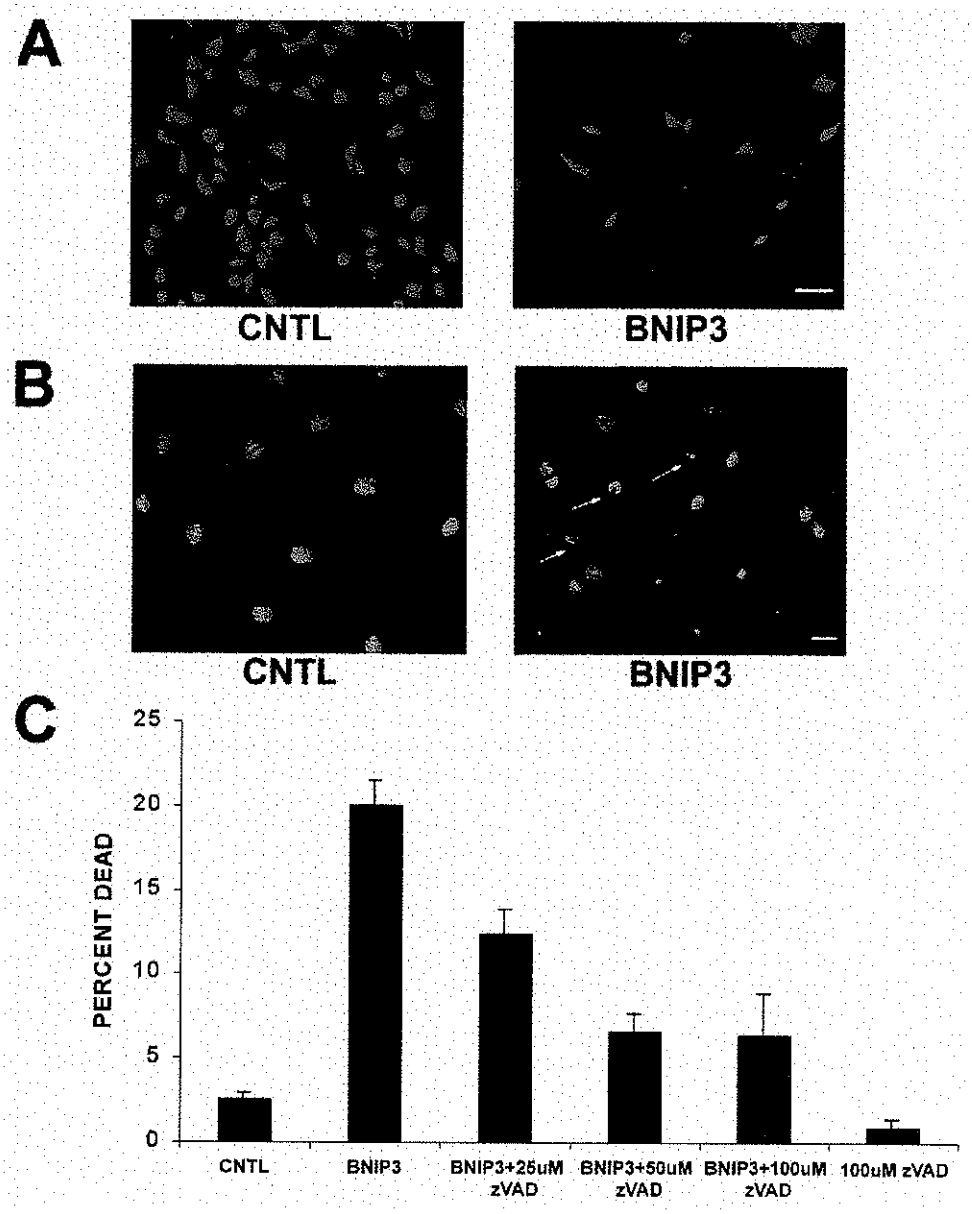

Figure 28: BNIP3 triggers apoptosis of ventricular myocytes.
Panels A&B) Fluorescent microscopic images of neonatal ventricular myocytes in the presence and absence of exogenous BNIP3. Panel A) Myocytes treated with the vital dyes calcein acetoxymethyl ester (green) and ethidium homodimer-1 (red) to identify the number of living and dead cells, respectively. CNTL (control treated myocytes); BNIP3 (myocytes expressing BNIP3). Bar = 100μM. Panel B) Myocyte nuclei stained with Hoechst 33258 (blue) to assess nuclear morphology. Bar = 10μM. Panel C) Histogram illustrating the percentage of dead cells; viability was assessed as described for panel A. BNIP3-induced cell death was suppressed by the pan caspase inhibitor z-Vad-fmk in a dose dependent manner (25μM - 100μM). Data represents mean ±SE ($P<0.001$) obtained from n=3 independent myocyte isolations with 2 replicates for each condition and >200 cells counted per condition.

3.4 BNIP3 does not modulate the expression of Bcl2 family members

To determine whether BNIP3 overexpression impacted upon the expression of other Bcl-2 family members, we performed western blot analysis of Bad, Bax and Bcl-2 in control and BNIP3 expressing cardiac cell lysate (Figure 29). As with hypoxia, there was no apparent change in the levels of Bad or Bax in cardiac cell lysate after BNIP3 overexpression relative to control treated cells. Similarly, the level of Bcl-2 protein was consistent between control and BNIP3 samples. The above data suggest that apoptosis due to BNIP3 overexpression is a primary consequence of BNIP3 and should not be attributed to BNIP3-mediated changes in the expression levels of other Bcl2 family members.

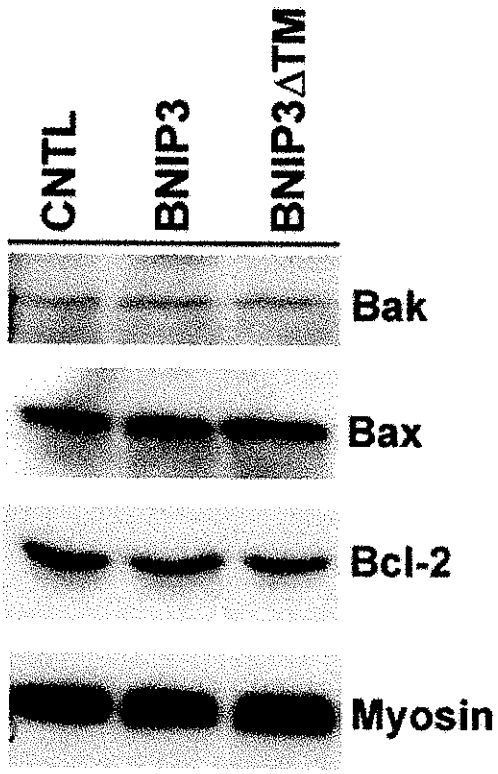

Figure 29: Protein levels of Bcl-2 family members in cells expressing BNIP3 proteins. Western blot analysis of cardiac cell lysate derived from ventricular myocytes in the presence and absence of BNIP3 and BNIP3ΔTM proteins. No apparent change in the expression levels of Bak, Bax, or Bcl-2 was detected in the presence or absence of BNIP3 or BNIP3ΔTM. Data were obtained from n=3 independent myocyte isolations. CNTL (normoxic cells); BNIP3 (myocytes expressing wildtype BNIP3 protein); BNIP3ΔTM (myocytes expressing BNIP3 which lacks the transmembrane domain of the protein).

3.5 Hypoxia provokes the integration of BNIP3 into mitochondrial membranes

In 1998 Shore and colleagues proposed a novel mechanism by which death-inducing proteins of the Bcl-2 family could impinge upon the mitochondria to transduce apoptotic signals ([257]). In their model endogenous Bax was found loosely associated with mitochondria but in response to a pro-death stimulus underwent integral mitochondrial membrane insertion. We observed BNIP3 in the mitochondrial fraction of neonatal ventricular myocytes (Figure 30). However, to determine the precise level of BNIP3 association with myocyte mitochondria in response to a hypoxic stimulus, we isolated mitochondria from normoxic and hypoxic cardiomyocytes and subjected them to alkali extraction. The purpose of alkali extraction is to dissociate and solubilize unintegrated mitochondrial proteins thus identifying proteins that are integrally associated with the organelle ([257]). Following alkaline extraction of isolated mitochondria, BNIP3 was undetectable in normoxic cell mitochondria indicating that it was alkaline soluble and thus only peripherally associated with mitochondria (Figure 31). However, in response to hypoxia, mitochondrial BNIP3 was no longer soluble at alkaline pH but was readily detectable in the alkaline treated mitochondrial fraction. This suggests that BNIP3 responds to hypoxia by firmly integrating into mitochondrial membranes.

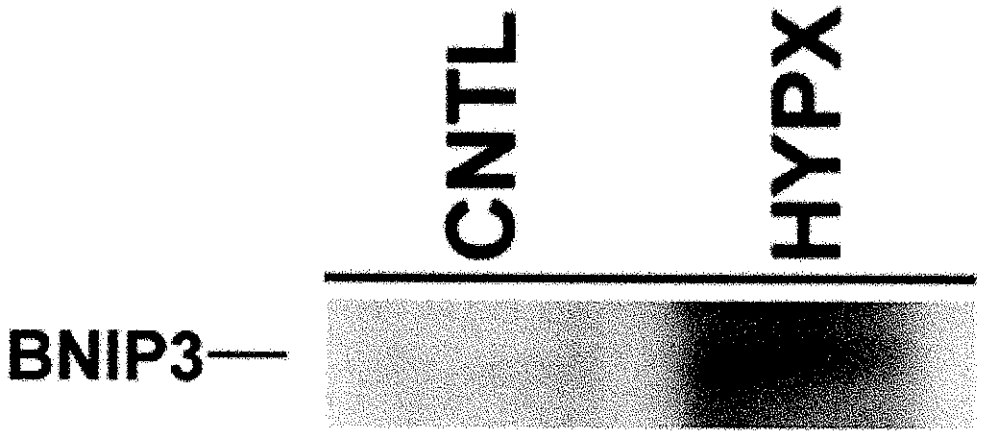

Figure 30: Hypoxia induces the accumulation of BNIP3 protein in the mitochondria. Whole mitochondrial fraction of cells subjected to hypoxia and probed for BNIP3. Isolation of mitochondria from normoxic and hypoxic myocytes (24hr) followed by western blot analysis for BNIP3. Elevated levels of BNIP3 protein are detectable in the mitochondria of myocytes subjected to hypoxia. CNTL (normoxic cells); HYPX (hypoxic cells).

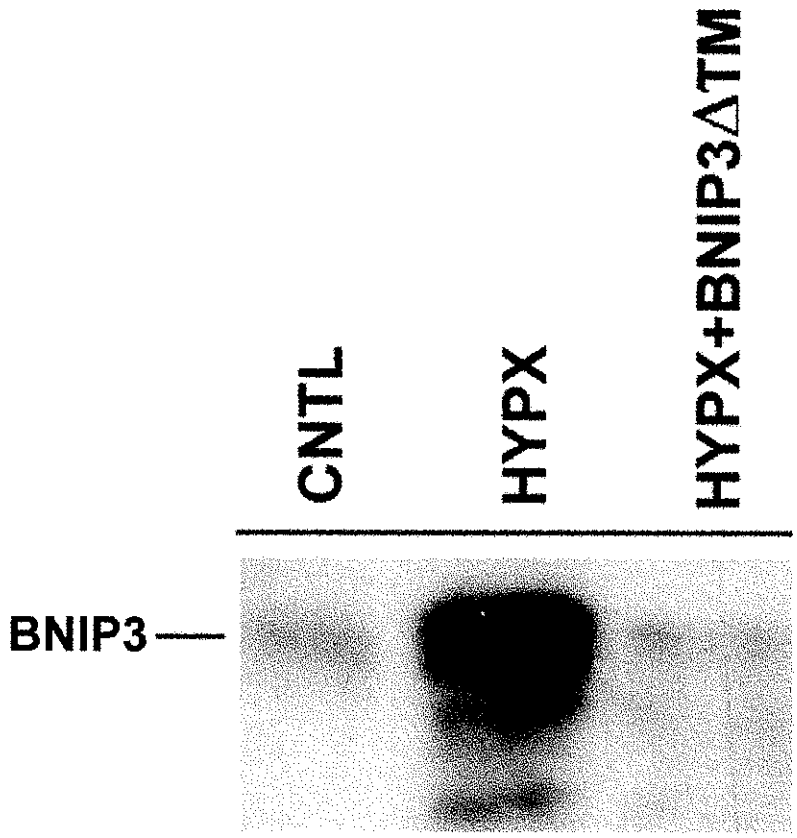

Figure 31: Hypoxia-induced mitochondrial integration of the endogenous BNIP3 is suppressed by BNIP3ΔTM. Alkaline extraction of mitochondria from normoxic and hypoxic myocytes (24hr) in the presence and absence of BNIP3ΔTM and subsequent western blot analysis for BNIP3. Absence of detectable BNIP3 in the normoxic group indicates that BNIP3 is alkaline soluble and not integrated into mitochondrial membranes. Presence of BNIP3 after alkaline treatment of mitochondria indicates that BNIP3 is integrated into mitochondrial membranes. In the absence of BNIP3ΔTM, endogenous BNIP3 is detectable in the alkaline-treated fraction, indicating it had integrated into mitochondrial membranes. In the presence of BNIP3ΔTM after alkaline treatment, the endogenous BNIP3 was not detected in the mitochondrial fraction, indicating it was not integrated into mitochondrial membranes. CNTL (normoxic cells); HYPX (hypoxic cells); HYPX+BNIP3ΔTM (myocytes expressing BNIP3ΔTM and subjected to hypoxia).

3.6 BNIP3 promotes mitochondrial PT

As we have shown, hypoxia triggers opening of the PT pore which potentiates the apoptotic signal. Therefore, we next ascertained whether BNIP3 would provoke mitochondrial PTP opening. Immunofluorescence microscopy of ventricular myocytes assayed for PT revealed that in contrast to control cells, a marked reduction in mitochondrial fluorescence was observed in cells over-expressing BNIP3 (Figure 32). To confirm that the BNIP3-induced reduction in Calcein fluorescence adequately represented PT, we pretreated myocytes with Bongkrekic acid to prevent PTP opening. As predicted, BA suppressed BNIP3-induced reduction in calcein fluorescence confirming the specificity of the assay for determining pore opening. We therefore conclude that BNIP3 causes PT opening.

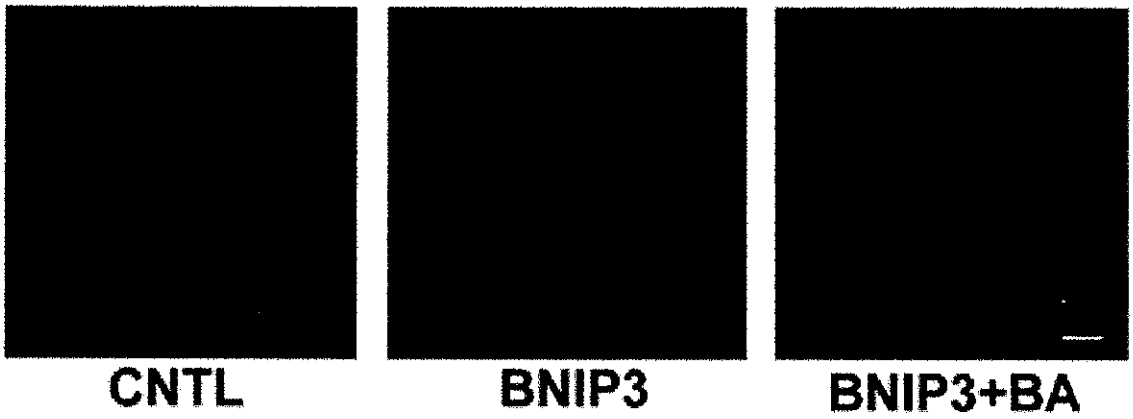

Figure 32: BNIP3 induces permeability transition pore opening. Mitochondrial permeability transition pore (PT) was monitored in ventricular myocytes using the membrane permeable dye calcein-acetoxymethyl ester in the presence of cobalt chloride to quench the cytoplasmic signal. Opening of the PT pore is discerned by a loss in green fluorescence. CNTL indicates normoxic control myocytes; BNIP3, myocytes infected with an adenovirus encoding BNIP3; BNIP3+BA, myocytes infected with an adenovirus encoding BNIP3 in the presence of Bongkrekic acid (50 μmol/L).

3.7 BNIPΔTM suppresses hypoxia- induced cell death of ventricular myocytes independent of changes in Bak, Bax or Bcl2 protein levels

To gain a better understanding of the mechanism of BNIP3 induced apoptosis, we tested whether a carboxyl terminal transmembrane domain deletion mutant, BNIP3ΔTM, defective for mitochondrial membrane targeting could suppress hypoxia-induced cell death of ventricular myocytes. As shown in Figure 33, cells expressing BNIP3ΔTM were indistinguishable from control cells with respect to cell viability suggesting that mitochondrial membrane targeting was important for the toxicity of BNIP3. A 5.6 fold increase ($p<0.05$) in myocyte cell death was observed in cells exposed to chronic hypoxia; remarkably, hypoxia-mediated cell death was significantly reduced in the presence of BNIP3ΔTM (Figure 33B). Furthermore, BNIP3ΔTM had no apparent effect on the expression level of Bax, Bak or Bcl2 in ventricular myocytes (Figure 29).

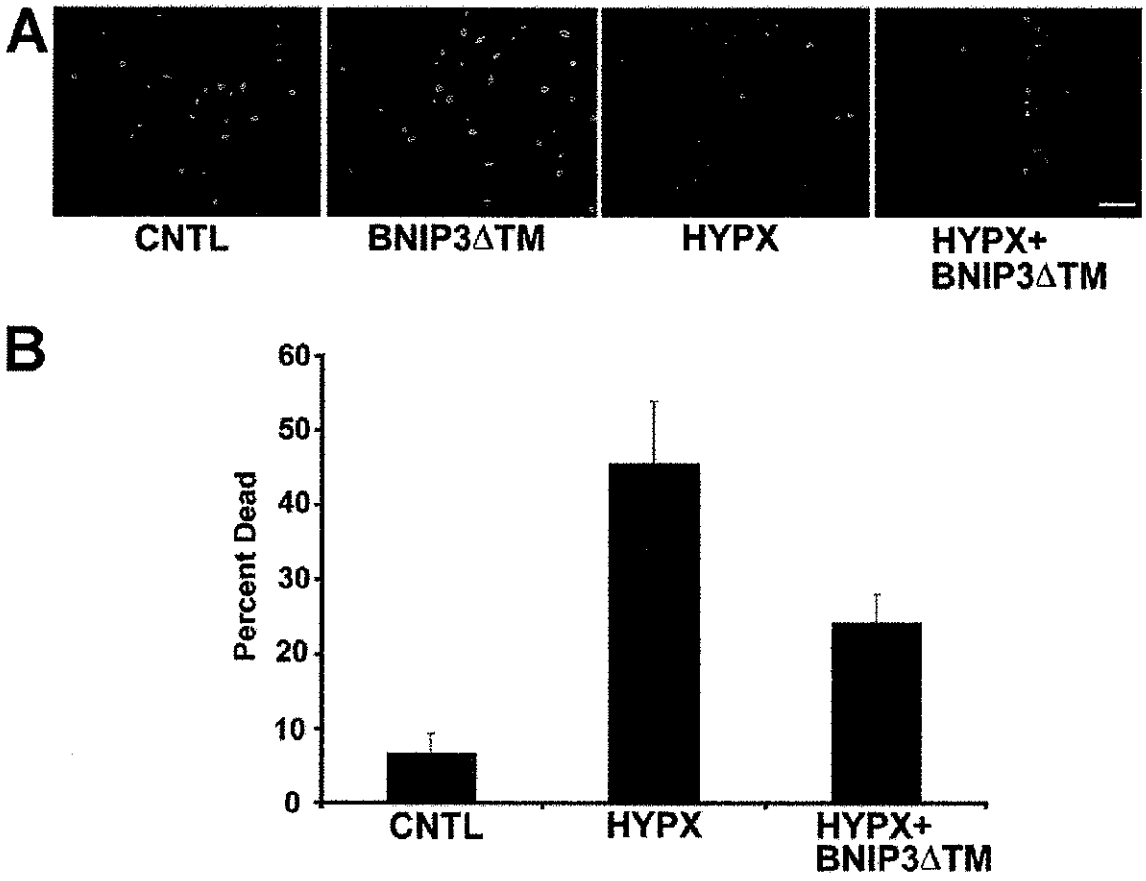

Figure 33: BNIP3ΔTM rescues hypoxia-mediated cell death.
Panels A) Fluorescent microscopic images of neonatal ventricular myocytes treated with the vital dyes calcein acetoxymethyl ester (green) and ethidium homodimer-1 (red) to identify the number of living and dead cells, respectively. CNTL (control treated myocytes); BNIP3ΔTM (myocytes expressing mutant BNIP3 lacking the transmembrane domain); HYPX (myocytes subjected to 24 hr of hypoxia); HYPX+BNIP3ΔTM (myocytes expressing BNIP3ΔTM and subjected to hypoxia). Bar = 100μM. Panel B) Histogram of data shown in B. Data were obtained from at least n=3 independent myocyte isolations with 2 replicates for each condition and >200 cells counted per condition.

3.8 BNIPΔTM blocks hypoxia-induced integration of endogenous BNIP3

A likely mechanism by which BNIP3ΔTM prevents hypoxia-mediated apoptosis is through dominant negative inhibition of the endogenous BNIP3 protein. As evidence in favor of this hypothesis, BNIP3 resistance to alkaline extraction during hypoxia was lost in the presence of BNIP3ΔTM (Figure 31). In other words, as a dominant negative inhibitor of the wild-type protein, BNIP3ΔTM was able to block hypoxia-mediated mitochondrial integration of the endogenous BNIP3 protein, an important component of hypoxia-induced cell death of ventricular myocytes.

V. DISCUSSION

Cardiac myocytes, like most cells, have the capacity to undergo apoptotic cell death. Toxic stimuli activate intracellular signaling pathways which lead to DNA fragmentation and cell death. While the loss of cells through an apoptotic program is relatively innocuous in a tissue with normal cell turnover, apoptosis is a potentially dangerous phenomenon in the heart because of its inability to regenerate. Notwithstanding, the programmed and predictable nature of apoptosis makes it a formidable target for genetic intervention. By identifying the molecular regulators of apoptosis in the heart and understanding the mechanisms by which they operate, it is possible to design therapeutic strategies to regulate apoptotic cell death and progress toward maintaining cardiac function after injury. In this thesis, I investigate the molecular mechanisms of hypoxia, BNIP3 and p53-mediated apoptosis in the heart.

1. Hypoxia-mediated apoptosis

Apoptosis has been detected in the myocardium during ischemia-reperfusion ([12;17]), myocardial infarction ([20]) and in patients with end stage heart failure ([26;80]). Our *in vitro* data suggest that prolonged oxygen deprivation is a trigger of cardiomyocyte apoptosis in these disease phenomena ([7]). To elucidate the molecular mechanisms of hypoxia-mediated apoptosis in the heart, we utilized a model of cultured neonatal ventricular myocytes. It has been previously reported that neonatal myocytes are more resistant to hypoxia compared to their adult counterparts. While the basis for low oxygen tolerance in the neonatal heart is unknown, it may be attributed to developmental differences in substrate availability and metabolism, calcium handling or reduced energy

demand (reviewed in ([363]). Nonetheless, I provide evidence of neonatal ventricular myocyte apoptosis after 24 hours of hypoxia, a time point consistent with other laboratories ([364-366]).

Caspases integrate signals from a diverse array of pro-apoptotic stimuli and coordinate the dismantling of the cell by cleaving a variety of intracellular targets. Early reports from our laboratory demonstrated that caspase 3, a distal death effector caspase, was active during hypoxia-mediated apoptosis of adult ventricular myocytes ([7]). Pre-treatment with Ac-DEVD-CHO, a specific inhibitor of caspases related to caspase 3, attenuated hypoxia-mediated apoptosis. These findings pointed towards caspases as an integral component of the cardiomyocyte cell death pathway triggered by hypoxia. It is generally accepted that caspase activation can occur by one of two predominant mechanisms: 1) self-cleavage or cleavage by other caspases and 2) recruitment to ligated death receptor complexes. In the first scenario, upstream initiator caspases such as caspase 8 are able to activate downstream death effector caspases such as caspases 3, 6, or 7 ([367;368]). In the latter case, CD95/Fas or TNFα receptor ligation activates a death inducing signaling complex (DISC) which is capable of directly activating caspase 8 ([165;369]). Interestingly, elevated levels of Fas mRNA have been observed in rat myocytes subjected to hypoxia ([4]). To elucidate a role for caspase 8 during hypoxia, I determined the status of caspase 8 by western blot and the level of caspase 8 activity in neonatal ventricular myocytes subjected to 24 hours of hypoxia. I report that caspase 8 is processed and proteolytically active in ventricular myocytes subjected to hypoxia. If caspase activation does in fact proceed in a hierarchical fashion then caspase 8, an apical initiator caspase, should have the ability to activate downstream caspases in the apoptotic

signaling cascade. Using an *in vitro* system, Stennicke et al. demonstrated that caspase 8 is capable of direct activation of caspase 3 ([367]). Along a similar vein, inhibition of apical caspases should prematurely halt the apoptotic signaling cascade and promote cell survival. In fact, YVAD, an upstream peptide caspase inhibitor was shown to block apoptosis induced by the Fas ligand or Fas receptor cross-linking ([370]). While synthetic caspase inhibitors appear effective for preventing caspase activation and apoptosis following brief exposure to pro-death stimuli, a limited cell permeability and short half-life question their utility in response to prolonged death signals. The cytokine response modifier A (CrmA) gene cloned from the cowpox virus encodes a naturally occurring caspase 8 inhibitor ([355]). Therefore to circumvent the limitation of peptide caspase inhibitors, I generated a replication defective adenovirus encoding CrmA to uniformly and efficiently express the CrmA protein in ventricular myocytes. CrmA effectively blocked caspase 8 activity following prolonged hypoxia. Importantly, CrmA was sufficient to block hypoxia-mediated apoptosis of ventricular myocytes. The anti-apoptotic role for CrmA is supported by several studies where CrmA was shown to block Fas/CD95 induced apoptosis ([371;372]).

The mitochondrion, once recognized for its role in energy metabolism, is now considered to be a central integration site for biological signals that promote cell life or cell death (reviewed in ([209])) ([31;33]). Since mitochondria contain the necessary apoptotic machinery to activate the cell death pathway, these important organelles are now appreciated as key decision makers for whether a cell will live or die following a noxious signal. Cytochrome c was one of the first mitochondrial proteins to be recognized as an important post-mitochondrial pro-death factor. Released into the cytosol following an

appropriate stimulus, cytochrome c interacts with Apaf-1, dATP and pro-caspase 9 to activate distal caspases and apoptosis ([34;246;373]). Caspase 8 has been reported to cause cytochrome c release and apoptosis in a variety of cell types ([374-376]). I demonstrate that cytochrome c is released from cardiomyocyte mitochondria during hypoxia. Reportedly, changes in $\Delta\Psi_m$ and PTP opening contribute to the outer mitochondrial membrane permeabilization necessary for protein release from mitochondria and post-mitochondrial apoptotic signal transduction ([217;218]). I demonstrate that hypoxia-mediated apoptosis of ventricular myocytes involves a reduction in $\Delta\Psi_m$ and PTP opening. Bongkrekic acid, an inhibitor of the PTP and CrmA are sufficient to block hypoxia-mediated PTP opening, loss of $\Delta\Psi_m$ and apoptosis of ventricular myocytes but intriguingly, CrmA does not prevent cytochrome c release. These data support a role for the PTP as a caspase inhibitable mitochondrial checkpoint for cell death induction and suggest that cytochrome c release may not be sufficient for hypoxia-mediated apoptosis. Our results also support the notion that cytochrome c release can occur through a caspase independent mechanism and may not be obligatorily linked to apoptosis ([7;124;356]).

It is currently unclear how the cardiomyocyte evades apoptosis in the presence of significant cytochrome c release. It is interesting to speculate that unidentified factors may cooperate with cytochrome c and orchestrate the mitochondrial death response ([203;377]). Whether hypoxia triggers the release of such factors from ventricular myocyte mitochondria in a caspase inhibitable manner is currently unknown but could explain the paucity of apoptosis in the presence of cytosolic cytochrome c. Alternatively, it is possible that CrmA inhibits caspase events, which occur downstream of cytochrome c release but proximal to DNA fragmentation. However, the ability of BA to rescue

apoptosis but not cytochrome c release at the level of the mitochondria would argue against this latter hypothesis. Yet another hypothesis might argue that the sites on cytochrome c that are necessary for interaction with and activation of caspase 9 are occluded and require active caspase 8 to undergo the appropriate conformational change.

2. p53-mediated apoptosis

Early studies documenting elevated levels of p53 during cardiac disease states noted that p53 expression was often associated with an increased incidence of apoptosis. p53 has been implicated as a central effector of apoptosis in a variety of cell types in response to stresses that also impact the heart ([293;378;379]). One of these common cellular stresses that trigger p53 accumulation is hypoxia ([358]). That p53 may be an integral component of apoptotic signaling in the heart was confirmed by our laboratory when it was unequivocally demonstrated that overexpression of p53 triggers apoptosis of neonatal ventricular myocytes ([42]). While the mechanisms of p53-mediated apoptosis in the heart were unknown, it seemed apparent that transcription of the death promoting gene Bax was involved, providing a tentative link between p53 and apoptosis ([42;324]). Since then evidence has emerged that pro-death transcription factors such as p53 may be able to induce apoptosis independent of *de novo* gene transcription ([380-384]). Here I test the hypothesis that DNA binding and *de novo* gene activation is *not* required for the death inducing ability of p53. I find that p53 is capable of transcription-independent apoptosis. I also demonstrate that p53-mediated apoptosis involves perturbations to mitochondria including loss of $\Delta\Psi_m$, cytochrome c release and caspase 3-like activation.

The p53 protein is structurally comprised of 4 domains, the N-terminal transactivation domain, DNA binding domain, tetramerization domain and C-terminal regulatory domain (reviewed in ([385])). The incidence of tumors that carry mutations in the p53 gene is high and the majority of defects are harbored in the DNA binding domain of the protein rendering it incapable of performing its transcriptional duties (reviewed in ([386])). This suggested that the transcriptional properties of p53 were necessary apoptosis and thus tumor suppression. By using a molecular genetics approach, I find that p53 can mediate apoptosis of ventricular myocytes independent of DNA binding and de novo gene transcription of death factors Bax and MDM2, known targets of p53. A higher incidence of apoptosis is observed in cells expressing wild-type p53 than the DNA binding mutant suggesting that wild-type p53 may activate cell death through multiple mechanisms.

I observe mitochondrial defects including changes in mitochondrial membrane potential and cytochrome c release associated with a transcription defective p53. This would suggest that p53 can directly impact upon the mitochondrial death pathway. Moreover, p53 has recently been found to localize to the mitochondria where it causes outer mitochondrial membrane permeabilization through a direct interaction with Bcl-2 and Bcl-XL proteins ([387]). Earlier work from our laboratory had demonstrated that Bcl-2 could rescue p53-mediated cell death in ventricular myocytes. It was proposed that Bcl-2 heterodimerized with and inactivated Bax, a notion which was in line with the titration model of apoptotic cell death ([42]). In light of this new information, Bcl-2 may rescue p53-mediated cell death by titrating out p53 at the level of the mitochondria, thus preventing mitochondria defects and maintaining the integrity of its membranes.

Although I did not test the effect of Bcl-2 on mutant p53-mediated cell death, it is probable that Bcl-2 would also be cytoprotective in this instance.

It is becoming increasingly apparent that mitochondria play a key role in cell death signaling. I propose that in p53-mediated cell death, activation of the mitochondrial death pathway through a direct effect of p53 may be the first line of attack in an apoptotic response. Unlike transcription dependent effects of p53, the direct action of p53 at the level of the mitochondria can presumably occur instantaneously. Subsequently, pro-death p53 response genes such as Bax, PUMA ([325]), Noxa ([388]) and more recently Pac1 ([389]) would converge on and amplify the death signal. Interestingly, Bax, Puma and Noxa have been reported to trigger mitochondrial defects suggesting that the death signal originates at the level of the mitochondria.

The cytochrome c/Apaf-1-dependent pathway appears to be active in p53-mediated cardiomyocyte cell death. I detect cytochrome c release by two independent methods – western blot analysis and immunocytochemistry. Similarly, Mihara et al. report p53-mediated cytochrome c release in irradiated thymocytes ([387]). As predicted by the hierarchy of caspase activation, the release of cytochrome c triggered by p53 would trigger apoptosome formation, caspase 9 processing and subsequent caspase 3 activation. In support of this notion, Cui et al., 2002 demonstrate that drug-induced cell death mediated through p53 can be prevented by expression of a dominant-negative mutant of caspase 9 ([390]). As a downstream target of activated caspase 9, I monitor myocyte cell lysate for caspase 3 activity. I observe caspase 3-like activation in response to wild-type and transcription defective p53. Intriguingly, the fact that induction of apoptosis is less with the mutant p53 compared to its wild-type counterpart, may reflect an additive effect

of transcription independent and transcription dependent mechanisms of caspase 3 activation for apoptosis.

3. Hypoxia and BNIP3-mediated apoptosis

Using a yeast-two hybrid approach I had found that BNIP3, a novel death-inducing mitochondrial protein is expressed in the heart. Interestingly, increased transcript levels of BNIP3 have been detected in immortalized cell lines during hypoxia ([45;291]). When myocytes are subjected to hypoxia, I observe a duration dependent increase in BNIP3 protein expression. Peak expression of BNIP3 occurs at 24 hours, a time point coinciding with peak hypoxia-mediated cell death. I reasoned that BNIP3 may be responsible for the cell death observed during hypoxia in myocytes given its reported ability to cause cell death in cancer cell lines ([44;278]). I employed adenoviral technology to express BNIP3 in ventricular myocytes and observed considerable apoptotic cell death, solidifying a functional link between hypoxia and cardiomyocyte cell death.

While it is becoming increasingly apparent that mitochondria play an important role in the transduction and amplification of apoptotic signals, the specific mediators of the mitochondrial death response are largely unknown. I postulate that as a mitochondrial protein, BNIP3 may mediate the mitochondrial defects associated with hypoxia-mediated cell death in the heart. Specifically, I consider that BNIP3 induces cell death through the mitochondrial PTP. I report several key observations that support this argument. First, hypoxia is sufficient to provoke integration of BNIP3 into mitochondrial membranes. Second, BNIP3 expression coincides with PTP opening, a BNIP3-induced mitochondrial defect which can be inhibited by BA. Finally, BNIP3ΔTM, which cannot insert into

mitochondrial membranes, suppresses hypoxia-mediated cell death of ventricular myocytes. Whether BNIP3 directly impinges upon the PTP to stimulate opening is currently unknown but plausible given the precedence for interaction of Bcl-2 family members with components of the pore. For example, Bim has been shown to interact with VDAC resulting in loss of membrane potential ([391]) whereas BAX reportedly interacts with the ANT causing a decrease in the exchange of ADP for ATP ([259]). It is also interesting to speculate that at its mitochondrial position BNIP3 may interfere with the ability of Bcl-2 to stimulate ANT activity ([392]).

Since BNIP3 is a potent effector of mitochondrial defects and apoptosis, deregulated BNIP3 activity could have profound consequences on cellular viability. I observe that BNIP3 inserts into mitochondrial membranes following prolonged hypoxia, but how is protein integration regulated in the absence of a pro-death signal? Other pro-apoptotic Bcl-2 family members are regulated by posttranslational modification. For example, phosphorylation of Bad by Akt, enables 14-3-3 to sequester Bad in the cytosol in an inactive state. On the other hand, Bid undergoes caspase dependent cleavage prompting t-Bid to translocate to the mitochondria and cause cell death. Prior to its cleavage, Bid is a relatively innocuous cytoplasmic protein. It is tempting to speculate that BNIP3 is also subject to post-translational modification. One or more modifications to the BNIP3 protein could either preserve its extra-mitochondrial localization prior to receipt of a death signal or conversely, stimulate its translocation following the appropriate trigger. Alternatively, BNIP3 activity may be regulated by an accessory protein which directly impacts upon its ability to translocate to the mitochondria.

Whereas deregulated activity of BNIP3 could have dramatic effects on cell viability so too would deregulated expression BNIP3. I report that BNIP3 is substantially induced in the heart following hypoxia in relation to other pro-death Bcl-2 family proteins. This distinction has implications for unique transcriptional control of BNIP3 gene expression. Interestingly, canonical binding sites for the hypoxia-inducible transcription factor HIF-1α have been identified in the BNIP3 promoter supporting a potential mechanism for transcriptional activation of BNIP3 during hypoxia ([45;281]). Interestingly, Mizutani et al. have reported a HIF-1-independant hypoxia responsive element in the BNIP3 promoter which is activated by the zinc finger protein pleomorphic adenoma gene-like-2 (PLAGL2) ([393]). It may be that PLAGL2 and HIF-1 synergize to activate BNIP3 following hypoxia. NO has been reported to suppress the expression of BNIP3 mRNA in hepatocytes ([283]). As NO stimulation is widely associated with apoptosis induction in the heart it is tempting to speculate that NO would stimulate rather than inhibit BNIP3 gene expression in cardiomyocytes. Whether HIF-1α, NO or other factors influence BNIP3 gene expression in the heart during hypoxia is unknown and awaits further investigation.

Previous reports describe the transmembrane domain of BNIP3 as necessary for membrane targeting and protein homodimerization. I demonstrate that BNIP3 lacking the transmembrane domain does not trigger cell death of ventricular myocytes but in fact, affords protection against hypoxia induced cell death. Given that the rescue imparted by BNIP3ΔTM occurs independent of changes in Bcl-2 family members suggests that it likely occurs through a mechanism that does not alter protein levels of related proteins. Insight into the mechanism of action of BNIP3ΔTM comes from the fact that

mitochondrial integration of endogenous BNIP3 protein is impaired in the presence of BNIP3ΔTM. This finding supports a role for BNIP3ΔTM as a dominant negative inhibitor wild-type BNIP3 possibly by competing with regulatory factors which are necessary for mitochondrial targeting.

I observe apoptotic cell death following BNIP3 overexpression in cardiomyocytes. Notwithstanding, necrotic mechanisms of cell death have also been attributed to BNIP3 ([44;275;277]). Vande Velde and colleagues describe a "necrotic-like", caspase independent mode of cell death by BNIP3 ([277]). To date, apoptosis and necrosis have been distinguished on the basis of a number of criteria including the occurrence of caspase processing; however, evidence in the literature suggests that apoptosis can occur through a caspase independent pathway. Thus, caspase activation may not be a suitable marker for characterizing the mode of cell death. From another perspective, apoptosis and necrosis may reflect elements of the same cell death pathway (i.e. the mitochondria) in a cell type and context specific manner. While Vande Velde et al. reported BNIP3 triggers cell death independent of caspase activation, I demonstrate that the pan caspase inhibitor z-VAD-fmk suppresses BNIP3-induced cell death in a dose dependent manner and corroborates our findings that hypoxia-mediated cell death is a caspase-mediated process. Further, expression of BNIP3 provoked cytochrome c release, a phenomenon not observed by Vande Velde et al. I believe that the differences between the two studies are due to the model system employed. While I use primary myocyte cell culture, the studies of Vande Velde et al. were conducted in immortalized cell lines which may not comprise the same signaling pathways as non-transformed cells. Because our studies

were conducted in primary cell culture I believe that our results depict the actions of BNIP3 in a physiologically relevant light.

VI. SUMMARY

Ventricular myocytes are susceptible to apoptosis by a variety of stimuli. The permanent loss of potentially viable myocytes by apoptosis may exacerbate the decline in cardiac function associated with cardiac injury. One strategy to maintain cardiac function following injury is to prevent or modulate cardiac apoptosis. For this to be a viable option, it is necessary to understand the molecular regulators and signaling pathways of apoptosis in the heart. Using neonatal ventricular myocytes I studied the impact of hypoxia, p53 and BNIP3 on cardiac cell apoptosis with an emphasis on caspase activation and the mitochondrial death pathway.

I found that hypoxia is sufficient to cause apoptosis of neonatal ventricular myocytes. An increase in caspase 8 activity was observed in neonatal ventricular myocytes subjected to hypoxia relative to normoxic control cells. Adenoviral-mediated delivery of the caspase inhibitor, CrmA, to myocytes inhibited hypoxia-mediated caspase 8 activation and apoptosis of myocytes. Hypoxia provoked myocyte mitochondrial defects including cytochrome c release, loss of mitochondrial membrane potential and permeability transition pore opening. CrmA expression prevented the hypoxia-mediated loss of mitochondrial membrane potential and opening of the permeability transition pore but not cytochrome c release. Our data suggest that hypoxia triggers apoptosis of ventricular myocytes through caspase 8-mediated mitochondrial defects. Further, I suggest that cytochrome c release in the context of myocyte hypoxia may be a caspase independent event.

Next I investigated the molecular mechanisms by which p53 triggers apoptosis of ventricular myocytes. p53, a known transcription factor, has the ability to transactivate

pro-apoptotic genes such as Bax. Transactivation of death effector genes was initially thought to be indispensable for p53-mediated cell death in some cell types. I probed the requirement of DNA binding and gene transcription for p53-mediated cell death in the heart. I found that p53 is able to trigger apoptosis of neonatal ventricular myocytes independent of DNA binding and *de novo* gene transcription albeit to a lesser extent. Caspase 3-like activation was detected in myocytes expressing wildtype or a transcription defective p53. Inhibition of caspase 3-like caspases with c-DEVD-CHO abrogated apoptosis by either protein. I also found that wild-type and transcription defective p53 provoked a reduction in $\Delta\Psi m$ and cytochrome c release. From these results, it appears that the ability of p53 to trigger apoptosis of myocytes is not contingent upon *de novo* gene transcription. Rather, transcription dependent and independent pathways of p53-mediated cell death may share elements of the same artillery including activation of the mitochondrial death pathway and caspase 3 activation.

I also evaluated the impact of BNIP3 during hypoxia-mediated apoptosis. BNIP3 is a prodeath Bcl-2 gene family member which localizes to intracellular membranes. Importantly, elevated levels of BNIP3 mRNA have been reported in transformed cell-lines subjected to hypoxia. I found that hypoxia induces expression and mitochondrial accumulation of BNIP3 in neonatal ventricular myocytes. Further, hypoxia triggered the integration of BNIP3 into the outer mitochondrial membrane. Transfection of BNIP3 in other cell types has been shown to cause cell death. Adenoviral-mediated delivery of BNIP3 but not BNIP3ΔTM to cardiomyocytes provoked wide-spread cell death which could be inhibited by pretreatment with the pan-caspase inhibitor z-VAD-fmk. Further, cells that expressed BNIP3 displayed mitochondrial defects consistent with permeability

transition pore opening. Expression of BNIP3ΔTM suppressed the mitochondrial integration of endogenous BNIP3 within the outer mitochondrial membrane and cell death induced by hypoxia. It is conceivable that cell death triggered by BNIP3 is contingent upon its integration into the outer mitochondrial membrane for initiation of the mitochondrial death pathway, caspase activation and cell death.

VI. CONCLUSIONS

Increased apoptosis and a limited regenerative capacity is a potentially crippling combination for the heart. It is therefore not surprising that apoptosis is a contributing factor for cardiac disease. A better understanding of the pathways leading cardiomyocyte cell apoptosis may lead to new strategies for the prevention and treatment of cardiac pathologies and possibly, unimpeded cardiac regeneration in the presence of appropriate growth factors. Given the intricacies of apoptotic signaling, multiple levels exist to interrupt the process. Obviously inhibition of apoptotic signaling at the source is preferable but not always feasible. Also it may be necessary to selectively inhibit specific apoptotic signaling pathways while others are left intact. This will require an understanding of both the mechanisms involved in cardiac myocyte apoptosis and the facets that are unique to each disease state. It is important to keep in mind that apoptosis serves a physiological role in the organism. How long-term maintenance of a population of irreversibly damaged cells will affect the function of neighboring healthy cells is unknown. But improvement of initial symptoms associated with apoptosis may still be important for short-term management of cardiac disease. In other contexts, prevention of cell death by apoptosis in otherwise healthy cardiac cells may slow the progression to heart failure. Overall it is hoped that understanding mechanisms of cardiomyocyte apoptosis will ultimately prove beneficial to those suffering from cardiac disease.

VII. FUTURE DIRECTIONS

The role of mitochondria in the context of cellular physiology is expanding. Long recognized for their involvement in energy metabolism and constituting 30% of the cardiomyocyte cell volume, cardiomyocyte mitochondria have now emerged as key players within the apoptotic signaling pathways of the heart and may well be the gatekeepers of cardiomyocyte apoptosis. At their central location within the apoptotic signaling cascade, mitochondria integrate biological signals to decide whether a cell will live or die. This makes the mitochondrion an ideal target for therapeutic interventions to modulate cardiomyocyte cell death and preserve cardiac function after injury. Future therapeutic strategies to modulate apoptosis in the context of heart failure will undoubtedly require a thorough understanding of the mitochondria as a central regulator of apoptosis.

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