MOLECULAR REGULATORS OF HYPOXIA MEDIATED APOPTOSIS IN VENTRICULAR MYOCYTES

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

MASTER OF SCIENCE

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Molecular Regulators of Hypoxia Mediated Apoptosis in Ventricular Myocytes

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A Thesis/Practicum submitted to the Faculty of Graduate Studies of The University of Manitoba in partial fulfillment of the requirements of the degree of

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ABSTRACT

While the molecular mechanisms regulating hypoxia-mediated apoptosis in the myocardium remain poorly defined, there is evidence to suggest important roles for the mitochondria and *caspases,* a class of cysteine proteases.

Methods: Adult and neonatal ventricular myocytes were subjected to hypoxia and then assayed for the incidence of apoptosis. Caspase activation was assessed by Western blot analysis and fluorometric assay. Mitochondrial perturbations including cytochrome c release and collapse of the membrane potential were monitored by Western blot and fluorescent microscopy respectively. Following caspase inhibition by Ac-YVAD-CHO, Ac-DEVD-CHO and adenoviral delivery of CrmA, a serpin protein known to prevent caspase activation, the effect on apoptosis and mitochondrial perturbations were examined.

Results: Compared to control, hypoxia induced a 3-fold increase ($P<0.05$) in the number of apoptotic myocytes. Furthermore, hypoxia was associated with caspase activation and mitochondrial cytochrome c release in both cell types. A direct role for caspase activation was determined using synthetic caspase inhibitors (adult myocytes) and adenoviral delivery of CrmA, (neonatal myocytes). While Ac-YVAD-CHO, (inhibitor of caspases related to caspase-1), prevented caspase 3 activation, apoptosis and partially attenuated cytochrome c release, Ac-DEVD-CHO (inhibitor of caspases related to caspase-3), prevented PARP (poly-ADP-ribose-polymerase) processing and apoptosis, but had no effect on hypoxia-mediated cytochrome c translocation. Furthermore, CrmA expression effectively inhibited caspase activation and apoptosis, but failed to inhibit cytochrome c translocation in neonatal myocytes. Mitochondrial defects including opening of the

mitochondrial permeability transition (PT) pore and loss of mitochondrial membrane potential, $\Delta\Psi_m$, were observed during hypoxia in neonatal myocytes, and effectively prevented by adenoviral delivery of CrmA. Moreover, pre-treating neonatal myocytes with Bongkrekic acid, a specific inhibitor of PT pore opening, prevented $\Delta\Psi_m$ collapse and apoptosis.

Conclusions: Together this data points to the direct involvement of caspases during the progression of apoptosis, and suggests that mitochondrial cytochrome c release may be a caspase-independent event. Moreover this data suggests a critical role for mitochondrial perturbations such as PT pore opening and $\Delta\Psi_m$ collapse during hypoxia. We can infer from these experiments that $\Delta\Psi_m$ collapse is in part controlled through a caspase-regulated pathway and that cytochrome c release occurs independently of $\Delta\Psi_m$ collapse and caspase activation.

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LIST OF ABBREVIATIONS:

Caspase	Cysteinyl aspartate specific protease
ICE	Interleukin-1-converting enzyme
Apaf-1	Apoptosis-activating-factor
$\Delta\Psi m$	Mitochondrial membrane potential
PT	Permeability transition
Ac-YVAD-CHO	N-acetyl-Tyrosine-Valine-Alanine-Aspartate Aldehyde
Ac-DEVD-CHO	N-acetyl-Asparate-Glutamate-Valine-Aspartate Aldehyde
CrmA	Cytokine response modifier A
TUNEL	Terminal-transferase-mediated dUTP-biotin nick end labeling
pO_2	Partial pressure of oxygen
PARP	Poly-(ADP)-ribose-polymerase
DISC	Death Inducing Signaling Complex
ANT	Adenine Nucleotide Translocator
BA	Bongkrekic Acid
AIF	Apoptosis Inducing Factor
HEK293	Human Embryonic Kidney cells 293
TMRM	Tetra-methyl rhodamine methyl ester
CNTL	Control
HYPX	Hypoxic

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I. INTRODUCTION

An intriguing issue in cardiovascular science is the concept of programmed cell death or *apoptosis* in the heart. Apoptosis is a mode of cell death that proceeds via a coordinated series of events, genetically programmed within cells. Apoptosis is an energy-dependent process involving the activation of endogenous endonucleases, which results in inter-nucleosomal chromatin cleavage (45). Apoptotic cells are often defined by distinct morphological changes including chromatin condensation, membrane blebbing and cell shrinkage (43). Cells undergoing apoptosis are condensed into "apoptotic bodies" which are subsequently phagocytosed by neighboring macrophages, thus evading the inflammatory response (1).

Apoptotic cell death widely occurs in nature. Normal development of tissue and organs in multi-cellular organisms depends on precise cell removal to ensure the correct number of cells is positioned in the fetus (40). A balance between cell life and cell death is therefore required to maintain tissue homeostasis and organisms survival. In this regard, deregulated apoptosis may lead to insufficient cell death manifested by proliferative disorders such as cancer (2). Alternatively, excessive apoptosis has been implicated in the pathogenesis of neurodegenerative disorders such as Alzheimer's (51), Parkinson's (52) and Huntington's diseases (3, 4).

Of substantial interest is the recent evidence for apoptosis in several cardiovascular disorders. In both experimental models of heart failure and in studies from explanted failed human hearts, evidence for myocardial apoptosis has been brought forward. Following acute myocardial infarction in rats, Kajstura *et al.* reported that apoptosis represented the predominant form of cell death, not necrosis (5). Further studies by Cheng *et al.* demonstrated increased levels of apoptosis in the myocardium adjacent to

the infarcted area, relative to tissue remote from the injury (6). Several groups reported that apoptosis plays a pivotal role in atherosclerosis and associated changes in the vessel wall (7). Gottlieb *et al.* demonstrated the occurrence of apoptosis in rabbit cardiomyocytes following ischemia-reperfusion (8). These studies and others demonstrate that myocardial apoptosis is a widespread phenomenon occurring in a variety of cardiac pathologies.

Moreover, recent evidence indicates that myocyte apoptosis occurs in human diseases, such as patients with end-stage heart failure and cardiomyopathies. Explanted hearts from patients with end-stage heart failure displayed evidence of DNA fragmentation identified by *in situ* end labeling and agarose gel electrophoresis (9). Olivetti *et al.* demonstrated a 232-fold increase in myocyte apoptosis using 36 hearts from cardiac transplant patients with congestive heart failure in comparison to hearts from patients who died from causes not related to cardiovascular disease (10). Moreover, Saraste *et al.* identified apoptotic myocytes in the border zone of hearts from patients who succumbed to acute myocardial infarction (11).

Myocardial apoptosis raises an intriguing issue in the field of cardiology, given the lack of myocyte replication soon after birth. This innate property of myocytes prevents any myocyte proliferation following cardiac injury. Moreover, the loss of viable myocytes via an apoptotic program is believed to contribute to ventricular re-modeling and further deterioration of myocardial function (131). The ability to modulate, or even prevent inappropriate cell loss would be of significant therapeutic value in maintaining cardiac function following injury. Because apoptosis proceeds through a pre-determined

program involving many checkpoints, pharmacological, biological or chemical interventions may enable the rescue of cells proceeding through an apoptotic program.

A better understanding of the molecular regulators of apoptotic cell death is required before a clinical application can be envisioned. Genetic studies in the nematode, *Caenorhabditis elegans*, have resulted in the discovery of several genes required for proper worm development. Two pro-apoptotic genes *ced-3* and *ced-4* are required for cell death, while *ced-9* acts to inhibit it (12). The mammalian homologues of these genes are interleukin-1 converting enzyme (ICE), apoptosis-activating-factor 1 (Apaf-1), and bcl-2 respectively (13, 14).

An important role for the ICE family has been implicated in apoptosis (15). As many as 20 different but related members have been identified and are collectively referred to as caspases (*c*ysteinyl *a*spartate *sp*ecific prote*ases*), denoting their specific ability to cleave their cellular substrates following an aspartic acid residue (70). Caspases exist in cells as zymogens and themselves must be cleaved following an aspartic acid residue before becoming active proteases. This property implies that caspases must be activated through cleavage by other caspases or via self-activation. A hierarchical cascade of caspase activation and cleavage has thus been proposed for the mechanistic pathway (Reviewed in 16, 17, 18)

A mitochondrial pathway has also been implicated as an important regulator of the apoptotic signaling pathway (19). Through an undefined mechanism, cells induced to undergo apoptosis release mitochondrial cytochrome c into the cytosol. Association with other cellular factors in the presence of dATP triggers the activation of downstream caspases resulting in cellular apoptosis (14, 20).

Further, defects in mitochondrial integrity resulting in loss of mitochondrial membrane potential ($\Delta\Psi_m$) have recently been proposed as critical apoptotic events (21). The asymmetrical distribution of ions across the inner mitochondrial membrane is required for normal cell function. Loss of the membrane potential is an event believed to be inseparable from cell death (22). The underlying event thought to be responsible for $\Delta\Psi_m$ collapse is the opening of a permeability transition (PT) pore that allows for the extrusion of proteins having a molecular weight <1500 Da (23).

To test the impact of mitochondrial regulation and caspase activation during normal and diseased conditions in the heart, we examined the impact of hypoxia on neonatal and adult ventricular myocytes. Hypoxia can be defined as an imbalance between oxygen supply and demand to the cell (24). Oxygen deprivation is an important facet of ischemia arising in many cardiac pathologies. Studies in ventricular myocytes demonstrated that myocytes subjected to hypoxia die via an apoptotic program (25, 26, 27). However, the molecular regulators of hypoxia-induced apoptosis are largely undefined and are currently under investigation in our laboratory.

We hypothesize that caspases play an integral role in hypoxia-mediated apoptosis of ventricular myocytes. Inhibition of caspase activation may aid in promoting survival in cells induced to undergo apoptosis. Synthetic aldehyde inhibitors have been engineered which mimic the substrate site of caspases, thus preventing caspase activation (28, 29). The tetra-peptide inhibitors Ac-YVAD-CHO and Ac-DEVD-CHO act to inhibit caspase-1-like and caspase-3-like activity respectively (18). Furthermore, novel molecular biology techniques have enabled scientists to construct replication-defective adenoviruses for in vitro delivery of genes into cell cultures (30, 31). We constructed an adenovirus

encoding the cowpox virus protein, CrmA, an inhibitor of caspase activity, specifically caspase-1 and caspase-8 (32). A direct role for caspase activation during hypoxia has not been formally established. Moreover, whether caspases can influence mitochondrial defects is further unknown in the context of myocardial hypoxia.

Therefore, the main goals of my research project are: **(I)** To demonstrate that hypoxia induces apoptosis in both adult and neonatal ventricular myocytes. **(II)** To examine the incidence of caspase activation and mitochondrial cytochrome c release during hypoxia-mediated apoptosis of ventricular myocytes. **(III)** To evaluate the impact of caspase-inhibition on hypoxia-induced mitochondrial cytochrome c release and **(IV)** To determine if hypoxia involves a loss of mitochondrial membrane potential and whether this is a caspase-dependent phenomenon.

For these studies, adult and neonatal ventricular myocytes were subjected to hypoxia then assayed for the incidence of apoptosis, caspase activation and mitochondrial cytochrome c release. Caspase activity was attenuated by tetra-peptide inhibitors or adenoviral delivery of CrmA, and the effect on apoptosis and cytochrome c release was ascertained. Furthermore, neonatal ventricular myocytes were analyzed for mitochondrial membrane potential collapse. Whether loss of $\Delta\Psi_m$ is a caspase dependent event and whether it plays a vital role in hypoxia-mediated apoptosis was also examined.

I. LITERATURE REVIEW

A. HEART FAILURE

One of the leading causes of morbidity in the North American population is heart failure. The most common consequence of heart failure is left ventricular dysfunction, which subsequently results in decreased perfusion of vital organs (135). Compensatory mechanisms such as compensatory left ventricular hypertrophy and dilation (136) and increased activity of the renin-angiotensin system (137) are recruited in an attempt to maintain blood flow and homeostasis. These in turn lead to a "vicious cycle", whereby the physiological mechanisms intended to help, actually become factors that aid in the further progression of LV dysfunction (33, 149). Eventually these compensatory mechanisms lead to further cardiac failure, multiple organ failure, and eventually death (34).

Some contributing factors to the pathogenesis of heart failure are oxidative stress, hypoxia and subsequent to myocardial infarction (35). The cellular mechanisms responsible for myocardial dysfunction are multifactorial and include altered calcium handling (36), impaired excitation-contraction coupling (37) and irreversible cell loss (5, 38, 39). There is increasing evidence to suggest that cell loss occurs via an apoptotic program, subsequently leading to deterioration of ventricular function and heart failure (33).

B. APOPTOSIS

1. Overview

In 1972, Wyllie and Kerr coined the phrase "apoptosis" to describe the evolutionary conserved mode cell death commonly referred to as programmed cell death (40). Cell death can occur by two distinct modes, necrosis or apoptosis. Necrotic cell death results following severe injury to the cell. External factors including toxins and viruses all cause cells to die by necrosis. Necrotic cell death is not an energy dependent process. It is characterized by exhaustion of intracellular ATP stores, cell swelling, membrane rupture and subsequent extrusion of cell content into the cytosol thus triggering the immune response (41).

In contrast, apoptosis is a pre-programmed, genetically regulated mode of cell death and is therefore described as "cell suicide". Cells undergoing apoptosis exhibit distinct morphologic characteristics including chromatin condensation and margination, giving the cell a crescent shaped nucleus. Cytoskeletal changes occur characterized by membrane blebbing and cell shrinkage. Activation of endogenous nucleases result in oligonucleotide DNA fragmentation and nuclear degradation into "apoptotic bodies" (42, 42). Phagocytosis is carried out by adjacent macrophages or monocytes and sometimes vascular smooth muscle, to remove these apoptotic bodies (44). Because apoptosis occurs without membrane rupture or release of the cytoplasmic contents the inflammatory response is not activated (41).

2. Biochemical detection of apoptotic cells

A variety of biochemical techniques exist to identify apoptotic cells. Self-activation of endogenous endonucleases is responsible for the internucleosomal chromatin cleavage that is characteristic of apoptotic cells (42). In this regard, apoptotic cells are typically cleaved into nucleosomal fragments of 180-200 base pairs. Following gel electrophoresis of extracted DNA, apoptotic cell are visualized as a distinct "ladder" of fragmented DNA, 200 base pairs in size (42). In contrast, due to the random activation of degradative enzymes during necrosis, necrotic cells are visualized as a "smear" of degraded DNA following electrophoresis (45). While this is reliable technique to differentiate between apoptotic and necrotic cells, a large sample ($>10^6$ cells) is required (unpublished observations). Moreover, this technique is limited when analyzing tissue samples containing different cell types. It would not be possible to determine if the apoptotic process is restricted to a specific cell population (1) and quantification is difficult.

Fluorescent microscopy can be used for examination of nuclear morphology. Using fluorescent dyes such as Hoechst 33258, visualization of the nuclei of isolated cells or tissue samples is possible (1). Healthy cell nuclei are uniformly stained blue while apoptotic cells typically display condensed, hyper-chromatic nuclei, indicative of condensed, fragmented chromatin. While this technique allows for the incidence of apoptosis to be quantified, scoring apoptotic cells based on nuclear morphology may be subjective, and should therefore be used in conjunction with another technique. Furthermore, Hoechst staining may underestimate the incidence of apoptosis as some apoptotic cells lose their adherence and may be lost during washing. However, counter-

staining with a cell-specific marker allows the incidence of apoptosis to be assigned to a specific cell population (69).

Another common technique used to identify and score apoptotic cells also relies on nuclear fragmentation. Apoptosis yields single stranded breaks in high molecular weight DNA as well as double-stranded, low molecular weight DNA fragments (46). A biochemical assay termed TUNEL (TdT mediated deoxy-triphosphate nick end labeling) utilizes TdT (terminal deoxynucleotidyl transferase) to catalyze polymerization of fluorescein labeled dUTP nucleotides to free 3'-OH DNA ends (46). Using fluorescence microscopy or flow cytometry, incorporation of fluorescein labels can be quantified. It should be noted that the exposure of 3' DNA ends is not a unique event to apoptosis and also occurs during DNA and non-specific DNA damage. Because TdT will process any type of free 3' end, caution should be exercised when utilizing this technique.

3. Apoptosis in normal and diseased states

Apoptosis is typically used by multi-cellular organisms to selectively remove damaged or unwanted cells from its tissues (48). Normal organism development depends on programmed cell death (40). For example, tissue regression via apoptosis is responsible for digit formation on the hands and feet of many mammals (49). The immune response is dependent on the correct selection of B and T lymphocytes through an apoptotic program (50). Recent evidence, however, has demonstrated that defects in regulatory pathways governing apoptosis result in a variety of clinical disorders. In this regard, insufficient cell death may lead to proliferative disorders such as manifested by many cancers (2). Alternatively, excessive or inappropriate apoptosis has been implicated

in neurodegenerative disorders including Alzheimer's disease (51), Parkinson's disease (52) and Huntington's disease (53).

4. Apoptosis in cardiac pathologies

Historically, apoptosis has been associated only with cells progressing through the cell cycle. It was believed that terminally differentiated adult cells such as cardiac myocytes and neurons did not die by an apoptotic program. However, recent studies in animal models of heart failure have provided evidence for the incidence of apoptotic cell loss. Kajstura *et al.* were among the first to demonstrate that myocyte apoptosis, not necrosis was the predominant form of cell death following acute myocardial infarction in rats (5). Studies from the Anversa lab confirmed the incidence of apoptotic cell death following myocardial infarction secondary to coronary ligation (6). In this study they demonstrated that the incidence of apoptotic cell death, analyzed by internucleosomal DNA strand breaks, was greater in the myocardium adjacent to the infarcted area compared to regions remote from the injury (6). Using a canine model of chronic heart failure produced by multiple sequential intracoronary microembolization, Sharov *et al.* identified apoptosis in cardiac myocytes (54). Myocytes from these dogs in heart failure were located in the left ventricular region bordering the infarct. They were around 10-fold smaller than the viable myocytes and were not associated with any inflammatory response. All of these observations are characteristic of apoptotic cells. The wide spread incidence of myocardial apoptosis was further demonstrated by Anversa *et al.* in a pacing-induced model of heart failure in dogs (55). In these studies they used the TUNEL assay to identify apoptotic myocytes and further examined the morphological changes in

the chromatin structure in these cells by confocal microscopy. They report that nuclei staining positive for dUTP were also characterized by nuclear fragmentation and chromatin margination.

More recently, apoptosis has been documented in human patients manifesting several cardiac disorders including atherosclerosis (7), end-stage heart failure (10), cardiomyopathy (9) and myocardial infarction (11). In 1996, Narula *et al.* examined explanted hearts from patients having severe chronic heart failure such as ischemic and idiopathic dilated cardiomyopathy (9). Examination of cardiac tissue by DNA ladder and *in situ* dUTP labeling revealed the incidence of apoptosis in these patients. They concluded that myocytes are lost via an apoptotic program in patients with end-stage cardiomyopathy.

Olivetti *et al.* studied explanted hearts from patients having either ischemic or idiopathic dilated cardiomyopathy (56). Based on dUTP labeling, a 232-fold increase in the incidence of apoptosis was seen in the patients with congestive heart failure compared to control. Moreover, nucleosomal DNA ladders were seen in the myocardium of patients with both forms of cardiomyopathy. These results substantiated the earlier demonstration of apoptotic cell death in failing human hearts.

Further evidence for apoptosis in the myocardium was demonstrated by Saraste *et al.* upon examination of myocardial samples from patients who had died of acute myocardial infarction (11). They concluded that during re-opening of the infarct-related coronary artery, myocytes undergo apoptosis as well as necrosis.

Together these results provide compelling evidence for the occurrence of apoptosis in cardiac disorders. The induction of an apoptotic pathway in the myocardium

presents a compelling clinical issue, given the limited ability for ventricular myocytes to regenerate soon after birth. Because apoptosis proceeds through a series of checkpoints and balances, it may be subject to pharmacological interventions.

C. HYPOXIA

To date, one of the most common clinical cardiac disorders is coronary insufficiency, resulting in hypoxia. Hypoxia can be defined as the imbalance between oxygen supply to the myocyte and the amount of oxygen required (24). The most common causes of hypoxia are: 1) cardiac ischemia (ischemic hypoxia) resulting from a reduction of coronary blood flow; 2) cardiac hypoxia (systemic hypoxia) characterized by adequate perfusion with a drop in partial pressure of oxygen (pO2) in the arterial blood; 3) anemic hypoxia where there is decreased oxygen transport capacity of the blood but normal arterial pO2; and 4) histotoxic hypoxia occurring from reduced intracellular utilization of oxygen in the presence of sufficient blood flow (c.f. 24).

While hypoxia is an important component of ischemia, the two terms should not be interchanged. In addition to a reduction in oxygen supply, ischemia is characterized by a marked reduction in metabolite clearance and a drop in intracellular pH due to the accumulation of glycolysis end products. During cardiac hypoxia, the acid products of glycolysis are washed out by perfusion.

Several in *vivo* models have been utilized to simulate ischemic conditions including deprivation of serum, glucose and notably oxygen deprivation (57, 58). Normoxic cells depend on oxidative-phosphorylation for the production of ATP. Hypoxia causes the inhibition of oxidative-phosphorylation and a subsequent switch to glycolytic metabolism. As such, hypoxia results in decreased levels of high energy phosphates, lowered intracellular pH levels and increased lactic acid production. Importantly, decreased oxygen supply leads to cardiomyocyte death and their subsequent replacement by fibrotic tissue (24).

Historically, it was believed that myocyte loss due to ischemia or hypoxia proceeded by necrotic cell death. Tanaka *et al.* were among the first to show that hypoxia induces myocyte loss via an apoptotic program (58). Using an *in vitro* system in neonatal rat cardiac myocytes, they demonstrated that 24 hours of hypoxia induces DNA fragmentation visualized as a DNA ladder following gel electrophoresis. Moreover, nick-end labeling *in situ* confirmed the incidence of DNA fragmentation in myocytes, typical of cells undergoing apoptosis.

Further studies on the impact of hypoxia of neonatal rat cardiac cells were carried out by the Crow laboratory. They reported that 48 hours of hypoxia resulted in intranucleosomal DNA cleavage typical of apoptosis (27). Quantitative analysis by the TUNEL assay indicated that over 60% of the myocytes displayed evidence of end-labeling following hypoxia, compared to about 11% of normoxic controls. Of interest they noted that cardiac fibroblasts were resistant to hypoxia-mediated apoptosis.

Using a model of ischemia-induced cardiac apoptosis in the mouse, experiments from the Kitsis lab confirmed the incidence of myocyte apoptosis (59). They also concluded that apoptosis was limited mainly to the myocytes and was localized to the hypoxic regions during acute myocardial infarction.

These experiments provide compelling evidence that hypoxia induces significance damage to the myocardium. However, the molecular mechanisms governing hypoxia-mediated apoptosis are relatively unknown.

D. MOLECULAR REGULATORS OF APOPTOSIS

Research concerning the molecular mechanisms of apoptosis in mammalian cells has resulted in the discovery of several key regulators that act to promote or prevent cell death.

1. p53 and the Bcl-2 FAMILY

The tumor suppressor protein p53 has recently been implicated to play an integral role in the apoptotic process (reviewed in 38). This proposition is largely based on the finding that over 50% of all human malignancies contain mutations in the p53 gene (60). Other studies have demonstrated that fibroblasts derived from human tumors and lacking p53, were resistant to apoptosis and exhibit unlimited growth potential (61). Moreover, replacement of p53 into these cells restored growth arrest and apoptosis. In other studies, introduction of p53 in the myeloid leukemia cell line M1, which is devoid of endogenous p53, induced apoptotic cell death (62). Several genes that act to regulate the cell cycle and apoptosis are regulated by p53 transactivation. These include Mdm2 (63), p21(64), and the death promoting gene *bax* (a member of the Bcl-2 family) (65).

The Bcl-2 family to date comprises over 15 genes having either pro- or anti-apoptotic activities. Some death promoting genes include Bax, Bak, Bad, Bik and Bim, while those preventing cell death include Bcl-2, Bcl_{xl}, Bcl-W and Bfl-1 (66). Most of the Bcl-2 family members share significant sequence homology and possess at least one of four conserved motifs termed the Bcl-2 homology domains (BH1-BH4). The anti-apoptotic members contain at least BH1and BH2 and those sharing the most homology to Bcl-2 have all 4 domains. Most pro-apoptotic Bcl-2 genes possess only a short BH3

domain and are referred to as the "BH3 domain" proteins (67). A proposed mechanism for operation is heterodimerization, where pro- and anti-apoptotic family members can titrate out one another's function (68).

Evidence for the involvement of p53 in cardiac disorders is supported by studies from the Crow laboratory (27). They reported that hypoxia-mediated apoptosis was accompanied by an increase in p53 expression and transactivation. Kajstura *et al.* found increased levels of p53 and Bax in the border zone of infarcted hearts (5). While these studies and others suggest the involvement of p53 in apoptosis, a causal relationship in cardiac myocytes had not been determined.

To formally establish whether p53 is sufficient to induce cardiac cell apoptosis, our lab constructed a replication defective adenovirus encoding the p53 gene to over-express p53 in ventricular myocytes (69). Relative to cells infected with a control virus, p53 induced a 7-fold ($P<0.05$) increase in myocyte apoptosis. Moreover, in this study bax transcription was significantly increased in the presence of p53. Adenoviral delivery of bcl-2 abrogated the apoptosis induced by p53. However, the pro-survival effect of bcl-2 occurred independently of altered p53 expression, but was accompanied by a 4-fold repression of p53-dependent bax transcription. These experiments were pivotal in the elucidation of the molecular mechanisms governing apoptosis in the myocardium. These results provided the first direct evidence that bcl-2 acts as an anti-apoptotic factor in the heart.

2. CASPASES

Further elucidation of the molecular regulators of apoptosis arose from genetic studies in the nematode *Caenorhabditis elegans, which* identified several genes responsible for regulating cell life and cell death. Both *ced-3* and *ced-9* have been shown to promote cell death, while *ced-4* has anti-apoptotic properties (13).

The mammalian homologue of *ced-3* was identified as Interleukin-1 β converting enzyme (ICE) (15). To date 20 different but related members of the ICE family have been identified and are now collectively referred to as caspases (70). The term caspase stands for *c*ysteine *a*spartic-acid *sp*ecific prote*ase*, denoting the specific ability of these proteases to cleave their cellular substrates following an aspartic-acid residue (71). Caspases contain a cysteine residue located within a highly conserved pentameric sequence, which replaces the serine residue commonly found in most proteases.

Caspase cleavage of cellular proteins results in either protein activation or inactivation. The list of proteins that are targets for caspase cleavage is vast. Substrates include nuclear proteins such as lamin (72), PARP (poly-ADP-ribose-polymerase) (73) and mdm2 (74); proteins involved in signal transduction, cytoskeletal proteins including fodrin (75), gelsolin (76) and actin (77); as well as the regulatory proteins mitogen activated protein kinase (MAPK) (78) and protein kinase C δ (PKC δ) (79).

Caspases exist in cells as inactive zymogens, that are themselves cleaved at the P1 position of internally conserved Asp residues (80). The protease zymogen is composed of a pro-domain and an enzymatic region. Heterogeneity exists among the amino-terminal prodomain, suggesting important functional differences among some caspases. Caspases

including 1, 2, 4, 5, 8, 9, 10 each contain long pro-domains ~ 15-25 kDa, while caspases 3, 6, and 7 each have prodomains <5 kDa (16). The enzymatic region consists of an interdomain linker connecting a large sub-unit (~20 kDa) and small sub-unit (~10 kDa) (132). Caspase activation requires cleavage following an aspartic acid residue in the interdomain region. This releases the large and small subunit, which re-associate to form the heterodimeric, active enzyme comprising 2 small and 2 large subunits. Both the large and the small chain have components essential for the catalytic activity while the N-terminal prodomain is released (Reviewed in16,18).

Caspase precursors are activated at internal aspartic acid residues, a unique characteristic shared only with the serine protease, Granzyme B (81). Therefore caspase activation can only occur via auto-activation, cleavage by another caspase or cleavage by granzyme B.

Caspases participate in several distinct signaling pathways. They are involved in the B and T cell selection in the development of the blood system (82) and pro-inflammatory cytokine signaling (133). However, most notable is their involvement in apoptosis. Because caspases can self-activate as well as activate other caspases, it is believed they function in a hierarchical cascade of cleavage and activation (16). In this regard caspases involved in apoptosis have been classified as either upstream "initiator" or downstream "effector/executioner" caspases (17). Upstream, initiator caspases include caspase-8, caspase-9 and caspase-10, all having large pro-domains presumably containing essential regulatory regions. Effector caspases such as caspase-3, caspase-6 and caspase 7, act downstream in the cascade and have small pro-domains (1, 16, 17, 18).

Alternatively, caspases can be grouped according to the sequence surrounding the aspartic acid residue in their target substrate. Caspases 6, 8 and 9 preferentially cleave their substrate at V/L-E-T/H-D (valine/leucine-glutamate-threonine/histidine-aspartate) while caspases 3 and 7 act on proteins containing the sequence DEVD (aspartate-glutamate-valine-aspartate (134). The preferred target for caspases 1, 4 and 5 is YVAD (tyrosine- valine –alanine- aspartate) or the tetrapeptide W/L-E-H-D (tryptophan/leucine-glutamate –histidine- aspartate) (134).

Caspase activation is believed to occur through two different mechanisms. Data suggests that either a death receptor pathway (83) or a mitochondrial pathway is activated (84). Caspase activation in the death receptor pathway begins when a cell surface receptor (death receptor) is bound with its cognate extracellular death signal protein (86). Each death receptor is characterized by the presence of an 80 amino-acid chain termed the "death domain" (DD) (85). The death domain is located within the cytoplasmic region and is believed to be the critical region for pro-apoptotic activity. Following the binding of a ligand to its cognate receptor a homotrimeric complex is formed whereby intracellular adapter proteins are recruited to the cell membrane by virtue of DD-mediated protein-protein interaction (86).

These events have been well characterized for the death receptor CD95 (APO-1/Fas). Upon stimulation, the CD95 death domains aggregate and recruit 2 signaling proteins. A complex termed the death-inducing signaling complex (DISC) is formed comprising the receptor and the signaling proteins. The adapter protein FADD (MORT-1) couples through its C-terminal death domain to the DISC (87). Importantly FADD also contains another protein interaction domain called the death effector domain. The DED

of FADD recruits caspase- 8 containing an N-terminal DED within its prodomain (88). The zymogen caspase is processed to remove their pro-domains and are released as active proteases to the cytosol. A cascade of caspase activation ensues including the activation of caspase-3, 6 and 7.

3. MITOCHONDRIA

An alternate pathway for the induction of caspase activation involves the mitochondria. Several general inter-related mechanisms are known for the involvement of mitochondria in cell death. Disruptions of the electron transport system (135), oxidative-phosphorylation or ATP supply all affect cell viability (66). Alterations in cellular redox potential will lead to the accumulation of superoxide anions (35). And finally, the mitochondria release proteins capable of triggering caspase activation (103).

Under normal physiologic conditions mitochondria contain cytochrome c bound to their inner membrane. Perturbation to mitochondria results in the release of cytochrome c into the mitochondrial inter-membranous space then into cytosol through a currently unknown mechanism (14, 89). This translocation has been implicated as an important event in the progression of apoptosis. In the cytosol cyto c complexes with Apaf-1 (apoptosis-activating-factor-1), the mammalian homologue of *C. Elegans* ced-4 and also with pro-caspase-9 (20). This complex termed the "apoptosome" activates caspase-9 in the presence of dATP. Activation of caspsase-9 triggers downstream events including activation of caspase 3, culminating in apoptosis (89).

Whether release of cytochrome c from the mitochondria is a caspase-dependent event remains controversial and is actively being investigated in our lab. Several studies

have demonstrated that caspase inhibition during apoptosis induced by a myriad of stimuli including UV irradiation (90), staurosporin (90) or BAX over-expression (91), has no effect of cytochrome c release. However, during Fas-mediated apoptosis, inhibition of caspases by zVAD-fmk also prevented cytochrome c release (92). Notably, in this system cytochrome c was able to increase the number of apoptotic cells by amplifying the effects of caspase-8 activation (93).

Other defects in mitochondrial integrity provide evidence that apoptosis may proceed via a mitochondrial death pathway. A transmembrane potential, $\Delta\Psi_{m,}$ exists across the inner mitochondrial membrane due to uneven distribution of protons and other ions (96). This asymmetrical charge distribution gives rise to both a chemical (pH) and electrical gradient. The inner side of the inner mitochondrial membrane has a negative charge. By virtue of the Nernst equation, cationic lipophilic fluorochromes can distribute to the matrix thus correlating with $\Delta\Psi_m$ (94). Loss of this transmembrane potential is believed to be an early event in the apoptotic process inseparable from cell death (22). Studies have demonstrated that $\Delta\Psi_m$ occurs before morphologic changes including nuclear DNA fragmentation (22) and exposure of phosphatidyl serine (95) in the outer cell membrane leaflet. Loss of $\Delta\Psi_m$ may indicate a point-of-no return in a cell's commitment to die. Once cells have exhibited a decrease in $\Delta\Psi_m$, they still proceed to die even following removal of the apoptotic stimulus (22). These studies point to the importance of $\Delta\Psi_m$ in the apoptosis pathway.

Loss of $\Delta\Psi_m$ involves changes in mitochondrial permeability transition (MPT). (96). PT is defined as a sudden increase in permeability of the inner mitochondrial membrane to solutes <1500 Da (97). Permeability transition is caused by the opening of

a voltage–dependent, high conductance, inner membrane channel the PT pore. The pore is composed of the protein adenine nucleotide translocator (ANT) in the inner mitochondrial membrane and porin/VDAC found in the outer mitochondrial membrane (98). At contact sites between the inner and outer membranes these proteins act together to create the non-specific channel about 3 nm in diameter.

Ion equilibration within the intermembrane space occurs as a result of pore opening. Consequently, the H^+ ion gradient across the inner mitochondrial membrane is dissipated. Furthermore, pore opening results in uncoupling of the respiratory chain (99). The mitochondria experience volume dysregulation due to the increased osmolality, which results in matrix expansion. Because the inner membrane has a larger surface area due to the folded cristae, volume expansion results in outer membrane rupture (66).

A variety of agents exist which act to inhibit PT pore opening. The cyclosporin family binds to cyclophilin D associated with ANT, preventing pore opening (100). Bongkrekic acid acts as a ligand of the adenine nucleotide translocator and directly inhibits ANT preventing $\Delta\Psi_m$ loss (96). A critical role for $\Delta\Psi_m$ loss and PT pore opening has been suggested in the pathogenesis of apoptosis. Thymocyte apoptosis induced by dexamethasone and etoposide (101) can be inhibited by BA suggesting that PT may be a pivotal event in apoptosis.

The caspase activating protein, apoptosis-inducing factor (AIF) is released from the mitochondria presumably following PT opening and membrane rupture. AIF is a 50 kDa intermembrane protein (102). In contrast to cytochrome c, AIF is capable of inducing nuclear apoptosis in the absence of cytosolic components (103). Although AIF

is not classified as a caspase, its pro-apoptotic abilities can be directly inhibited by zVAD.fmk.

3. CASPASE INHIBITION

Given the important role of caspase activation in the execution of apoptosis, the ability to prevent caspase processing would be beneficial to preserving cell viability. Synthetic caspase inhibitors have been engineered which mimic the conserved aspartic acid region within the caspase active site. Aldehyde inhibitors Ac-YVAD-CHO and Ac-DVED-CHO have been engineered which act to inhibit activation of caspases related caspase-1 and caspase-3 respectively (117). Alternatively zVAD-fmk has been used as a global caspase inhibitor (122). Potential difficulties arise using these compounds due to the inability to test for their presence and their potential toxicity. Moreover whether the inhibitors are subject to degradation during prolonged exposure to apoptotic stimuli, such as chronic hypoxia, is equally unknown.

Unfortunately, current knowledge of known intrinsic proteins capable of caspase inhibition is limited. Recently a cellular homologue to viral FLIP, termed cFLIP (cellular FLICE-inhibitory-protein), has been identified (104). cFLIP overexpression was discovered in human melanoma cells and is believed to protect cells from CD95(APO-1/Fas)-induced apoptosis (104). Moreover, bcl-2 has been shown to prevent downstream caspase activation by inhibiting cytochrome c translocation (105).

Several viral inhibitors can also prevent caspase activation. Inhibitors of apoptosis (IAPs), are an evolutionary conserved family of homologous proteins with the ability to prevent cell death induced by ICE or ICE homologues (106). Recently IAP's have been

shown to selectively inhibit caspase 3 and 7 (107). Furthermore, the anti-apoptotic protein p35, a viral protein from baculovirus (108), acts as a general inhibitor of caspase activation (109). Another viral inhibitor of caspases is CrmA (cytokine response modifier A) a serpin protein from the cowpox virus. While inhibition of caspase 2, 3, 7 and 10 by crmA is weak, crmA has been shown to be a strong inhibitor of caspase 8 activation (32). CrmA has been shown to prevent apoptosis in a variety of cell types including fibroblasts (15), neurons (110), and HeLa cell (111). However, the ability for CrmA to suppress apoptosis in myocytes has never been examined. Moreover, whether CrmA is an effective inhibitor of hypoxia-mediated apoptosis is equally unknown and is currently under our investigation.

E. GENE TRANSFER

Molecular biology techniques have allowed for rapid transfer of foreign DNA into cultured cells. The ability for over-expression of genetic material has greatly contributed to our understanding of signaling pathways. However, conventional techniques utilized for gene transfer are limited with respect to cardiac myocytes (31). The technique of lipofection involves the incorporation of DNA within lipid particles to form an artificial liposome (139). Upon fusion with the cell membrane, the DNA is taken up by endocytosis. During electroporation, a process in which an electrical pulse is used to transiently open pores through which plasmid DNA can pass into the cell directly (140), the potential exists for cell damage. Other methods for gene transfer include DEAE-dextran (141), calcium-phosphate co-precipitation (142) and direct injection (143). All

above mentioned techniques are limited by a low infection efficiency of 3-10% for neonatal myocytes or lower for adult myocytes and some degree of toxicity (31).

The use of transgenic animals has been gaining increased attention in recent years. The ability to have stable over-expression of a gene in the germ line of an animal has tremendous potential in the dissociation of signaling pathways, but requires extensive experience and specialized equipment. Molecular biologists have devised several systems using viral vectors for the delivery of foreign DNA (113, 144). The retrovirus is a small RNA based virus commonly used for gene transfer. They are dependent on reverse transcription for conversion to DNA to deliver and integrate DNA into the host genome. Because the DNA can insert itself anywhere, this raises the possibility of positional mutations. Moreover, efficient gene delivery requires high titers, but retroviruses can only be grown to titers of 10^4-10^6 PFU/ml. The limiting factor for the use of retroviruses in cardiac myocytes is the requirement of a replicating cell type for retroviral delivery (144, 145).

Attention has been focused on the use of adenovirus for the delivery of foreign DNA into cardiac cells. Over 40 adenoviral serotypes are known, but types 2 (Ad2) and type 5 (Ad5) are most commonly utilized for the construction of recombinant adenoviral vectors (146). Ad5 contains linear, double-stranded DNA ~36 kbp, contained within an icosahedral, non-encapsulated virion. The early region gene (E1), containing essential sequences required for viral is removed from the Ad5 genome and replaced with exogenous, foreign DNA (113). This renders the virus incapable of replication outside of human embryonic kidney cells (HEK293) which contain the complementary E1 gene in trans. A two plasmid system for the generation of adenoviral vectors capable of

transferring foreign DNA, was engineered by Dr. F. Graham and colleagues (112, 113). One plasmid contains a bacterial origin of replication and an ampicillin resistant gene within the adenovirus genome. This 40 kbp plasmid is incapable of packaging into infectious particles. A second plasmid, often referred to as a "shuttle vector" contains overlapping adenoviral sequences with the cDNA insert of choice replacing a deleted E1 gene. Upon co-transfection into HEK293 cells, in vivo homologous recombination rescues the foreign cDNA insert into the deleted E1 region. This replication defective adenovirus is capable of infecting both replicating and non-replicating cell types with high efficiency and uniformity. There is no genetic or mutational instability upon infection as the adenoviral DNA is separate from host DNA as replicates as an episome.

The ability to over-express regulatory proteins in cardiac myocytes would have profound implications in many areas of cardiovascular sciences. Kirshenbaum *et al.* established techniques that allow both neonatal and adult cardiac myocytes to be transfected with an adenoviral vector (30, 31). Their efforts demonstrated that protein expression will occur with uniformity and high efficiency in >95% of the infected myocytes.

III. MATERIALS AND METHODS

Adult myocyte isolation

Male Sprague-Dawley rats (250-300g) were utilized for the isolation of adult ventricular myocytes (30, 114). Animals were anesthetized by an intra-peritoneal injection of ~. 07 ml/gm of a ketamine/ xylazine (1:10) mixture. Excised hearts were mounted on a modified Langendorff perfusion apparatus which allowed for switching between single-pass and recirculating perfusions at 37°C. The perfusate consisted of modified Joklik minimum essential medium supplemented with (mM) taurine, 60; glutamate, 8; carnitine, 2; magnesium, 3.4; glucose, 15; BSA 0.1%, calcium chloride, 25 μM; collagenase 0.1%, and hyaluronidase 0.1% (pH=7.4). The tissue and cell suspension was gently triturated and resuspended in culture medium M199 (Gibco, BRL).

Primary culture of adult ventricular myocytes

Freshly isolated adult ventricular myocytes were pre-plated for 1 hour to minimize contamination by non-myocytes. Myocytes were then submitted to primary culture overnight on plastic 35mm dishes precoated with laminin 20 μg/ml. Cells were plated at a density of 1 x 10^6 cells per dish in serum free media M199 (Gibco, BRL) supplemented with (mM) carnitine, 5; taurine, 5; creatine, 5; glutamine. 0.68; insulin, 1ug/ml; LiCl, 1nM; streptomycin 100μg/ml, pH 7.4. Cells were maintained in tissue culture incubators at 37°C under 5% CO_2 atmosphere (115).

Neonatal myocyte isolation and cell culture

Ventricular myocytes from 1-2 day old Sprague-Dawley rats were isolated (116). The rats were sacrificed by cervical dislocation and immediately placed in 70% ethanol. The hearts were excised and minced in phosphate buffered saline to 1-3 mm sections on ice. Cells were digested with collagenase CLSII, trypsin and DNase then purified by Percoll gradient centrifugation. To minimize contamination from non-myocytes, cells were pre-plated for 1 hour. Myocytes were submitted to primary culture and incubated overnight in Dulbecco's modified Eagle medium (DMEM)/Ham's nutrient mixture F-12 (1:1) supplemented with (mmol/L) Hepes, 17; Na2HCO3, 3; l-glutamine, 2; 50μg/mL gentamicin and 10% fetal bovine serum. Myocytes were plated at a density of 1 x 10^6 cells per 35mm dish. The following day media was removed and myocytes were transferred to serum free medium containing (μg/ml) insulin, 1; transferrin, 5; ascorbic acid, 25; 1nM Na_2SeO_4, 1nM LiCl, 1 nM thyroxine.

Hypoxia of adult and neonatal cardiac myocytes

Primary cultures of adult ventricular myocytes were subjected to hypoxia for 1 hour in an airtight chamber under conditions of constant nitrogen (95% N_2-5% CO_2) at 37°C (147). Neonatal myocytes were maintained in this environment for 24 hours (25). To achieve hypoxic conditions, myocytes were incubated in medium M199 (adult myocytes) or DMEM/F12 serum-free media (neonatal myocytes), pH 6.7, pre-gassed with 95%N_2-5% CO_2, pO_2 level <33mmHg. Myocytes held under normoxic conditions, pO_2 160 mmHg, served as controls.

Caspase inhibition

To ascertain whether a block to caspase activation would prevent hypoxia-mediated apoptosis of adult ventricular myocytes, synthetic aldehyde caspase inhibitors were utilized (117). Myocytes were pre-incubated 5 hours prior to the induction of hypoxia with 100-150 μM of the protease inhibitor Ac-DEVD-CHO (N-acetyl-Asp-Glu-Val-Asp-aldehyde) an inhibitor of caspases related to caspase-3, or with Ac-YVAD-CHO (N-acetyl-Tyr-Val-Ala-Asp aldehyde) an inhibitor of caspases related to caspase-1 (Biomol Research Laboratories). Our studies suggest this to be the optimal time and concentration to effectively inhibit caspase activation.

Recombinant Adenovirus

To inhibit caspase-activation in neonatal ventricular myocytes, we generated a replication-defective adenovirus encoding the cowpox serpin protein, CrmA (AdCMVCrmA). The 1.4 kb CrmA cDNA (generously provided by Dr. David Pickup, Duke University) (148) was excised by digestion with EcoRI and ligated into PCA3 vector to yield CrmA-PCA3. The adenovirus AdCMVCrmA, was generated by homologous recombination in HEK293 cells by co-transfection of CrmA-PCA3 and the PJM17 plasmid encoding the viral genome. The virus was harvested and propagated in human embryonic kidney cells (HEK293) as previously described (30).

The CrmA virus was diluted in DMEM/F12 serum free media and delivered to the myocytes using 20 plaque-forming units of AdCMVCrmA per cell. Myocytes were infected for 24 hours with $2x10^8$ pfu/ml of the viral stock. We have previously

established that this viral titer is sufficient to effectively deliver the gene to >95% of the myocytes (30).

Assays of apoptosis

i) Hoechst 33258 staining

Fluorescent microscopy was utilized to visualize nuclear changes associated with cells undergoing apoptosis. Adult and neonatal cardiac myocytes were grown on 12mm glass coverslips. For the identification of cardiac nuclei, myocytes were fixed in 70% ethanol and stained with sarcomeric myosin antibody MF-20 (provided by D. Bader, Vanderbilt University) followed by 10 μg/ml rhodamine-conjugated anti-mouse IgG. Myocytes were stained with (1 μg/ml) Hoechst dye 33258 (Molecular Probes, Eugene, Oregon) for the visualization of nuclear morphology. Slides were then washed with phosphate buffered saline (PBS) and mounted on glass slides (69).

ii) TUNEL analysis

To assess the incident of apoptotic nuclei *in situ*, adult myocytes were subjected to TdT mediated deoxy-triphosphate nick end labeling (TUNEL) assay (46). Myocytes were fixed in 4% paraformaldehyde then incubated at 37°C for 1 hour in TdT buffer containing 140mM sodium cocodylate, 1mM cobalt chloride, 30mM Tris –HCl, pH 7.2, 50 units of terminal deoxynucleotide transferase and 1 nmol of fluorescein-conjugated dUTP (Roche Diagnostics). Following 3 washes in PBS, coverslips were mounted on glass slides for analysis. Cell analysis was conducted on an Olympus AX70 Research Microscope equipped with excitation and emission filters set for detection of Hoechst 33258 and fluorescein isothiocyanate. Data was obtained from at least n=3 independent

myocyte cultures scoring ≥ 200 cells for each condition. Results were compared by Student's T-test, with a significance level of $P<0.05$.

iii) DNA ladder

Genomic DNA from neonatal ventricular cardiac myocytes was isolated and subjected to gel electrophoresis on 1.2% agarose gel for the analysis of nucleosomal DNA fragmentation (116).

Immunofluorescence Microscopy for cytochrome c translocation

For the *in situ* detection of cytochrome c during hypoxia, adult cardiac myocytes were fixed in 4% paraformaldehyde. Myocytes were incubated overnight at 4°C with 1μg/ml of a murine antibody directed towards mitochondrial cytochrome c (Pharmingen), followed by a 1 hour incubation with anti-mouse conjugated fluorescein IgG (0.5 μg/ml) (Roche Diagnostics).

To monitor the translocation of cytochrome c in neonatal ventricular myocytes, following adenoviral infection and hypoxia, cells were incubated with 0.1 μM MitoTracker Red CMX-Ros (Molecular Probes) for 30 minutes at 37°C for the detection of intact mitochondria. Detection of cytochrome c proceeded as described above for the adult cells. Both neonatal and adult cells were visualized using an Olympus IX 70 Research microscope equipped with an Olympus Fluoview Laser Scanning Module.

Mitochondrial and cytosolic fractionation.

Myocytes were fractionated into mitochondrial and S-100 (cytosolic) compartments for detection of mitochondrial cytochrome c release into the cytosol. In brief, myocytes were rinsed once with ice-cold PBS & glucose, then harvested in 200µl of isotonic buffer A (250mM sucrose, 20mM HEPES, 10mM KCl, 1.5mMMgCl2, 1mM EDTA, 1mM EGTA, 1mM dithiothreitol, 1mM PMSF, 8µg/ml aprotinin, 2µg/ml leupeptin, 5µg/ml pepstatin, pH=7.4). Following a 15 minute incubation on ice to allow cell swelling, myocytes were dounce homogenized 15 strokes on ice, then centrifuged twice at 750xg for 10 minutes at 4°C. Following aspiration of the supernatant and centrifugation at 10,000 x g 4°C, the mitochondrial pellet was re-suspended in 50µl of buffer A. To ensure the removal of all mitochondrial contamination, the supernatant (S-100) was centrifuged at 100,000 x g at 4°C.

Western blot analysis

For immunodetection of caspase-3 and PARP protein from adult hearts, normoxic and hypoxic cardiac myocytes were harvested in NP-40 lysis buffer (1.0% NP-40, 0.5% SDS, 150 mM NaCl, 50mM Tris-HCl, pH 7.4). Following denaturation in Laemmli buffer at 100°C, protein (50 µg) was resolved on a 12.5% SDS gel at 140V for 3h then electrophoretically transferred to a PVDF membrane. For detection of caspase-3, the filter was incubated for 3 hours, 4°C with a rabbit anti-body directed towards the p17 fragment (clone R280, 1 µg/ml) (provided by D.Nicholson, Merck Frosst Laboratories. Montreal, Canada). Detection of PARP was achieved using a rabbit polyclonal antibody

directed towards the parental 116kd and cleaved 85kd fragment of PARP (Roche Diagnostics). Proteins were visualized with 0.5 μg/ml horseradish-peroxidase-conjugated donkey antibody (Roche Diagnostics) directed against rabbit IgG.

For detection of cytochrome c, mitochondrial and cytosolic fractions were denatured as described above and resolved on a 15% polyacrylamide gel. Cytochrome c was detected with a mouse antibody directed toward mitochondrial cytochrome c (1.0 μg/ml: Pharmingen). To ensure pure separation of the fractions, immunodetection was performed using a murine antibody against cytochrome c oxidase subunit 1 (1.0 μg/ml: MP) and a murine antibody directed towards sarcomeric α-actin M4401 (1.0 μg/ml; Sigma). Proteins were visualized with 0.5 μg/ml horseradish-peroxidase-conjugated donkey antibody (Roche Diagnostics) directed against mouse IgG.

In neonatal cardiac myocytes, following adenovirus-mediated gene transfer of CrmA and 24 hours of hypoxia, cells were harvested in RIPA lysis buffer (1% Triton-X, 0.1% sodium dodecyl sulfate, 140mMNaCl, 10mM Tris and 1% deoxycholate) supplemented with a protease inhibitor cocktail. Whole cell lysates (50μg) were subjected to electrophoresis on a 12.5% SDS-polyacrylamide gel as described above. The PVDF filter was incubated overnight at 4°C with a rabbit polyclonal antibody directed towards CrmA (generously provided by Dr. David Pickup, Duke University). For detection of caspase-8 (FLICE), 1μg/ml of a anti-human mouse monoclonal antibody directed toward the 55kD parental form of caspase-8 was used (Pharmingen). Cytochrome c release into the cytosol was detected as above for adult myocytes. Proteins were visualized with horseradish-peroxidase-conjugated goat antibody (Caltag) directed

against rabbit (CrmA) or mouse (FLICE) IgG. All proteins were visualized using enhanced chemi-illuminesence (ECL) reagents (Amersham).

Fluorogenic detection of caspase activity.

Caspase-8 (FLICE) activity was assayed using ApoAlert Fluorescent Assay Kit (Clontech) as described by the manufacture. A fluorometer was set with excitation wavelength of 400 nm and emission wavelength at 505 nm. In brief, a shift in fluorescence emission of 7-amino-4-trifluoromethylcoumarin (AFC) can be detected when active capases-8 proteolytically cleaves the AFC-IETD (isoleucine-glutamate-threonine-aspartate) substrate. Data was obtained from at least n=3 independent myocyte cultures.

Mitochondrial membrane potential ($\Delta\Psi_m$) and permeability transition (PT)

To detect loss of mitochondrial membrane potential, $\Delta\Psi_m$, cells were loaded with 1 μM JC-1 dye (5,5',6,6'-tetraethylbenzimidazolylcarbocyanine iodide) or 50 nm tetramethyl rhodamine methyl ester (TMRM) (Molecular Probes, Eugene, Oregon) for 30 minutes at 37°C (23). To monitor changes in mitochondrial permeability transition (PT), ventricular myocytes were loaded with 0.4 μm calcein-acetoxymethylester (Molecular Probes, Eugene, Oregon) which will stain viable cells. In the presence of 5mM cobalt-chloride (Fisher), the cytoplasmic signal will be quenched. Viable mitochondria will be visualized as green organelles against a dark background. Alternatively, cells having an increase in membrane permeability will leak the calcein-AM from the mitochondria and appear colorless (23). Following dye loading, cells were incubated for 30 min at 37°C. All hypoxic conditions were maintained in the hypoxic

chamber under constant nitrogen pressure for the duration of the incubation period. Cells were imaged on an Olympus AX-70 Research microscope and analyzed using Image-Pro plus software to compare relative intensities for each condition relative to control cells.

IV. RESULTS

Apoptosis of adult ventricular myocytes:

Adult ventricular myocytes were subjected to 1 hour of hypoxia (95%N_2-5%CO_2, pO2<33mmHg), and assayed for the incidence of apoptotic cell death (Figure 1). Distinct morphological differences between control myocytes (panel A) and hypoxic myocytes (panel B) were detected by phase contrast imaging. While normoxic myocytes exhibit rod-shape morphology, hypoxic myocytes display cytoplasmic condensation, membrane blebbing and cell shrinkage, all characteristics of cells undergoing apoptosis. Compared to control myocytes, hypoxia invoked a 3-fold ($P<0.05$) increase in apoptotic cell death, as demonstrated by TUNEL assay. Apoptotic nuclei are tagged with fluorescein conjugated dUTP (panel D vs. panel C). A 3-fold ($P<0.05$) increase in hypoxia-mediated apoptosis was verified by Hoechst 33258 nuclear staining. Control cells are uniformly stained blue (panel E) while hypoxic myocytes display hyper-chromatic, condensed nuclei (panel F). Results are summarized in Figure 2, panels A and B.

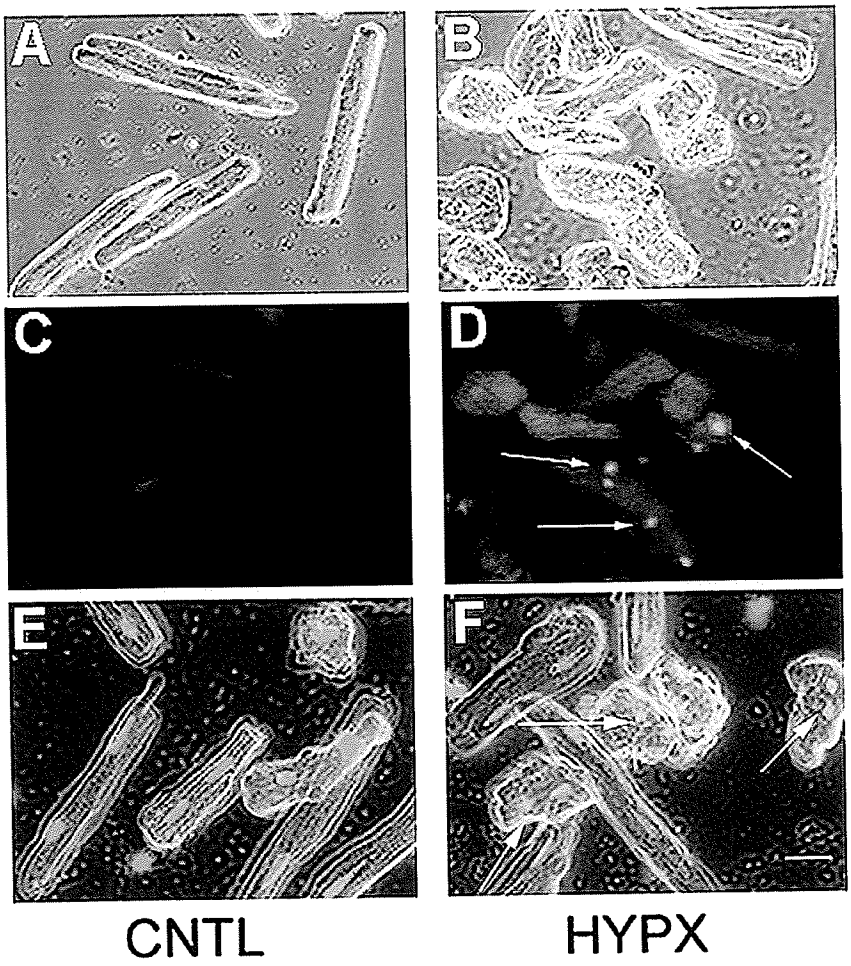

Figure 1: Hypoxia induces apoptosis of adult ventricular myocytes.

Representative microscopy images of normoxic and hypoxic adult ventricular cardiac myocytes.

CNTL (control) (A), (C) and (E) and HYPX (hypoxia) (B), (D) and (F). Phase contrast images panels (A) and (B); Fluorescent microscopy images for TUNEL assay, panels (C) and (D); and fluorescent microscopy images of Hoechst 33258 nuclear staining panels (E) and (F). Arrows point to apoptotic nuclei. Bar = 25 μm.

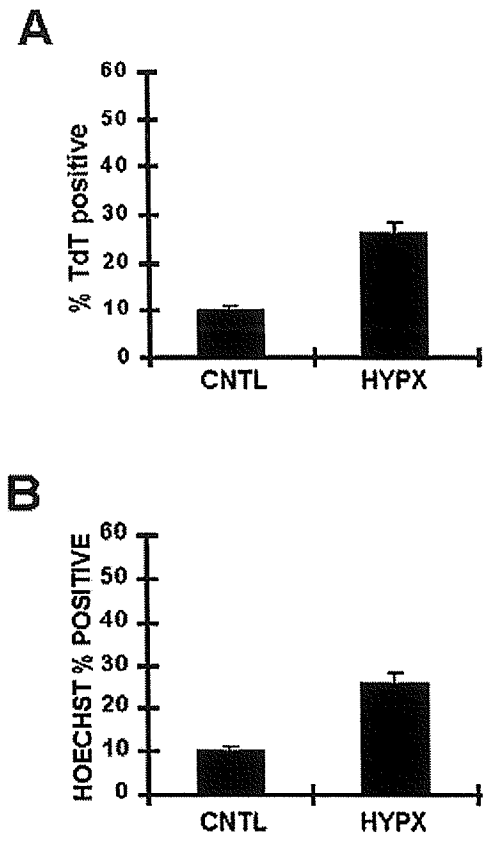

Figure 2: Histogram of cell analysis for hypoxia-mediated apoptosis

Histograms representing percent myocyte apoptosis determined by (A) TUNEL assay and (B) Hoechst 33258 staining.

Data is obtained from at least n=4 independent myocyte cultures, scoring >200 cell per condition. Data = mean ± SE ($P<0.05$).

Hypoxia induced caspase activation

To assess whether capases are activated during hypoxia, we examined the processing of caspase-3. Caspase-3 exists in cells as a 32-kDa pro-form, which is proteolytically cleaved to its active 17-kDa form by apoptotic stimuli. Western blot analysis with an anti-body specific for the active p17 fragment of caspase-3 was used on whole cell lysate from control and hypoxic myocytes. In contrast to cells held in normoxic conditions, hypoxia induced capase-3 cleavage to p17 (Figure 3, panel A). Moreover a concomitant cleavage in the caspase-3 substrate PARP from p116 to p85 preferentially occurred during hypoxia (panel B). Together these observations suggest that caspase-3 is both processed and activated during hypoxia.

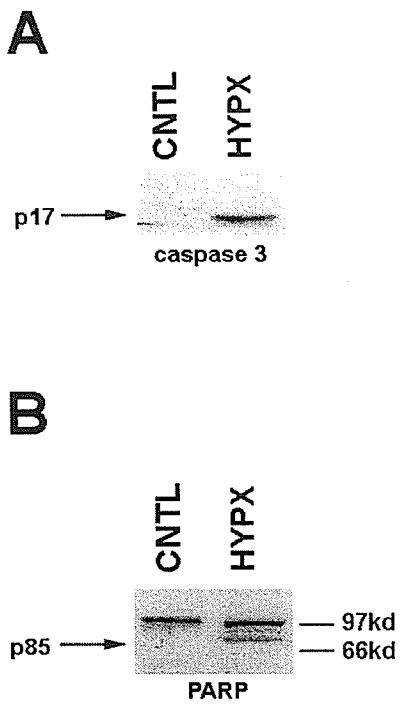

Figure 3: Hypoxia activates caspase-3.

Western blot analysis of whole cell lysate from normoxic (CNTL) and hypoxic (HYPX) myocytes. The filter was probed with an antibody directed towards the active p17 fragment of caspase-3 (panel A) and an antibody towards the 116kD parental and 85kD cleavage product of the caspase-3 substrate PARP (panel B).

Hypoxia induces mitochondrial cytochrome c release.

It has been proposed that mitochondrial release of cytochrome c is an important apoptotic event (20). To test whether hypoxia induces cytochrome c release, we fractionated normoxic and hypoxic myocytes into cytosolic and mitochondrial compartments. Western blot analysis was used to detect the presence of cytochrome c in the cytosol. In contrast to normoxia, hypoxia provoked significant translocation of cytochrome c from the mitochondria to the cytosol (Figure 4, panel A). To verify the purity of the cytosolic compartments, and to ensure the absence of mitochondrial contamination, both fractions were probed with antibodies specific for mitochondrial and cytosolic proteins. Cytochrome oxidase subunit I, a mitochondria specific protein was detected only in the mitochondrial fraction while the cytoplasmic protein α-sarcomeric actin was detected solely on the S-100 fraction (Figure 4, panel B). These results demonstrate that complete separation of the fractions was achieved during the isolation procedure and that cytochrome c detected in the cytosol is in fact due to hypoxia and not mitochondrial contamination.

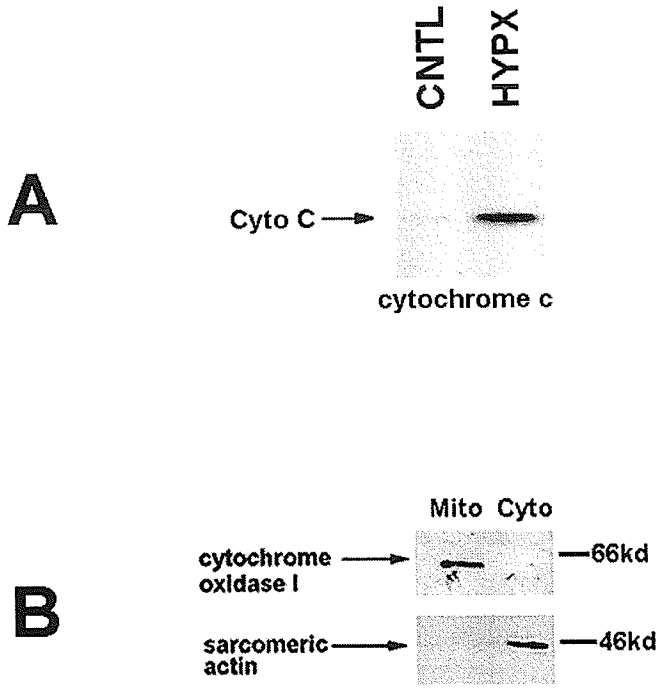

Figure 4. Hypoxia induces cytochrome c translocation.

The cytosolic fraction of cardiac cell lysate from control and hypoxic myocytes was subjected to Western blot analysis with an antibody directed towards cytochrome c. Hypoxia induces the release of mitochondrial cytochrome c (panel A).

Mitochondrial and cytosolic extracts were probed for the presence of mitochondrial and cytosolic specific markers (panel B). Upper panel demonstrates mitochondrial cytochrome oxidase I is present only in the mitochondrial not in the cytosolic fraction. Bottom panel illustrates the presence of sarcomeric actin in the cytosol and its absence in the mitochondria.

The effect of caspase inhibitors on caspase activation and mitochondrial cytochrome c release.

Since caspase activation has been implicated as an important event during apoptosis, we examined the effect of caspase inhibition on mitochondrial cytochrome c release and apoptosis. Prior to the induction of hypoxia, myocytes were treated with tetrapeptide caspase inhibitors which block the activation of caspases related to caspase-1 (Ac-YVAD-CHO) or caspase-3 (Ac-DEVD-CHO). To verify the effectiveness of these inhibitors, Western blot analysis was performed with antibodies toward active caspase-3 and its substrate PARP. Treatment of myocytes with the YVAD-CHO inhibitor prevented hypoxia mediated caspase-3 processing (Figure 5, panel A). Moreover caspase-3 activation was prevented during hypoxia with both YVAD-CHO and DEVD-CHO, demonstrated by the inhibition of PARP processing to p85 (Panel B). Lower panels depict Western blot analysis of the filters with a sarcomeric actin antibody to demonstrate equivalent levels of protein loading.

To test the involvement of caspases during hypoxia-mediated cytochrome c release, we ascertained whether caspase inhibition would effect cytochrome c translocation. The cytosolic fractions of normoxic myocytes and hypoxic myocytes pretreated with YVAD and DEVD were subjected to Western blot analysis with an antibody towards cytochrome c. The caspase inhibitor Ac-YVAD-CHO suppressed cytochrome c release during hypoxia relative to untreated controls. Interestingly, myocytes pre-treated with Ac-DEVD-CHO, inhibitor of caspases related caspase-3, failed to prevent any mitochondrial cytochrome c release and were indistinguishable from untreated hypoxic samples (panel C).

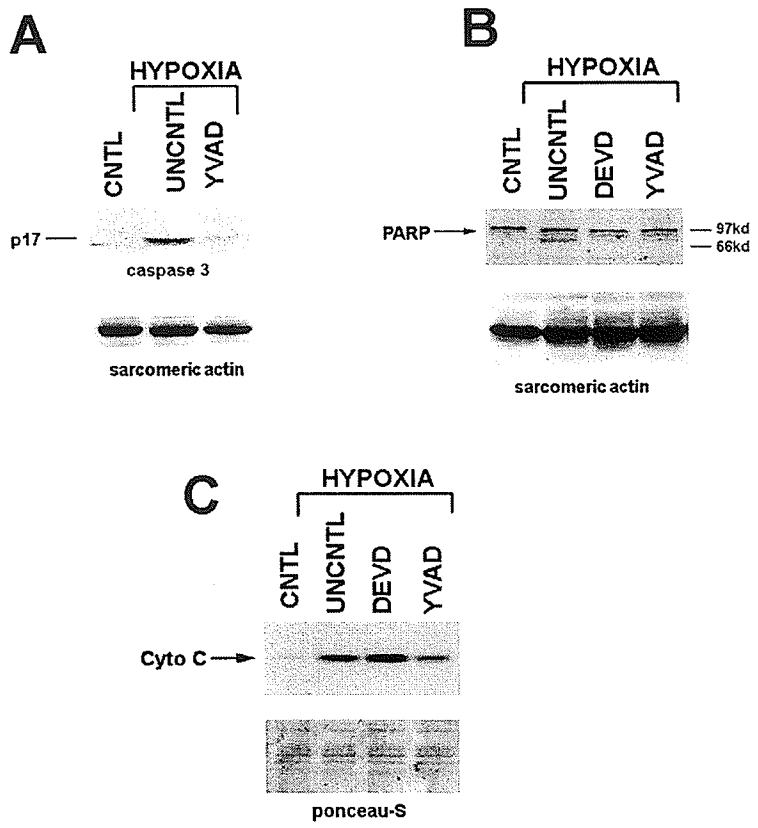

Figure 5: The effect of caspase inhibitors Ac-YVAD-CHO and Ac-DEVD-CHO on caspase activation and mitochondrial cytochrome c release.

Western blot analysis of control myocytes (CNTL), and myocytes subjected to hypoxia untreated (UNCNTL), and pretreated with the caspases inhibitors Ac-DEVD-CHO (DEVD) or Ac-YVAD-CHO (YVAD) prior to induction of hypoxia. Panel A: Caspase-3 processing during hypoxia is inhibited by pre-treatment with Ac-YVAD-CHO. Equivalent protein is verified by sarcomeric actin. Panel B: Both DEVD and YVAD prevent PARP cleavage demonstrating their effectiveness of inhibiting caspase-3 activation. Panel C: Hypoxia-mediated cytochrome c release is attenuated by pre-treatment with Ac-YVAD-CHO but not Ac-DEVD-CHO. Lower panel depicts ponceau-S staining to verify equivalent protein loading.

Caspase inhibitors attenuate hypoxia-mediated apoptosis

To test the direct involvement of caspase activation during hypoxia-mediated apoptosis, we ascertained whether caspase inhibition by Ac-DEVD-CHO or Ac-YVAD-CHO is sufficient to prevent cell death. As determined by Hoechst 33258 nuclear staining and TUNEL assay, both YVAD-CHO and DEVD-CHO significantly suppressed hypoxia-mediated apoptosis to a level comparable to control cells (Figure 6).

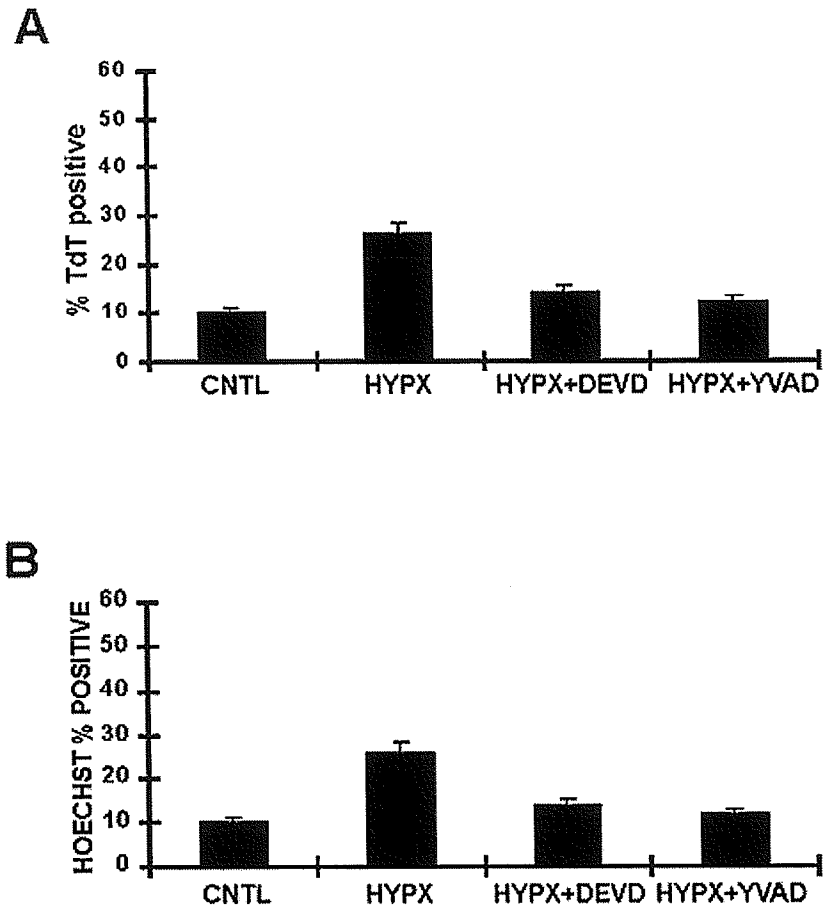

Figure 6: Hypoxia-mediated apoptosis is inhibited by caspase inhibitors Ac-YVAD-CHO and Ac-DEVD-CHO.

Histogram representation of the percentage of apoptotic cells, as determined by TUNEL analysis (A) and Hoechst 33258 nuclear staining (B). For each conditions, >200 cells were scored from at least n=4 independent myocyte cultures ($P<0.05$).

CNTL (control), HYPX (hypoxia), HYPX + DEVD (pretreatment prior to hypoxia with Ac-DEVD-CHO), HYPX + YVAD (Hypoxia plus Ac-YVAD-CHO).

Hypoxia-mediated apoptosis of neonatal cardiac myocytes

To establish that hypoxia induced apoptosis is not confined to adult ventricular myocytes, we subjected neonatal ventricular myocytes to the same hypoxic conditions utilized for adult myocytes. Consistent with other reports, we report that 24 hours was the minimal time required to induce apoptosis in neonatal myocytes (25, 27). A hallmark feature of cells undergoing apoptosis is nucleosomal fragmentation of DNA into 180-200 base–pair fragments. In contrast to normoxic cells, ventricular myocytes subjected to hypoxia displayed evidence of nucleosomal DNA fragmentation and DNA laddering, indicative of cells undergoing apoptosis (Figure 7). To confirm that the mode of cell death during hypoxia was by apoptosis, we utilized Hoescht 33258 dye to assess nuclear morphology *in situ* (Figure 8, panels A and B). Quantitative analysis revealed that 48% of hypoxic myocytes were apoptotic versus 3% of normoxic control cells ($P<0.001$) (Figure 8, panel C).

Figure 7: Hypoxia-mediated apoptosis of neonatal ventricular myocytes

Hypoxia induces nucleosomal DNA laddering following gel electrophoresis. CNTL (normoxic controls); HYPX (myocytes subjected to hypoxia).

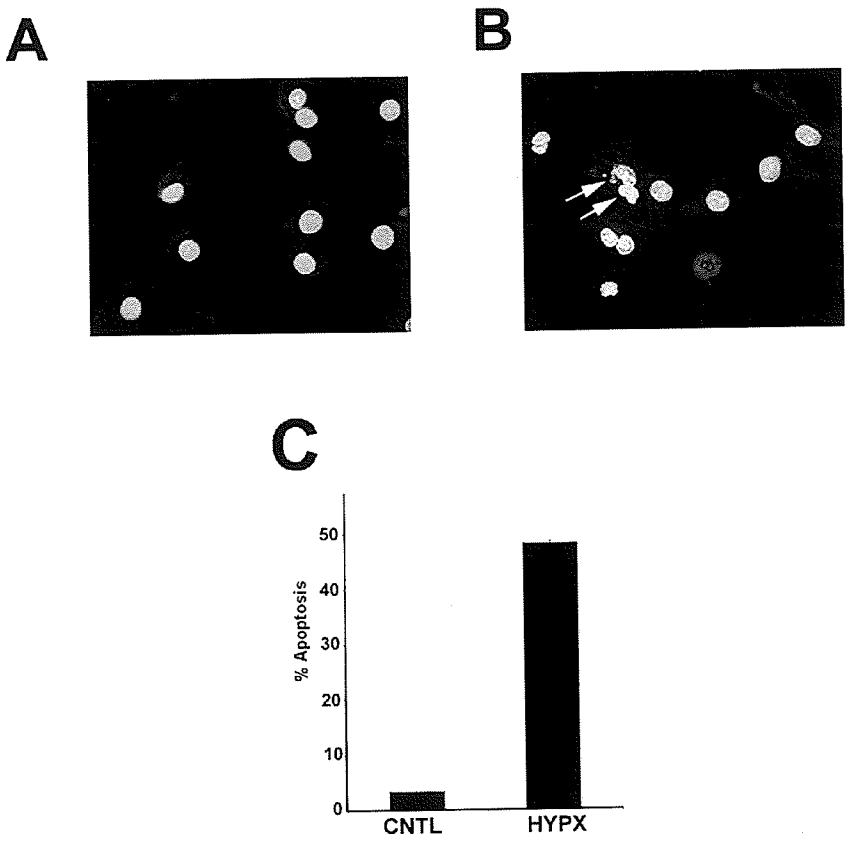

Figure 8: Quantitative analysis of hypoxia-mediated apoptosis of neonatal ventricular myocytes.

Fluorescent microscopy images of myocytes double stained for sarcomeric actin (red) and Hoechst 33258 nuclear staining (Blue).

Panel A) Normoxic control myocytes. Panel B) Hypoxic myocytes. Panel C) Histogram representing percent myocyte apoptosis determined by Hoechst 33258 staining.

Data represents mean ±SE ($P<0.001$) obtained from n=3 independent myocyte isolations with replicates of 2 for each condition counting >200 cells per condition.

Caspase-8 activation

It has been previously reported that caspase-8 represents the apical caspase in a cascade that leads to subsequent activation of down-stream caspases and apoptosis (118). To establish whether hypoxia is sufficient to provoke caspase 8 activity, we quantitatively assessed caspase-8 activity. Analysis of caspase-8 activity was measured in a fluorometric assay where the active protease cleaves the AFC-substrate conjugate emitting a yellow-green fluorescence at 505 nm. Activation of caspase-8 results in the cleavage of IETD-AFC releasing the fluorescent substrate (AFC). A 1.5 fold increase ($P<0.05$) in caspase 8 activity was observed in the hypoxic myocytes compared to normoxic control cells, indicating that hypoxia is sufficient to provoke caspase 8 activation (Figure 9).

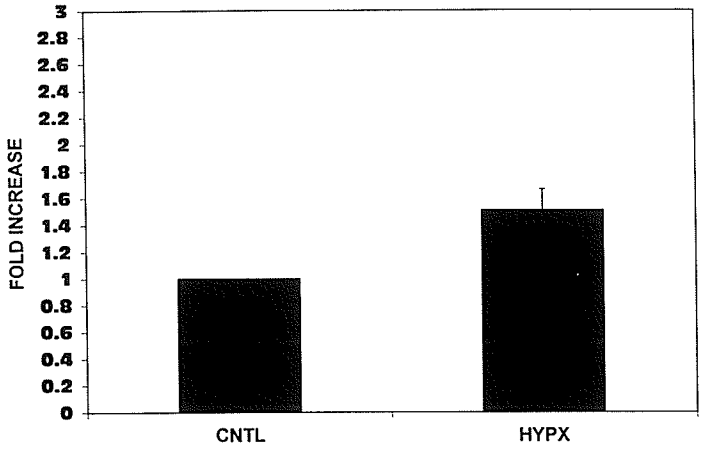

Figure 9: Hypoxia induces caspase-8 activation

Normoxic cells (CNTL); Hypoxia (HYPX)

Fluorometric analysis of caspase-8 activation during hypoxia. Data are obtained from at least n=3 independent myocyte isolations. Results are presented as percent increase from control ± SE ($P<0.05$).

Caspase inhibition by expression of the crmA gene:

To test the involvement of caspase-8 activity during hypoxia mediated apoptosis, we generated a replication defective adenovirus encoding CrmA, a serpin protein from the cowpox virus and a selective inhibitor of caspase-8 activity (32). Neonatal ventricular myocytes were infected for 24 hours with the CrmA recombinant adenovirus or incubated in serum-free media. Following exposure to hypoxia, cell lysates (50ug) were subjected to Western Blot analysis for the detection of CrmA protein. The 38kD CrmA protein was detected in the virally infected cells but not in uninfected control cells (Figure 10), confirming efficient delivery and expression of the cow pox virus CrmA protein in ventricular myocytes. Similarly, no CrmA was detected in cells infected with a control virus lacking the crmA c-DNA insert (data not shown). Importantly, hypoxia-induced capases-8 activity was significantly ($P<0.05$) attenuated in myocytes expressing the CrmA protein (Figure 11), verifying that CrmA was functionally active in myocytes and sufficient to prevent the proteolytic processing of caspase-8 during hypoxia.

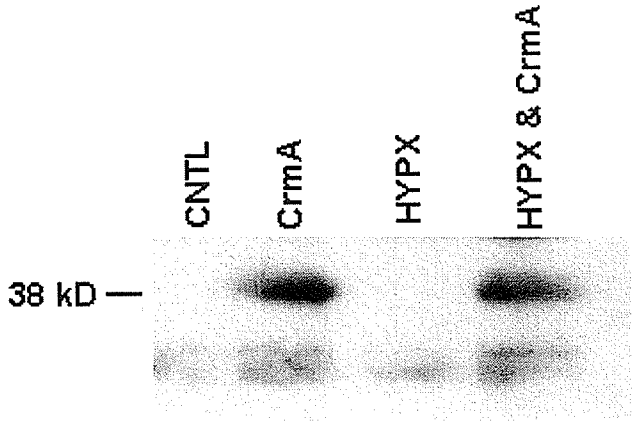

Figure 10: Expression of CrmA protein following adenoviral delivery of AdCMV CrmA

Control myocytes (CNTL); CrmA infected myocytes (CrmA); hypoxia (HYPX); myocytes expressing CrmA and subjected to hypoxia (HYPX & CrmA).

Western blot analysis with antibody directed toward CrmA demonstrating that CrmA (38kD) is efficiently expressed in myocytes infected with the AdCMVCrmA virus.

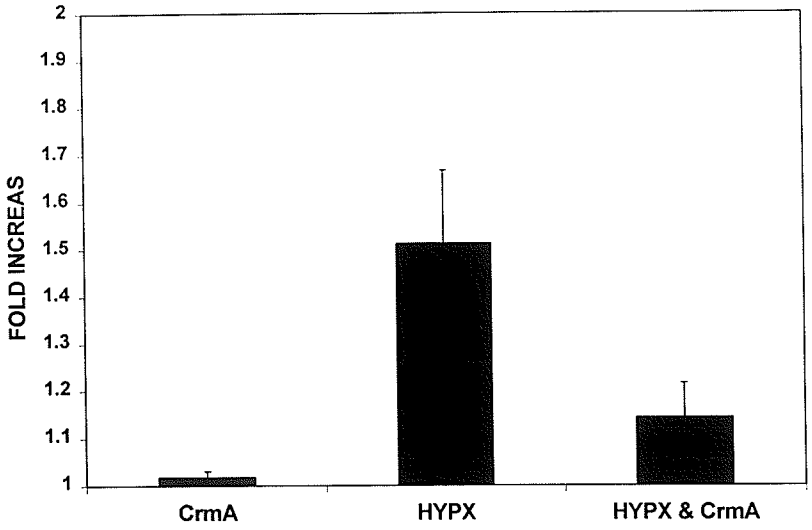

Figure 11: CrmA attenuates hypoxia-mediated caspase-8 activity.

Fluorometric analysis demonstrating that CrmA suppresses hypoxia-induced caspase-8 activity. Data was obtained from n=3 independent myocyte cultures. Results are presented as fold increase from control ± SE ($P<0.05$).

CrmA suppresses hypoxia-mediated apoptosis of neonatal ventricular myocytes

To assess a direct involvement for caspase-8 in hypoxia-mediated apoptosis we ascertained whether CrmA expression is sufficient to inhibit hypoxia-mediated apoptosis. As determined by Hoechst 33258 nuclear staining, myocytes infected with CrmA prior to the onset of hypoxia displayed only 8% apoptotic nuclei, compared 48% of untreated hypoxic cells ($P<0.05$) (Figure 12, panel A-D; summarized in panel E). Nucleosomal DNA laddering during hypoxia was also prevented by CrmA (Figure 13). Taken together these results verify that hypoxia-mediated apoptosis proceeds through a CrmA-inhibitable caspase pathway. Moreover there was no difference in the number of apoptotic cells between the control group and CrmA infected cells, verifying that adenoviral delivery is non-toxic to the myocytes.

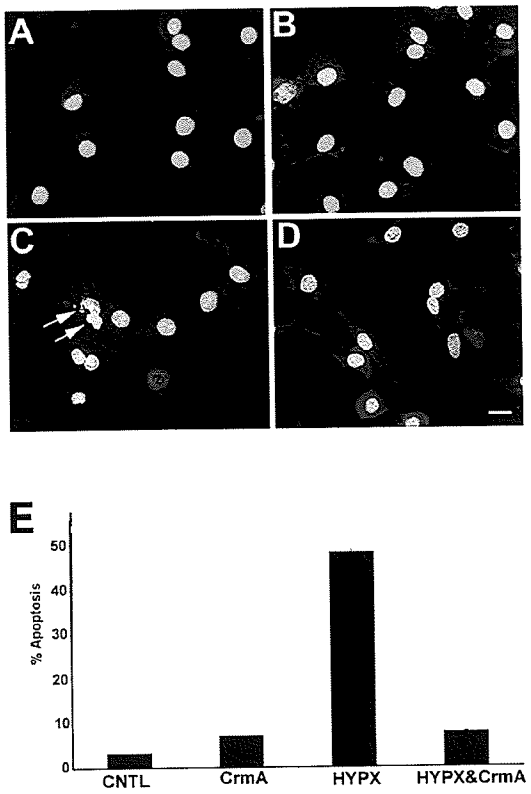

Figure 12: Adenoviral delivery of CrmA attenuates hypoxia-mediated apoptosis

Fluorescent microscopy images of myocytes double stained for sarcomeric actin (red) and Hoechst 33258 nuclear dye (Blue).

Panel A) Control myocytes (CNTL); Panel B) CrmA infected myocytes (CrmA); Panel C) Myocytes subjected to hypoxia (HYPX); Panel D) Myocytes expressing CrmA and subjected to hypoxia (HYPX & CrmA); Panel E) Histogram representing percent apoptosis following Hoechst staining. Data represents mean ± SE ($P<0.05$) obtained from n=3 independent myocyte isolations with replicates of 2 for each condition. Bar = 10 μm.

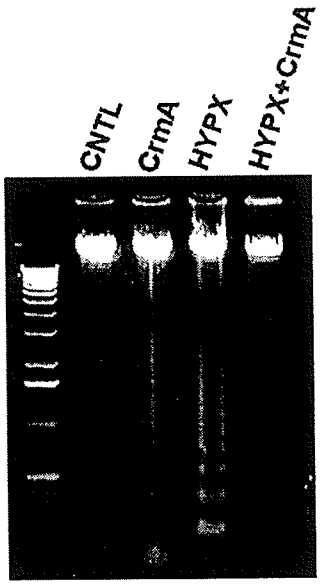

Figure 13: CrmA expression prevents nucleosomal DNA fragmentation.

Gel electrophoresis of genomic DNA demonstrating that CrmA prevents hypoxia-mediated nucleosomal laddering. Control myocytes (CNTL); CrmA infected myocytes (CrmA); Myocytes subjected to hypoxia (HYPX); myocytes expressing CrmA and subjected to hypoxia (HYPX & CrmA).

Mitochondrial cytochrome c release.

Fluorescent immunocytochemistry was used for the analysis of cytochrome c translocation during hypoxia mediated apoptosis of neonatal ventricular myocytes. Myocytes were stained with Mitotracker red CMXRos for the visualization of mitochondria (red) and an antibody directed toward cytochrome c (green) then analyzed by confocal microscopy. Control cells displayed discrete "granules" of intense green staining representative of cytochrome c (Figure 14, panel A), which were completely localized to the mitochondria (Figure 14, panel B). This was visualized as a yellow fluorescent overlay from the green (cytochrome c) and red (mitochondria) fluorescent signals (panel C). Following 24 hours of hypoxia, no discrete granules could be visualized. In contrast, the cytoplasm was diffusely stained green, representative of mitochondrial release of cytochrome c into the cytosol (panel E). Examination of the overlay (panel F), indicates that cytochrome c is de-localized from the red mitochondria, suggesting that hypoxia-mediated apoptosis of neonatal myocytes also involves the release of cytochrome c from the mitochondria. Of interest, CrmA did not attenuate mitochondrial release of cytochrome c (panels G-I). Western blot analysis confirmed these observations, demonstrating that normoxic cells have little cytochrome c in the S-100 fraction while hypoxic myocytes with or without CrmA contain increased amounts of this mitochondrial protein.

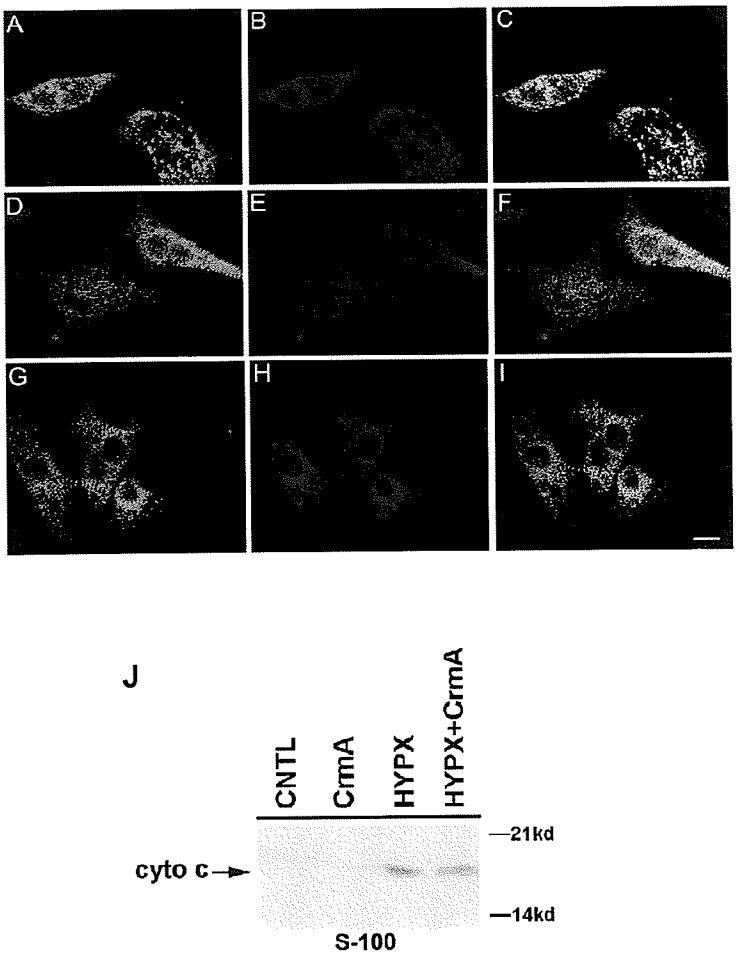

Figure 14: Mitochondrial cytochrome c release during hypoxia

Control myocytes (panels A-C); Myocytes subjected to hypoxia (panels D-F); Myocytes transfected with AdCMVCrmA and subjected to hypoxia (panel G-I).

Myocytes were probed with an antibody directed toward cytochrome c (green) (Panels A,D,G). MitoTracker CMXRos staining for mitochondria (red) (Panels B, E, H). Overlay images of cytochrome c and CMXRos (Panels C,F,I).

Panel J (Western Blot) Cytosolic (S-100) fraction of myocytes subjected to Western Blot analysis with an antibody directed toward cytochrome c. Bar=100μm.

Mitochondrial transmembrane potential

The mitochondria have been implicated to play an important role in the apoptotic pathway. A mitochondrial transmembrane potential ($\Delta\Psi_m$) exists across the inner mitochondrial membrane as a result of asymmetrical proton distribution. For normal mitochondrial function, an electrical gradient, with a negatively charged inner mitochondrial membrane is required. Loss of this transmembrane potential is believed to be an early apoptotic event (22, 94).

We tested the possibility that loss of membrane potential is associated with hypoxia-mediated apoptosis using the dual emission fluorescent dye JC-1 (Molecular Probes). At low potentials JC-1 exists as a monomer, emitting a green fluorescence. At higher potentials, JC-1 forms "J-aggregates" and a corresponding red fluorescence. Visualization of control myocytes stained with JC-1 predominantly revealed the presence of red mitochondria (Figure 15, panel A). Addition of CCCP (carbonyl cyanide 3-chlorophenylhydrazone), a protonophore that dissipates the H^+ ion gradient generated by the electron transport chain, was used a positive control to demonstrate the change in fluorescence of depolarized mitochondria. As expected, myocytes incubated with 5 μM CCCP displayed a decrease in red fluorescence and an increase in green fluorescing mitochondria typical of a low $\Delta\Psi_m$, demonstrating dye sensitivity to reduced $\Delta\Psi_m$ (Figure 15, panel B). This pattern of green mitochondria was also evident in myocytes exposed to 24 hours of hypoxia, suggesting that loss of mitochondrial membrane potential occurs during hypoxia mediated apoptosis (panel C). Of greater interest was the observation that CrmA was able to preserve membrane potential in hypoxic myocytes, visualized as an

abundance of red mitochondria (Figure 15, panel D). Red fluorescent intensity relative to control was analyzed with ImagePro Plus Software (Figure 15, panel E). These observations were further verified using TMRM, a cationic, red fluorescent dye that distributes to the negatively charged inner mitochondria membrane of polarized mitochondria (23). Hypoxia-induces a reduction in $\Delta\Psi_{m,}$ as evidenced by a loss of red fluorescence in hypoxic myocytes relative to normoxic controls (Figure 16, panels A vs.B). Infection of CrmA prior to the induction of hypoxia maintains the membrane potential indicated by the red fluorescence (Figure 16, panel C). Fluorescent intensities are summarized in Figure 16, panel E.

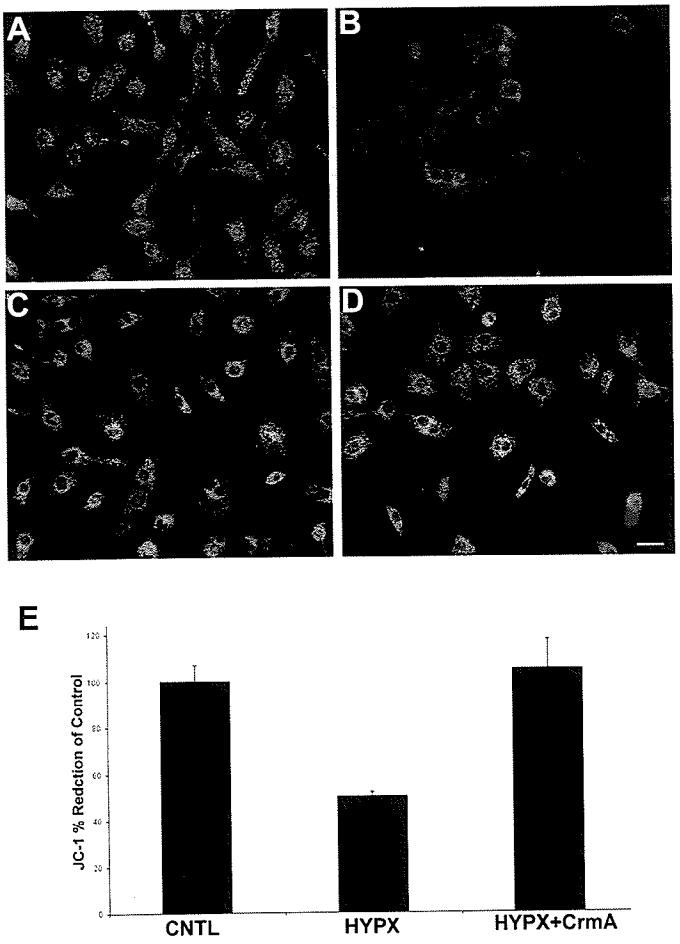

Figure 15. Hypoxia induces a loss in mitochondrial membrane potential, $\Delta\Psi_m$, indicated by JC-1 staining

A) (CNTL) Control myocytes loaded with JC-1 dye exhibit red fluorescent mitochondria indicative of high mitochondrial transmembrane potential; B) (CCCP) Dye sensitivity was tested with CCCP which abolished the transmembrane potential indicated by the loss of red fluorescence; C) (HYPX) Hypoxia resulted in the loss of $\Delta\Psi_m$, which was rescued by D) CrmA (HYPX& CrmA); E) Histogram representing percent reduction in JC-1 red fluorescence relative to control cells. Data are expressed as mean ± S.E. ($P<0.05$). Results are obtained from n=3 independent myocyte cultures counting >100 cells per condition. Bar=25μm.

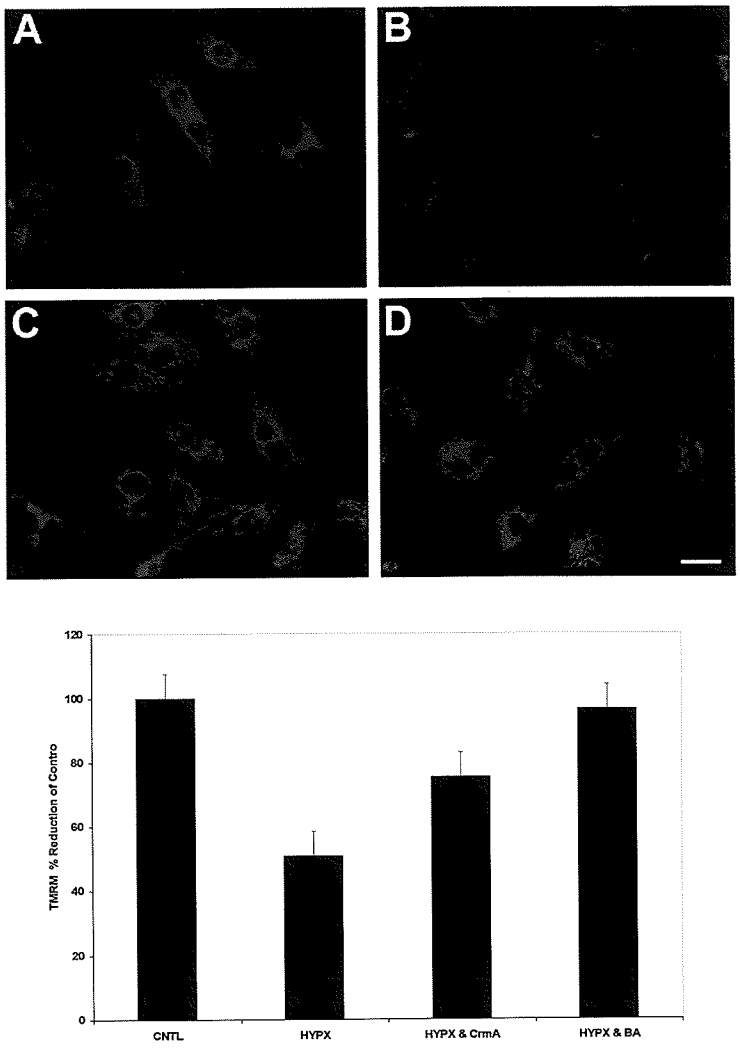

Figure 16: Collapse of mitochondrial membrane potential, $\Delta\Psi_m$, during hypoxia

Myocytes were loaded with TMRM to assess $\Delta\Psi_m$ loss during hypoxia. TMRM is a red-fluorescing dye which exclusively stains the mitochondria and is not retained upon $\Delta\Psi_m$ collapse. A) Normoxic control cell (CNTL); B) Hypoxic myocytes (HYPX); C) Myocytes expressing CrmA and subjected to hypoxia (HYPX & CrmA); D) Bongkrekic acid treated myocytes subjected to hypoxia (HYPX & BA).

Opening of the mitochondrial permeability transition pore during hypoxia

Loss of mitochondrial membrane potential often occurs following the opening of the mitochondrial permeability transition pore (PT pore). To assess the incident of PT pore opening during hypoxia, we loaded the myocytes with calcein-AM in the presence of cobalt-chloride. The AM ester form of calcein is non-fluorescent until it de-esterified inside living cells. Calcein-AM emits a green fluorescence indicative of a viable cell. Co-incubating calcein-loaded cells with Co^{+}, quenches the fluorescent signal in the cytosol. Upon imaging this method produces the appearance of green fluorescent mitochondria over a dark background. Opening of the PT pore results in the leakage of calcein from the mitochondria into the cytosol. Subsequent quenching by Co^{+} produces colorless images of the cells.

To assess whether hypoxia induces the opening of the mitochondrial transition pore, myocytes were loaded with calcein-AM in the presence of cobalt-chloride and imaged by fluorescent microscopy (23). Control cells exhibited green fluorescing mitochondria indicating the integrity of the mitochondria (Figure 17, panel A). In contrast, hypoxic myocytes emitted no fluorescent signal, indicative of an open PT and the inability for the mitochondria to retain calcein (panel B). Of greater interest was the observation that myocytes expressing CrmA and subjected to hypoxia maintained punctate green mitochondria (panel C). These observations suggest that opening of the PT pore may be regulated by a caspase-dependent pathway.

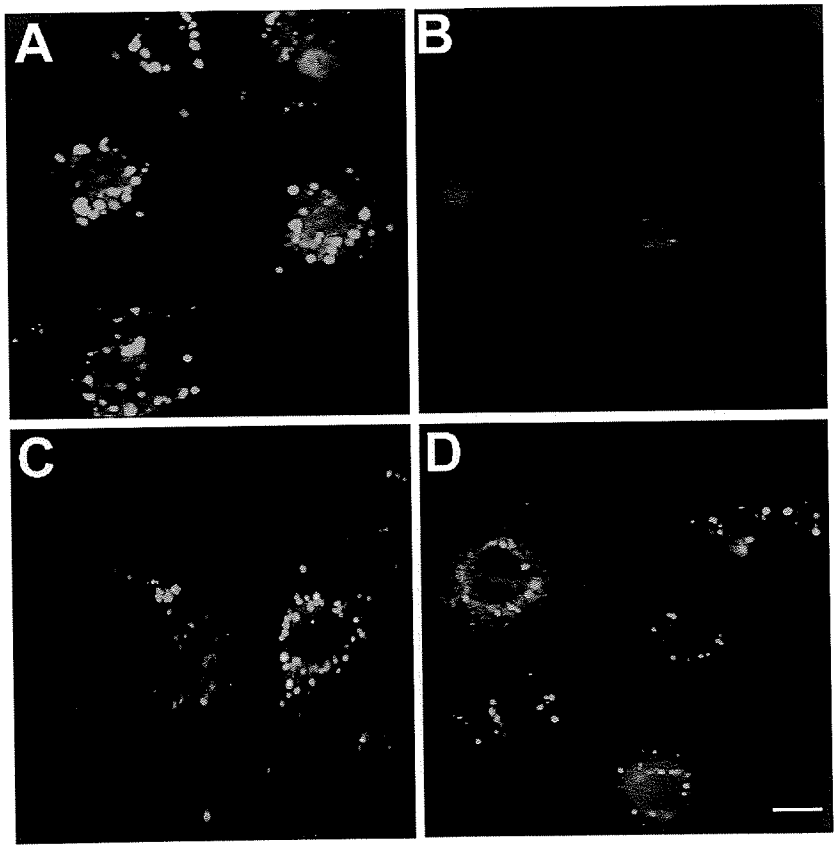

Figure 17: Mitochondrial permeability transition pore is opened during hypoxia and regulated by a CrmA-inhibitable caspase pathway.

A) Normoxic controls exhibit punctate green fluorescing mitochondria, indicating membrane integrity and the ability to retain of calcein-AM. B) Hypoxia induces the opening of PT pore and the subsequent release of calcein into the cytosol where it is quenched by Co^{+}. C) CrmA prevents the opening of PT pores during hypoxia. D) Bongkrekic acid maintains mitochondrial membrane integrity in myocytes subjected to hypoxia. Bar = 10μm.

The role of mitochondrial PT pore opening and loss of membrane potential during hypoxia

The significance of mitochondrial membrane potential loss during hypoxia is unknown. To examine the effect of $\Delta\Psi_m$ collapse during hypoxia we incubated the myocytes with 50μM Bongkrekic acid (BA), prior to the onset of hypoxia. Bongkrekic acid is a specific inhibitor of mitochondrial membrane potential loss, acting as a ligand of the ANT receptor. Analysis of cells loaded with calcein-AM and cobalt-chloride revealed that BA is sufficient to prevent pore opening during hypoxia (Figure 17, panel D). Moreover, analysis with the TMRM dye revealed that the fluorescent intensity of cells treated with BA prior to hypoxia was comparable to that of control cells. (Figure 16, panel A vs. D). These observations demonstrate that BA was effective in preventing PT pore opening and $\Delta\Psi_m$ collapse during hypoxia.

To ascertain the impact of maintaining mitochondrial membrane potential, we examined the effect of Bongkrekic acid on the incident of hypoxia-mediated apoptosis. As demonstrated by Hoechst 33258 staining for nuclear morphology, Bongkrekic acid suppressed hypoxia-mediated apoptosis to a level comparable to control cells (Figure 18). These results point to an important role for the mitochondria in the progression of apoptosis.

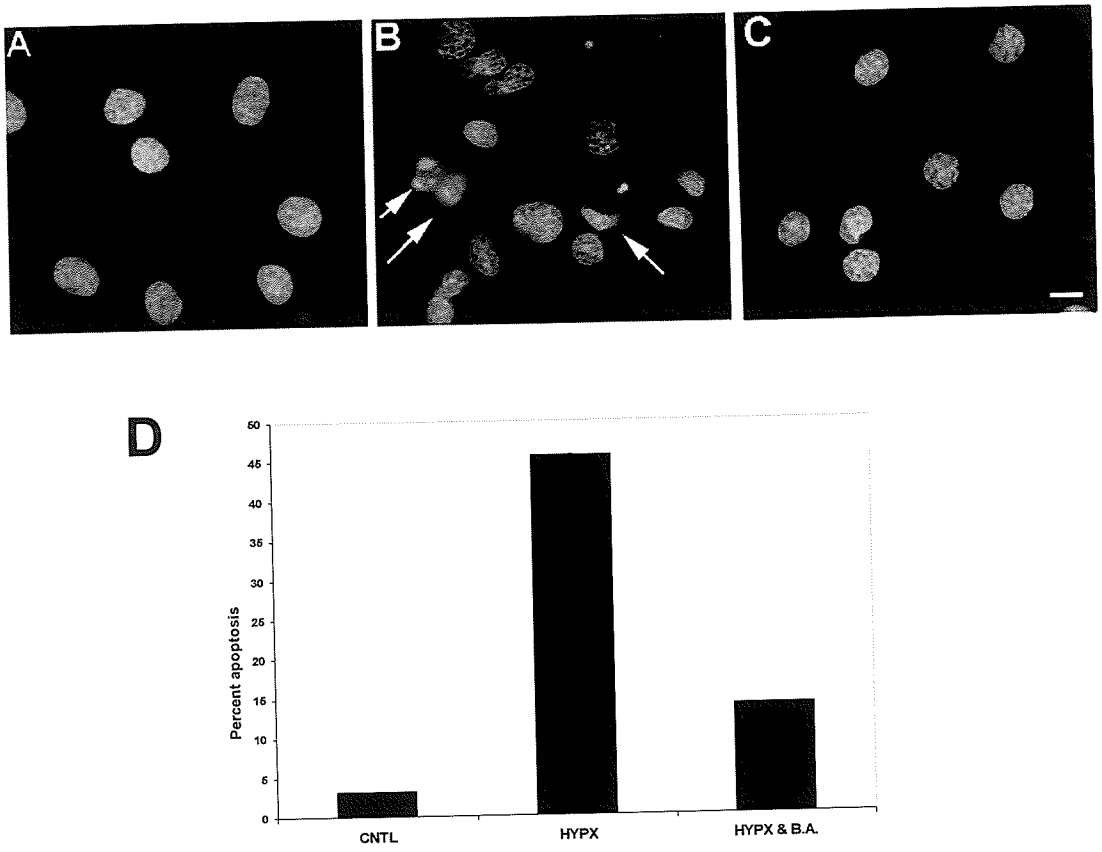

Figure 18: Hypoxia-mediated apoptosis is attenuated by preservation of the mitochondrial membrane potential.

Bongkrekic acid prevents hypoxia-mediated apoptosis. Ventricular myocytes were stained with Hoechst 33258 dye for nuclear morphology and analyzed for the presence of fragmented nuclei. A) CNTL (normoxic controls); B) HYPX (hypoxic myocytes); C) Myocytes treated with 50 μM Bongkrekic acid prior to the induction of hypoxia (HYPX & BA); D) Histogram representing percent apoptosis based on Hoechst 33258 nuclear staining. Data obtained from n=2 myocyte isolation scoring >300 cells per condition. Data expressed as mean ± S.E. ($P<0.05$). Bar = 10μm.

V. DISCUSSION

Apoptosis is a genetically conserved mode of cell death that proceeds as a coordinated series of events. Evidence suggests that the loss of viable myocytes from the myocardium proceeds via an apoptotic program (5, 56). Therefore the possibility exists to target the molecular components regulating apoptosis in disease conditions.

For these experiments we examined the impact of hypoxia on ventricular myocytes. Our findings provide evidence that hypoxia alone, without re-oxygenation, is sufficient to induce apoptotic cell death. Following 1 hour of hypoxia, nearly 30% of adult myocytes displayed evidence of apoptosis, compared to less than 10% of controls (Figures 1 & 2). It has been previously documented that neonatal myocytes are more resistant to both hypoxia and ischemia than adult myocytes both in culture and in whole heart experiments(27, 150). Consistent with other reports (25, 26), we required 24 hours of hypoxia to induce apoptosis in neonatal ventricular myocytes. Compared to normoxic myocytes, neonatal myocytes held under hypoxic conditions experienced nuclear fragmentation (Figure 7) corresponding to a 45% increase in cell death as measured by Hoechst staining (Figure 8).

Whether oxygen deprivation alone is sufficient to invoke apoptosis is a controversial topic. Gottleib *et al.* report that ischemia alone does not induce apoptosis. A reperfusion period is required to invoke apoptosis in rabbit cardiomyocytes (8). However our finding that hypoxia alone induces apoptosis in ventricular myocytes is substantiated by several reports (25, 26, 27). Importantly, all findings report that an extended period of oxygen deprivation is required in neonatal myocytes for the onset of apoptosis.

Although evidence exists for caspase activation during cardiac pathologies, a direct involvement for caspase activity during hypoxia-mediated apoptosis has not been formally tested. Following 1 hour of hypoxia in adult myocytes, we demonstrated by Western blot analysis that caspase-3 is processed and is proteolytically active, by virtue of processing the caspase substrate PARP. Pre-treatment with both Ac-YVAD-CHO (inhibitor of caspases related to caspase-1) and Ac-DEVD-CHO (inhibitor of caspases related to caspase-3) prevented hypoxia-induced activation of caspase-3. We can propose that during hypoxia-mediated apoptosis of adult myocytes, caspase-3 activity lies downstream of caspase-1 like activity. This finding is consistent with other reports stating that caspases operate in a hierarchical manner, with caspase-3 acting downstream in the pathway as an executioner of apoptosis (90,119).

Caspase-3 activation has recently been documented during several cardiac injuries. Using an in vivo model of myocardial ischemia and reperfusion in rat, Black *et al.* reported that the levels of caspase-3 were increased and localized to the ischemic/re-perfused region (120). Moreover they discovered that caspase-3 was co-localized to TUNEL positive myocytes. Clinically, Narula *et al.* found evidence for caspase-3 activity in explanted hearts from patients with end-stage cardiomyopathy (121).

An important observation from our study is that hypoxia-mediated apoptosis of adult myocytes is attenuated by both caspase inhibitors, Ac-YVAD-CHO and Ac-DEVD-CHO. This finding directly implicates the involvement of caspases during apoptosis induced by oxygen deprivation. The ability for caspase inhibitors to prevent apoptosis is consistent with a report from Yaoita *et al.* that demonstrated that zVAD-fmk effectively attenuated infarct size and apoptosis following ischemia-reperfusion injury (122).

To confirm this finding and demonstrate that it is not restricted to the adult myocardium, we assayed for caspase activation following hypoxia-mediated apoptosis of neonatal ventricular myocytes. Tanaka et al reported that 24 hours of hypoxia induced apoptosis in rat myocytes with an increase is Fas mRNA (123). Current understanding of the events surrounding apoptosis induced by the Fas receptor propose that the Fas ligand and the adapter molecular FADD recruit and activate pro-caspase-8 (124). Using a fluorometric assay kit specific for caspase-8 activity, we report that 24 hours of hypoxia induces a 1.5 fold increase in caspase-8 activity relative to normoxic controls.

It has been proposed that caspases function in a hierarchical manner with caspase-8 acting as the apical caspase (118). Caspase-8 has the intrinsic ability for auto-activation and it can also process other caspases (ex. caspase-3) *in vitro* (125). Thus, inhibiting activation of caspase-8 should prevent the initiation of a caspase-8-dependent apoptotic pathway, promoting cell survival.

The cytokine response modifier A (CrmA) is cowpox virus gene having anti-apoptotic properties. Several studies have implicated CrmA as an inhibitor of apoptosis mediated by the Fas/CD95 signaling pathway (126, 127). A study to determine the specificity of CrmA as a proteinase inhibitor revealed that CrmA does not inhibit all caspases equally and is quite selective in its substrate choice. While CrmA has the highest affinity for caspase-1, inhibition of caspase-8 activity was the next potent (32). A role for caspase-1 activity has not been established during apoptosis. Therefore, to exploit the unique property of CrmA as a caspase inhibitor, we constructed a replication defective adenovirus encoding the CrmA gene to examine the effects of caspase-8 activity in neonatal myocytes. Myocytes expressing CrmA protein suppressed caspase-8

activity relative to untreated hypoxic myocytes. Moreover, CrmA infected myocytes were more resistant to apoptosis induced by hypoxia, as shown by the absence of DNA laddering and a significant reduction in fragmented nuclei compared to hypoxia alone. That CrmA reduces the incidence of apoptosis suggests that caspases are involved in the pathway. Moreover, it also demonstrates a potential therapeutic use for CrmA in the prevention of apoptosis.

During hypoxia mediated apoptosis of both adult and neonatal cardiac myocytes, we demonstrate that mitochondrial release of cytochrome c into the cytosol occurs. This translocation is partly attenuated in the adult heart using Ac-YVAD-CHO, inhibitor of caspases related to caspase-1. However, neither the caspase-3 inhibitor Ac-DVED-CHO, nor adenoviral mediated CrmA expression had any effect on mitochondrial cytochrome c release. However, both methods of caspase inhibition effectively prevented apoptotic cell death. These observations suggest that cytochrome c release itself is not sufficient to induce apoptosis.

While several groups have reported that mitochondrial cytochrome c release is obligatorily linked to apoptosis (20, 128), there have also been reports of mitochondrial cytochrome c release where cells do not undergo apoptosis. Using UV irradiation and staurosporin induced apoptosis, Bossy-Wetzel *et al.* report that zVAD-fmk effectively prevented caspase activation and cell death, but cytochrome c translocation from the mitochondria to the cytosol was not prevented (90). Moreover, experiments using serum and glucose deprivation to induce apoptosis in neonatal myocytes confirmed that Z-VAD–fmk will prevent cell death but not cytochrome c release (57). These findings substantiate our observations from both adult and neonatal cardiac myocytes, suggesting

that a factor or event other that cytochrome c release is necessary and sufficient for hypoxia-mediated apoptosis.

To further elucidate what this event may be, we examined mitochondrial defects including mitochondrial membrane potential $\Delta\Psi_m$, and the membrane permeability transition pore (MPT pore). The role of the mitochondrial membrane potential has never been examined during hypoxia, although several groups have demonstrated its importance in apoptosis. In TNFα-induced apoptosis, Bradham *et al.* (129) demonstrated that MPT and depolarization is required for nuclear fragmentation, caspase-3 activation and cytochrome c release from the mitochondria. Bossy-Wetzel *et al.* demonstrated that cytochrome c release occurs before a reduction in $\Delta\psi_m$ in UVB-induced apoptosis. Moreover they also discovered that while zVAD-fmk prevented cell death and suppressed loss of $\Delta\psi_m$, it had no effect on cytochrome c release (90). In contrast, studies from the Greenberg lab indicate that zVAD-fmk prevented Granzyme-B induced apoptosis and cytochrome c release, but it did not affect loss of $\Delta\psi_m$ (130). These findings suggest that $\Delta\psi_m$ may not be a conserved step in the process of apoptosis and may depend on activation of upstream caspases as well as the inducing stimulus or cell type. While the Kitsis lab reported loss of $\Delta\psi_m$ during glucose and serum deprivation in cardiac myocytes, the effect on cell viability was not established (57).

In this study we report that hypoxia-mediated apoptosis of neonatal ventricular myocytes involves a loss of mitochondrial membrane potential. A reduction in the red fluorescence of mitochondria relative to control was detected in hypoxic myocytes, utilizing two independent potential sensitive dyes. We further demonstrate that the PT pore is open during hypoxia, utilizing calcein-AM in the presence of cobalt-chloride. Of

greater interest is the finding that adenoviral delivery of CrmA, a specific inhibitor of caspase-8, prevented the collapse of $\Delta\psi_m$ and PT pore opening during hypoxia. These results concur with the finding of Doug Green, in that while CrmA maintained mitochondrial membrane potential and prevented hypoxia-mediated apoptosis, the loss of cytochrome c from the mitochondria was still evident (90). Taken together these results suggest that loss of mitochondrial membrane potential is caspase-dependent event while the loss of cytochrome c from the mitochondria can occur independently of caspase activation.

To assess the direct involvement for mitochondrial integrity during hypoxia-mediated apoptosis, we treated myocytes with Bongkrekic acid (BA), a specific inhibitor of $\Delta\psi_m$ disruption, prior to the induction of hypoxia. Analysis by fluorescent microscopy revealed that hypoxia induced PT opening and loss of mitochondrial membrane potential were both prevented by BA treatment. We ascertained the effect of preserving mitochondrial membrane potential during hypoxia. Analysis by Hoechst 33258 stain for nuclear morphology revealed that Bongkrekic acid suppressed hypoxia-mediated apoptosis to a level comparable to control myocytes. This finding points to a pivotal role for the mitochondria during the progression of apoptosis. Although $\Delta\psi_m$ is regulated by caspases, direct inhibition of $\Delta\psi_m$ collapse is sufficient to prevent hypoxia-mediated apoptosis in ventricular myocytes.

IV. CONCLUSIONS

These studies focus on the examination of the molecular mechanisms governing hypoxia-mediated apoptosis in ventricular myocytes. We verified that hypoxia alone, in the absence of re-oxygenation, is sufficient to induce apoptosis in both adult and neonatal myocytes.

Western bolt analysis of adult myocytes indicated that caspase-3 and its substrate PARP are proteolytically processed during hypoxia. Hypoxia induced an increase in caspase-8 activity relative to normoxic controls, as detected by fluorometric analysis in neonatal myocytes. Furthermore, mitochondrial release of cytochrome c into the cytosol was detected in both the adult and neonatal myocytes following exposure to hypoxia. Together these findings suggest that hypoxia involves the activation of a caspases which may act through a mitochondrial death pathway.

To assess the role of caspase activation, adult myocytes were treated with the tetra-peptide caspase inhibitors Ac-YVAD-CHO and Ac-DEVD-CHO prior to the induction of hypoxia. Hypoxia mediated caspase-3 activity and apoptosis were effectively prevented by both inhibitors. This result demonstrates a pivotal role for caspases during hypoxia-mediated apoptosis. Moreover, adenoviral delivery of CrmA to neonatal myocytes inhibited hypoxia-induced caspase-8 activity and apoptosis, verifying a global role for caspase activity during hypoxia-mediated apoptosis of ventricular myocytes.

While Ac-YVAD-CHO was capable of partially inhibiting cytochrome c translocation, both Ac-DEVD-CHO and CrmA expression had no effect. The ability to

prevent apoptosis in the presence of cytochrome c release suggests that mitochondrial cytochrome c translocation may be a caspase-independent event.

A collapse in mitochondrial membrane potential and the opening of the permeability transition pore were associated with hypoxia-mediated apoptosis in neonatal ventricular myocytes. Moreover, both events were attenuated by CrmA expression, suggesting that a caspase-dependent pathway regulates these mitochondrial defects. Furthermore, direct inhibition of $\Delta\Psi_m$ loss and PT pore opening prevented apoptosis, pointing to a mitochondrial death pathway for the progression of hypoxia-mediated apoptosis.

In summary, the conclusions from these studies are:

1) Hypoxia triggers apoptosis of adult and neonatal ventricular myocytes.

2) Caspases, specifically caspase-3 and caspase-8, are activated during hypoxia.

3) Hypoxia induces mitochondrial defects including release of cytochrome c, mitochondrial membrane potential loss, and opening of the permeability transition pore.

4) Caspase inhibition by Ac-YVAD-CHO, Ac-DEVD-CHO or CrmA prevents caspase activation and apoptosis.

5) Mitochondrial cytochrome c release occurs via a caspase-independent mechanism.

6) Loss of mitochondrial membrane potential and opening of the permeability transition pore are caspase-regulated events pivotal for hypoxia-mediated apoptosis

7) Therapeutic interventions designed to prevent caspase processing may prove beneficial at preventing apoptosis during pathological states in the heart.

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