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Exploring the Non-Death Function of Caspase Activity During Cardiac Hypertrophy

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EXPLORING THE NON-DEATH FUNCTION OF CASPASE ACTIVITY DURING CARDIAC HYPERTROPHY.

by

Sergei Kalynych

A thesis submitted to the Faculty of Graduate and Post-doctoral studies of the University of Ottawa in partial fulfillment of the requirements of the degree of

Masters of Science

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I would also like to thank my dear parents for everything.

ABSTRACT

Various pathological conditions that exert stress on cardiac muscle severely compromise the supply of oxygenated blood to peripheral tissues. To maintain steady cardiac output during adverse times, the myocardium is forced to undergo tissue remodeling in a process known as cardiac hypertrophy. The loss of cardiac myocytes during the transition to heart failure has been a subject of intense investigation, and this loss is hypothesized to originate from apoptosis/programmed cell death. In particular, caspase proteases have been implicated as primary components in this process. However, caspase activity has been linked to many cellular remodeling events independent of inducing cell death. Therefore, the possibility that alterations in caspase activity precipitate the cellular alterations that give rise to pathological cardiac hypertrophy was investigated in the current study:

Using cultures of primary neonatal cardiomyocytes and a combination of pharmacological and biological inhibitors it is demonstrated that:

a.) Physical growth of a myocyte is not dependant on activity of the apoptotic caspases;

b.) Caspase activity is not necessary for the transcriptional control of natriuretic peptide production (ANF and BNP);

c.) Transcriptional activity of a general stress-responsive transcription factor Nf-*k*B is dependent on caspase enzymatic activity in the neonatal cardiomyocyte.

LIST OF ABBREVIATIONS

ANF	Atrial natriuretic factor
BNP	B-type natriuretic factor
ATP	Adenosine triphosphate
BSA	Bovine Serum Albumin
CT-1	Cardiotrophin-1
cDNA	Complementary Deoxyribonucleic acid
CASPASE	Cysteinyl-aspartate specific protease
DAPI	4,6-Diamidino-2-phenylindole
DMEM	Dulbecco's modified Eagle medium media
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
FBS	Fetal Bovine Serum
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
HDAC	Histone Deacetylase
IgG	Immunoglobulin G
JMEM	Joklik's Modified Eagle's Medium
JNK	c-Jun N-terminal Kinase
kDa	Kilodalton
Mef2	Myocyte enhancer factor 2
MHC	Myosin heavy chain
$MgCl_2$	Magnesium Chloride

Na_3VO_4	Sodium Orthovanadate
NaCl	Sodium Chloride
NaF	Sodium Fluoride
LIF	Leukemia inhibitory factor
NFAT	Nuclear factor of activated T-cells
NF-*k*B	Nuclear factor kappa B
PAGE	Polyacrylamide gel electrophoresis
PARP	Poly (ADP-ribose) polymerase.
PCR	Polymerase chain reaction
PBS	Phosphate buffered saline
PI3K	Phosphotidylinositol-3-OH kinase
PMSF	Phenylmethylsulfonyl fluoride
RNA	Ribonucleic acid
RT-PCR	Reverse-transcriptase-polymerase chain reaction
SDS	Sodium Dodecyl Sulfate
STAT	Signal Transducer and Activator of Translation
SERCA	Sarco/Endoplasmic Reticulum Ca^{2+} ATPase
TBS	Tris Buffered Saline
Wt	Wild type
Z.VAD.FMK	Benzyl-oxyl-carbonyl-Val-Ala-Asp-fluoro-methyl-ketone

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CHAPTER 1. INTRODUCTION

1.1 Heart physiology.

Survival of vertebrate animals requires that the peripheral tissues constantly receive oxygenated blood and return deoxygenated blood back to the lungs in a timely uninterrupted fashion (Srivastava and Yu 2006). Seamless blood flow circulation is accomplished by the rhythmic contractions of the heart. From an evolutionary perspective the heart is an ancient organ and all members of the animal kingdom possess analogous structures. *Drosophila melanogaster* with its open circulation system utilizes a linear heart tube termed the dorsal vessel, fish have evolved a two-chambered heart composed of a single ventricle and a single atrium, amphibians contain two atria and one ventricle (Olson 2006), while mammals have a four chambered heart, comprising two atria and two ventricles.

The mammalian heart is a highly complex organ composed of multiple cell types which make up the 4 chambers: right atria and right ventricle separated by a septum from a left atria and a left ventricle. Unidirectional flow of blood is mediated by the heart valves, which coordinately open or close with contractions of the corresponding heart chambers. Deoxygenated blood enters the right atria and is pumped into the pulmonary artery by the right ventricle. It is then carried to the lungs, where erythrocytes will become loaded with freshly inhaled oxygen. Pulmonary veins will carry newly oxygenated blood to the left atria, and high contractile force generated by the left ventricle will send the oxygenated blood into the systemic circulation through the aorta (Fig 1-1).

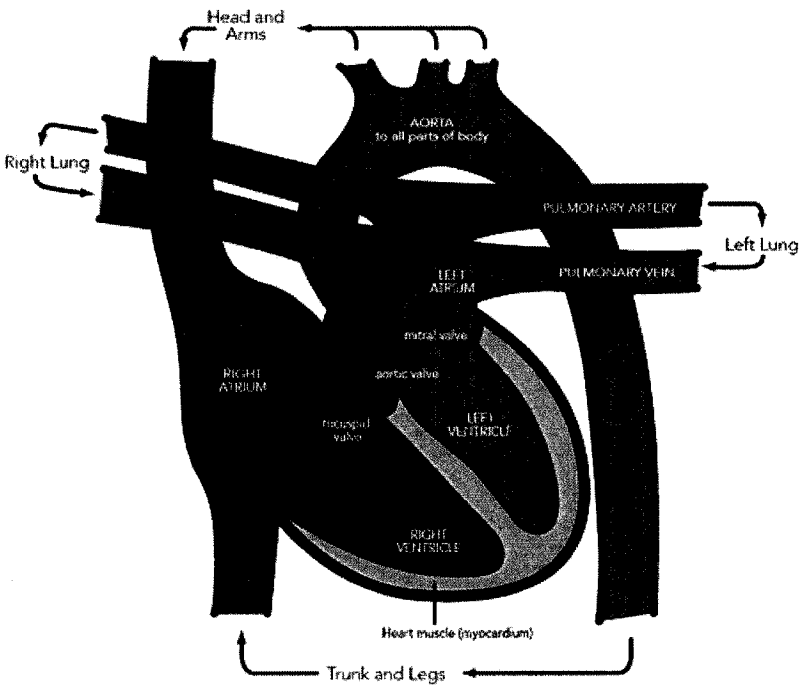


Fig 1-1. Schematic of blood flow through the four chambered mammalian heart.

The principal cell type responsible for maintaining rhythmic contraction of the heart is the cardiac muscle cell or cardiomyocyte. Similar to skeletal muscle, most of the volume of these cells is occupied by highly complex molecular motors responsible for the generation of contractile force referred to as sarcomeres. Because efficient cardiac output relies on the rhythmic contractions of the myocardium, cardiomyocytes are tightly electrically coupled, forming a syncitium that allows for free propagation of ion currents. This enables simultaneous depolarization and repolarization of the myocardium in a controlled manner, which confers a remarkable degree of precision to the timing of each heartbeat. Proper distribution of a contractile signal throughout the myocardium as well as the frequency of the heart beat itself is controlled by a network of highly specialized cells termed the cardiac conduction system (CCS) (Mikawa and Hurtado 2007; Moskowitz, Kim et al. 2007).

1.2 Hypertrophy of the heart muscle.

Most industrialized countries recognize heart failure as a leading cause of morbidity and one of the heaviest burdens on the modern health care system (Hill and Olson 2008). Although the condition represents the end-stage of various disease etiologies, an increase in left-ventricular mass known as pathological cardiac hypertrophy almost always precedes this pathology (Lips, deWindt et al. 2003).

Historically, pathological hypertrophy of the myocardium has been considered to be an adaptive response, which allows the heart to provide steady cardiac output in

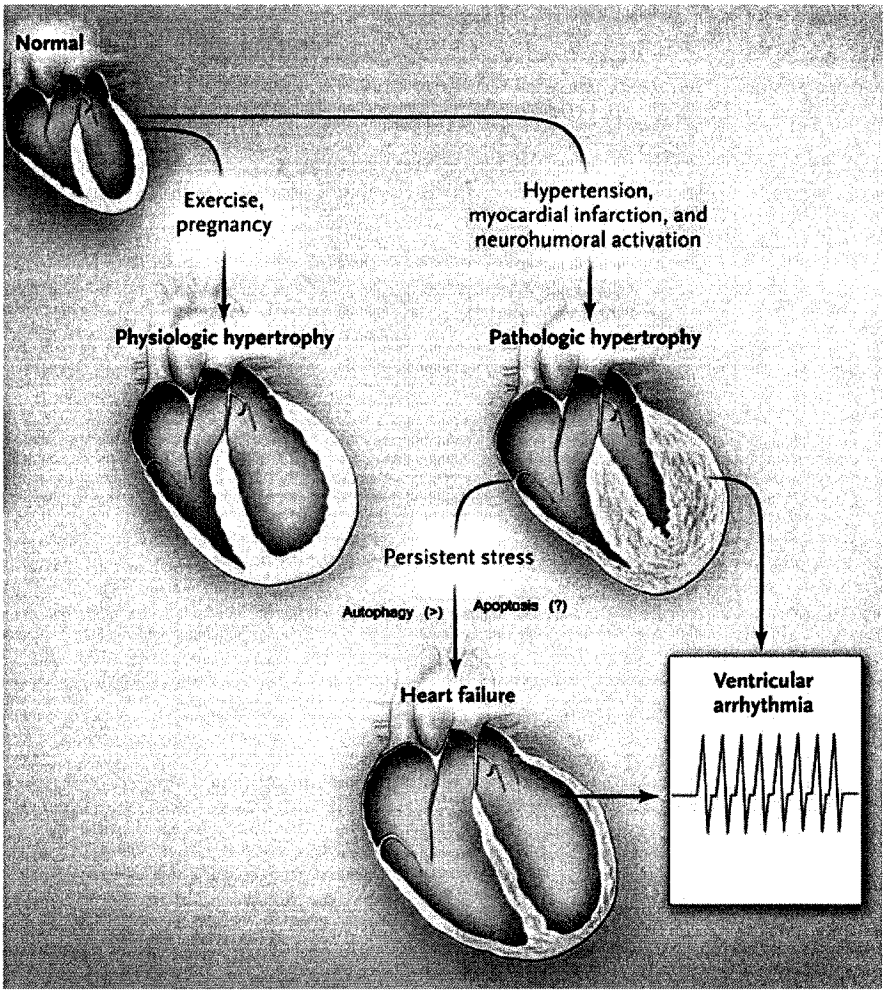


Figure 1-2. Schematic representation of the distinct changes to heart morphology that occur in response to stress (*pathological hypertrophy*) and those invoked by physiological cues (*physiological hypertrophy*) (Hill and Olson 2008).

conditions of abnormal stress. Stimuli such as hypertension, myocardial infarction, aortic stenosis, myocarditis, and mutations in genes encoding various sarcomeric components will compromise effective contractile function of the heart. Such remodeling of the myocardial tissue is accompanied by collagen accumulation and has drastic effects on the whole organ architecture: most commonly thickening of ventricular wall and septum take place without the matched increase in the chamber dimension, giving rise to so-called concentric hypertrophy (Heineke and Molkentin 2006). Long-standing hypertrophy will progress to dilation and thinning of ventricular walls associated with the cardiomyocyte loss. The mechanisms by which this transition occurs are not well understood, but both apoptosis (Olson and Schneider 2003) and autophagy (Hill and Olson 2008) have been implicated as causative agents (Figure 1-2).

In addition to disease pathology, ventricular hypertrophy may arise from various physiological processes and hence are compositely described by the term physiological hypertrophy (Dorn 2007). For example, any cardiac growth that happens post-natally must occur by an increase in size of individual cells and not by the hyperplasia, as cardiomyocytes exit the cell cycle shortly after birth and permanently lose their ability to divide ((Li, Wang et al. 1996; Dowell, Field et al. 2003). Cues that stimulate physiological hypertrophy include prolonged physical exercise, pregnancy, as well as post-natal heart growth.. While significant progress has been made in understanding molecular networks underlying pathological hypertrophy, mechanisms mediating the physiological adaptation of the heart muscle are just beginning to be unraveled. Several recent studies in rodents indicate that components of the IGF-1-PI3-Kinase signaling axis appear to play central roles in

physiologic hypertrophy (McMullen, Shioi et al. 2003; McMullen, Shioi et al. 2004; Dorn 2007; McMullen, Amirahmadi et al. 2007).

In addition to the increase in cell size, activation of hypertrophic signaling leads to several prominent hallmarks at the cellular level. Rapid growth response of cardiomyocytes is coupled to the addition of new sarcomeres to the myofibrils. Accelerated protein synthesis is thus necessary to allow for rapid production of new sarcomeric components. To accomplish this, activities of both RNA Pol. I (McDermott, Carl et al. 1991; Hannan and Rothblum 1995; Hannan, Luyken et al. 1996; Luyken, Hannan et al. 1996) and RNA Pol. III (Goodfellow, Innes et al. 2006) transcription machineries are elevated, leading to up-regulation in synthesis of large ribosomal RNAs, tRNAs, and 5S rRNAs during hypertrophic adaptation. Transcription of numerous messenger RNAs is also enhanced through phosphorylation of the C-Terminal domain of large sub-unit of RNA Polymerase II by cyclin dependant kinases (cdk9 and cdk 7) (Sano, Abdellatif et al. 2002). In addition, pathological hypertrophy is accompanied by a switch to the fetal genetic program where there is suppression of adult-specific isoforms of proteins involved in contractility, calcium handling, and metabolism and re-expression of their pre-natal-specific gene counterparts (McKinsey 2007)

With each contraction, the cytosol of a cardiomyocyte is subject to a 10-fold change in Ca^{2+} concentrations. Normally, at diastole (relaxation), excess Ca^{2+} is reabsorbed back into the sarcoplasmic reticulum by way of sarcoplasmic reticulum Ca^{2+}-ATPase (SERCA) and is pumped out of the cell by Na^{+}/Ca^{2+} exchanger (Molkentin 2006). Since Ca^{2+} is an important activator of several hypertrophic signaling modules, altering the expression of ion channels, is presumed to shift cytosolic Ca^{2+} balance towards the activation of pathways driving hypertrophic

program (McKinsey 2007). These include Ca^{2+}-calmodulin dependent protein kinases, calcineurin phosphatase, and conventional (typical) members of the PKC kinase family (McKinsey 2007).

Another important feature of the hypertrophic heart is the reliance on glycolytic metabolism (Dufour, Wilson et al. 2007). During the first postnatal week of life, a metabolic transition occurs in such a way that the preferred energy substrate is switched from carbohydrates to lipids (Alaynick, Kondo et al. 2007). From this point on, the heart satisfies the extremely high ATP demand by relying on beta-oxidation of fatty acids (Lehman and Kelly 2002). Hypertrophic signaling events alter metabolic control, making glucose the principal nutrient of choice (Alaynick, Kondo et al. 2007).

In addition to alterations in gene expression and metabolism, pathologic hypertrophy stimulates the ventricles of the heart to behave as an endocrine gland, secreting peptide hormones such as ANF (atrial natriuretic factor) and BNP (brain natriuretic factor). Natriuretic peptide hormones signal through receptors processing guanylyl cyclase activity, activating protein kinase G and primarily target kidney and systemic vasculature to regulate blood volume and blood pressure (Molkentin 2003; Hall 2004). ANF and BNP also exert a more direct effect on cardiac homeostasis: they act on cardiomyocytes themselves activating counter-regulatory mechanisms aimed at attenuating and opposing hypertrophic growth (Calderone, Thaik et al. 1998; Horio, Nishikimi et al. 2000; Kuhn, Holtwick et al. 2002).

1.3 Overview of signaling pathways controlling cardiac hypertrophic response

Hypertrophy of the left ventricle is the most accurate predictor for the development of heart failure (Lips, deWindt et al. 2003), as such much effort has been directed at understanding the molecular pathways mediating this response.

Cardiomyocytes utilize a highly complex network of signaling cascades to drive the pathological enlargement of the myocardium. Stimuli that promote cardiac growth do so predominantly by two principal mechanisms : i.) the signal is propogated through the cytosol by protein kinases to activate nuclear transcription factors; ii.) cytosolic transcriptional regulators are targetted to the nucleus via phosphorylation / dephosphorylation events (Clerk, Cullingford et al. 2007). Four MAP kinase family members are known to be the principal effectors of the first mechanism. ERK1/2, c-Jun N-terminal kinases (JNKs), p38-MAPK, and ERK5 are all activated in cardiomyocytes in response to most hypertrophic stimuli (Wang, Huang et al. 1998; Nicol, Frey et al. 2001; Bueno and Molkentin 2002) and their inhibition severely attenuates some aspects of the pathological hypertrophic response. A number of intracellular pathways have been implicated in the activation of MAP kinase modules including receptor tyrosine kinases (IGF-1 receptors (Teos, Zhao et al. 2008), FGF receptors (Kardami, Jiang et al. 2004), receptor serine/threonine kinases (TGFβ) (Watkins, Jonker et al. 2006) gp130 receptor , and G-protein coupled receptors (Clerk, Michael et al. 1998). It has also been proposed that cardiomyocytes activate $p38_{\alpha}$ and JNKs via intracellular sensors capable of detecting deformation or stretch (Nyui, Tamura et al. 1997; Sadoshima and Izumo 1997). Phosphoinositol -3 Kinase- Akt/Pkb signaling represents another important cytosolic signaling component associated with cardiac myocyte response to stress. Akt activity is thought to be necessary for the proper increase in the protein synthesis rate (via activation of the mTOR complex (Hahn-Windgassen, Nogueira et al. 2005)) as well as for the phosphorylation-dependant inactivation of GSK3-beta (Morisco, Zebrowski et al. 2000). The ultimate outcome of signal propagation through the cytosol is the activation of an ensemble of nuclear transcriptional regulators, which induce fetal gene expression.

Cardiac hypetrophy is also driven by signal-transduction pathways that enable nuclear migration of otherwise latent cytosolic transcriptional regulators such as STATS, NF-kB, and NFATs. Activation of STAT (Signal Transducers and Activators of Transcription) transcription factors primarily occurs through GP130 receptor, which responds to various IL-6-related cytokines (CT-1, LIF, etc.) (Terrell, Crisostomo et al. 2006). Cytokine binding induces activation of Jak kinases, which phoshorylate the receptor creating docking sites for STAT binding. Once recruited to the receptor, STATs are phosphorylated by Jak kinases and form active homo and heterodimers, which eventually accumulate in the nucleus (Terrell, Crisostomo et al. 2006). Various members of the Nf-κB family have also been demonstrated to be important hypertrophic mediators both in isolated cardiomyocytes (Purcell, Tang et al. 2001) and in several genetic models of hypertrophy (Dawn, Xuan et al. 2001; Li, Ha et al. 2004; Kawano, Kubota et al. 2005). Normally, RelA/p65 is sequested in the cytosol by forming a complex with IkB proteins. In response to a broad range of stress stimuli, this inhibition is relieved via Ikk-mediated phosphorylation and proteasomal degradation of IkB, allowing Nf-kB to translocate into the nucleus and to activate its target genes (Tergaonkar 2006).

Transcriptional regulators of the NFAT family represent crucial pro-hypertrophic factors and are also subject to shuttling between the cytosolic and nuclear compartments (Rao, Luo et al. 1997) . They serve as substrates for calcineurin phosphatase, which carries out dephosphorylation on selected serine and/or threonine residues. Removal of these posttranslational modifications results in conformational changes leading to exposure of the nuclear localization signal and subsequent nuclear import. Activation of this signaling pathway is proposed to occur primarily through increased cytosolic Ca^{2+} levels brought about mostly by stimulation of adrenergic

receptors of both α and β classes (Heineke and Molkentin 2006). Angiotensin-II, Endothelin-I, and α-adrenergic receptors act through trimeric $G_{\alpha/\alpha 11}$ proteins, which stimulate phosholipase C activity culminating in activation of PKC and in inositol-3-phosphate-mediated release of Ca^{2+} from the sarcoplasmic reticulum (Clerk, Cullingford et al. 2007).

The universal outcome of sustained cardiac hypertrophy is the progression to cadiomyopathy, characterized by dilation and thinning of the myocardium (Olson and Schneider 2003). This transition severely compromises cardiac output most frequently leading to heart failure. An increase in programmed cell death has been proposed to be the principal cause of cardiomyopathy, although relative contributions of apoptotic and autophagic components remain a subject of continuous investigations (Sanchis, Llovera et al. 2008). It has also been sugeested that hypertrophic signaling increases the sensitivity of cardiomyocytes to apoptosis (Kang, Yue et al. 2004). Hypertrophy induced by transgenic over-expression of the gene $G_{q\alpha}$ leads to dilated lethal cardiomyocyte in adult animals and increases apoptosis in response to isoproterenol stimulation of isolated cardiomyocytes (Adams, Sakata et al. 1998). In addition, increased cytosolic cytochrome c levels and activated caspase-3 have been detected in the hypertrophied heart muscle of guinea pigs following aortic constriction (Sharma, Dhingra et al. 2007). Furthermore, it was reported that chronic administration of caspase-3-specific chemical inhibitor (Ac.DEVD.CHO) significantly attenuates pathological heart growth in rensponse to partial abdominal aortic constriction in rats (Balakumar and Singh 2006). Interestingly, caspase-3 is also activated in human end-stage cardiomyopathy but fails to induce PARP cleavage and DNA fragmentation, which indicated the possibility of a non-apoptotic role for this protease (Narula,

Pandey et al. 1999; Haider, Narula et al. 2002).As such, caspase activation may represent a conserved feature of the pathological hypertrophic program.

1.4 Enzymatic characteristics of caspases.

Caspases are a highly conserved family of proteases, with enzymatic activity exerted through a catalytic dyad consisting of cysteine and histidine residues. These catalytic cysteines are found within the consensus sequence QA*C*xG conserved among all caspase family members (Shi 2006). Another unifying feature of this group of proteases is their exquisite specificity for targeted cleavage on the C-terminal side of aspartic acid residues (Timmer and Salvesen 2007).These cleavage sites are usually located on solvent exposed (surface accessible) loops of their corresponding targets (Mahrus, Trinidad et al. 2008)and are comprised of a 4 amino acid consensus designated as p1-p4 where p1 residue is always an aspartate most frequently followed by a glycine, serine or alanine (Salvesen and Riedl 2008). The generalized proposed enzymatic mechanism of caspase-mediated peptide bond hydrolysis is shown in Figure 1-3. The reaction can be loosely divided into two steps. First, cysteinyl thiol within the active site is deprotonated by the nitrogen of the imidazole ring of the catalytic histidine, allowing nucleophilic attack to occur on the alpha-carbon of the peptide bond. A tetrahedral acyl-caspase intermediate is formed during the first step of the reaction, followed by an interaction of an incoming water molecule to complete peptide bond hydrolysis and return the caspase protease to the initial ground state (Stennicke and Salvesen 1999).

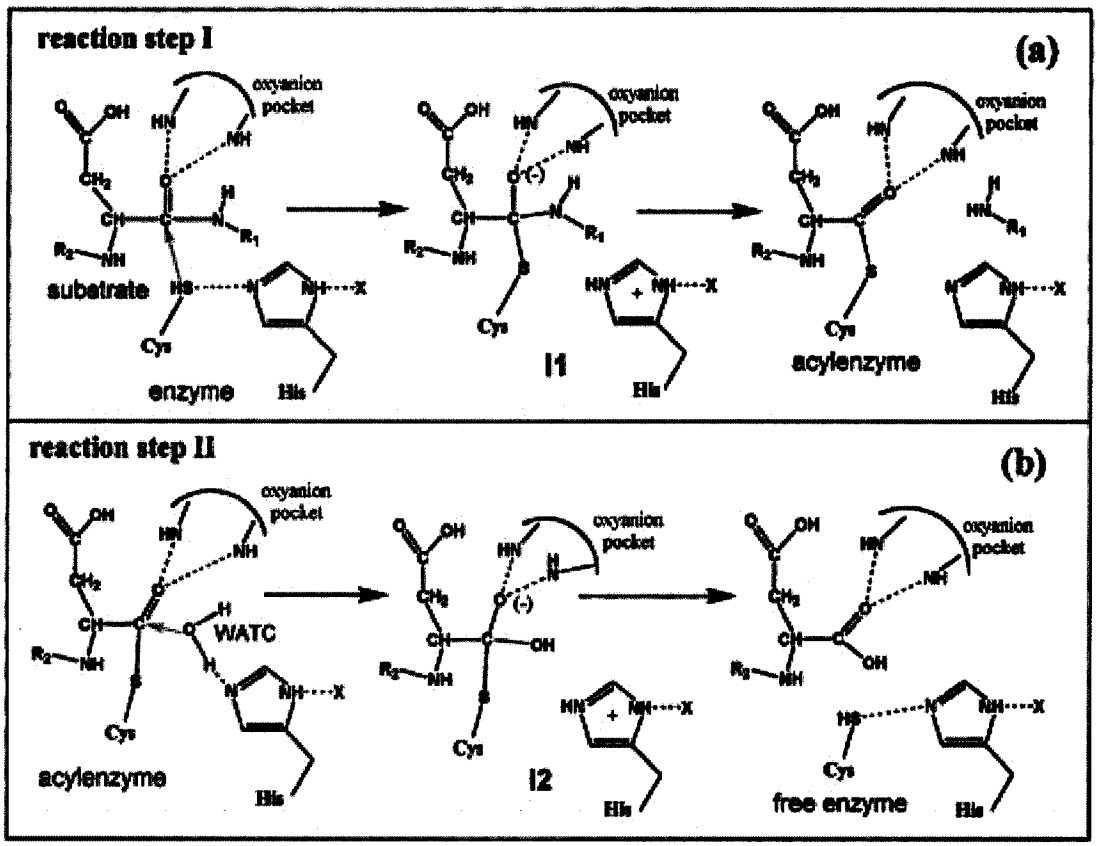


Figure 1-3. Proposed catalytic mechanism of caspase-mediated proteolysis.

(Adapted from Los and Walczak, 2002).

Traditionally, caspases have been divided into two functional groups: apoptotic and those involved in cytokine maturation, although certain members of the latter group are now also known to contribute to programmed cell death in several cell types (Stennicke and Salvesen 1999). The apoptotic caspases are further classified into the initiator and executioner sub-classes based on the hierarchical mode of activation of different family members in various forms of programmed cell death.. Initiator caspases (-2, -8, -9, and -10) exist in the cytosol as monomers with long N-terminal pro-domains. The pro-domains are required for clustering and oligomerization which are necessary for establishing enzymatic competence (Boatright, Renatus et al. 2003). Executioner caspases (-3, -6, -7) are found as obligate dimers, and their enzymatic maturation is dependant on cleavage as directed by the upstream initiator caspases. Interestingly, both classes of caspase enzymes have drastically different zymogenicity properties (the ratio of the activity of a processesed protease to the activity of the zymogen on any given substrate) (Timmer and Salvesen 2007). While executioner caspases have very high values i.e. their zymogens are very effectively constrained, initiator caspases retain enzymatic activity even in the unprocessed state, with a 10 fold variation in activity for processed and unprocessed caspase 9 (for comparison, caspase-3 is at least 10,000 times more active than its zymogen) (Timmer and Salvesen 2007). Once enzymatically mature, both initiator and executioner caspases form a tetramer consisting of two p20/p10 heterodimers (Riedl and Shi 2004) with two active sites positioned at the opposite ends of a molecule (Figure 1-4).

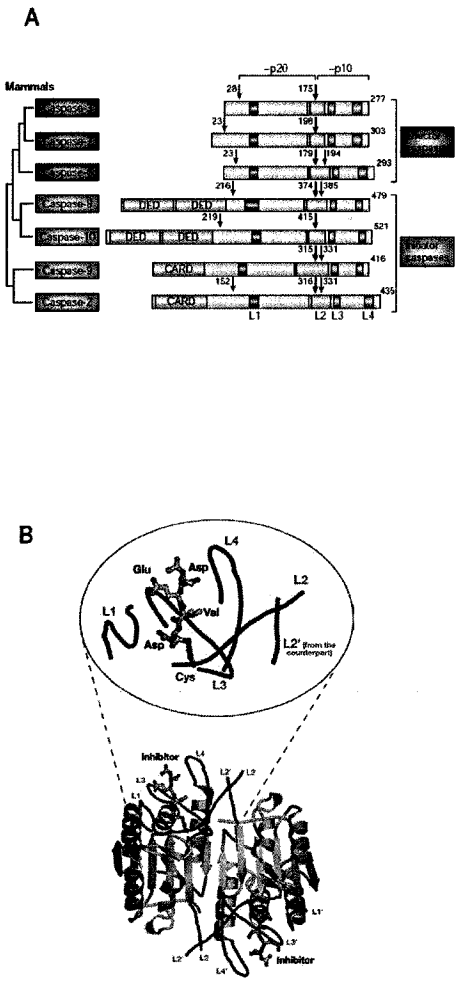


Figure 1-4. a.) Structural features of mammalian apoptotic executioner (purple) and initiator (red) caspases. Arrows indicate proteolytic cleavage sites necessary for zymogen maturation. b.) Crystal structure of a mature caspase-3 bound to a synthetic chemical inhibitor Z.DEVD.FMK. (Adopted from (Riedl and Shi 2004)).

1.5 Caspases as mediators of non-death signal transduction cascades.

Although initially discovered and characterized as apoptotic/programmed cell death components , recent observations suggest that caspase proteases influence an array of vital physiological events, including cardiac development. *Caspases 8 -/-, FADD -/- and cFLIP -/-* mice all have remarkably similar phenotypes as they fail to develop proper ventricular musculature (Varfolomeev, Schuchmann et al. 1998; Yeh, Pompa et al. 1998; Yeh, Itie et al. 2000; Kveiborg, Albrechtsen et al. 2008). Homozygous Caspase 3 -/- mice also exhibit pronounced and visible hyperplasia of neuronal tissues, which has historically been attributed to defective morphogenetic apoptosis (Kuida, Zheng et al. 1996). In addition, *Caspase-3-/-* 7-/- double mutants fail to form proper myocardium and are reported to have non-compaction of ventricular cardio-myocytes (Lakhani, Masud et al. 2006) .

These observations were initially interpreted as a requirement for caspase induced apoptosis in the normal developmental program. As such, the loss of caspase activity would lead to accumulation of cells that were normally destined to die, causing inappropriate developmental outcomes. However, more recent studies suggest that caspase activity may have beneficial cell autonomous effects independent of invoking cell death. For example, caspases are now known to be cell cycle regulators (Frost, Delikat et al. 2001; Woo, Hakem et al. 2003), promoters of cell migration (Helfer, Boswell et al. 2006) and most interestingly, indispensable components of molecular programs responsible for establishment of cell fates in various tissues (Fernando and Megeney 2007). Indeed, differentiation of a broad range of mammalian cells have been shown to be dependent on caspase activation (Fernando and Megeney 2007). These include cells that engage a differentiation program that may arguably resemble incomplete apoptosis such as keratinocytes, red

blood cells and lens fiber cells (Weil, Raff et al. 1999; Zermati, Garrido et al. 2001; Weber and Menko 2005). Moreover, other cell types such as skeletal myoblasts, neural stem cells, monocytes, ES stem cells appear to also rely on transient caspase-dependant proteolytic actions to initiate or complete their respective differentiation paths (Fernando, Kelly et al. 2002; Cathelin, Rebe et al. 2006; Fujita, Crane et al. 2008)..

Collectively, these observations suggest that caspase proteases are highly conserved regulators of various cell fate outcomes. However, the mechanism by which caspases perform such diametrically opposite tasks as differentiation and death remain unknown. One mechanism by which caspases may direct alternative cell fate choices is through the targeting of discrete signal pathways. Indeed, a striking percentage (38%) of identified caspase cleavage sites are reported to occur between various regulatory domains of substrates involved in signaling pathways (Mahrus, Trinidad et al. 2008). Furthermore, many of the substrates that have been identified to be *bona fide* caspase targets under non-apoptotic scenarios appear to be either carriers or final acceptors of biological signals such as kinases, phosphatases, and transcriptional activators/repressors. During skeletal muscle differentiation, inhibition of caspase-3 activity drastically affects cell wide kinase activity, with a specific loss in the cleavage activation of a major hub kinase Mst1, which is required for the differentiation process to occur (Fernando, Kelly et al. 2002). Several tyrosine kinases also appear to be dependent on activation by caspase-3: both Fyn & Lyn are caspase-3 substrates under apoptotic conditions in hematopoietic cells (Luciano, Ricci et al. 2001). In addition, caspase-3 has also been implicated in signaling events controlling cell cycle regulation. For example, caspase cleavage of the potent cell-cycle regulator p27Kip1 contributes to the maintenance of the proliferative capacity of

immortalized B-cells (Frost, Delikat et al. 2001). Caspases can also regulate cellular functions through cleavage of transcriptional regulators. Recently, differentiation of mouse embryonic stem cells was shown to be mediated by caspase-3-dependant inactivation of Nanog, one of the four master-regulators of pluripotency (Fujita, Crane et al. 2008). In addition, a screen conducted to identify novel caspase-8 substrates identified HDAC-7 as the most readily cleaved substrate of caspase-8 as described to date. This cleavage event completely eliminated the transcriptional repression function of this histone deacetylase family member (Scott, Fuchs et al. 2008).

Interestingly, caspases have also been shown to influence signal transduction events independent of their enzymatic activity. In these circumstances, the caspase acts as a scaffold aiding in the assembly of signaling complexes. This is best illustrated by the interaction of pro-caspase-8 with the p85α sub-unit of PI3Kinase in response to EGF treatment in NB7 neuroblastoma cell line. This interaction appears to be necessary for the activation of Rac subsequently influencing cell adhesion and motility (Senft, Helfer et al. 2007). The importance of caspase signaling scaffolds is further underscored by the evidence that pro-domains of caspases -2, -8, and -10 serve as sites of recruitment of numerous NfKappaB activators (TRAF2, RIP1, NEMO/IKK-gamma) (Chaudhary, Eby et al. 2000; Shikama, Yamada et al. 2003; Lamkanfi, D'Hondt et al. 2005). Finally, like many other signaling platforms, caspase-8 has been recently shown to be associated with lipid rafts during T-cell proliferation in response to antigen activation (Koenig, Russell et al. 2008).

1.6 Caspases as possible players in pathological cardiac hypertrophy.

Based on the collective observations outlined above, the following possibility was considered: non-apoptotic functions of some members of the caspase family may mediate the homeostasis of the adult heart and in particular contribute to signaling

events that give rise to pathological cardiac hypertrophy. Indeed, well-established hypertrophic pathways such as calcineurin-NFAT signaling, have been linked to caspase responsiveness in non-cardiac systems. Calcineurin-mediated dephosphorylation of NFAT leads to NFAT nuclear translocation, followed by interaction with other heart-specific transcription factors to re-activate embryonic genes (Molkentin, Lu et al. 1998). Interestingly, in Jurkat T-Cells, calcineurin was shown to be cleavage-activated by caspase-3 and this cleavage event was necessary for NFAT-dependent IL-2 expression (Mukerjee, McGinnis et al. 2001). Therefore caspase activation of calcineurin may be operating within the myocardium to give rise to cardiac hypertrophy.

Histone Deasetylase-4 (HDAC-4) was also reported to be a caspase-3 substrate under various apoptotic conditions (Liu, Dowling et al. 2004). HDAC-4 is a class-II histone deacetylase that is involved in the transcriptional repression of pathological cardiac genes by forming a complex with a transcription factor myocyte-enhancer factor-2 (MEF2) (McKinsey, Zhang et al. 2000). Current hypothesis states that in response to hypertrophic stimuli, HDAC-4 is phosphorylated by members of CaM-II kinase superfamily and is exported out of the nucleus (McKinsey and Olson 2004). Once liberated of HDAC-4 and -5 suppression, Mef-2 can orchestrate transcription of "pathological" cardiac genes. It is plausible to assume however, that local caspase-3 activation could result in the cleavage of HDAC-4 as an additional mechanism to engage mef2- mediated transcription. Initiator caspases may also influence activation of a general stress-responsive transcription factor NF-κB during cardiac hypertrophy, in a manner similar to that shown in other cell types. As noted previously, Nf-κB is one of the central mediators of cardiac hypertrophy in both cardiomyocyte cell culture models (Purcell, Tang et al. 2001) and in various

genetically modified rodent strains (Dawn, Xuan et al. 2001; Li, Ha et al. 2004; Freund, Schmidt-Ullrich et al. 2005; Kawano, Kubota et al. 2005). Uncovering novel mechanisms for NfKappaB activation in the heart may therefore potentially yield new opportunities for modulating certain components of the undesired hypertrophic signaling.

As noted above, a role for caspase proteases have been identified in murine heart development. *Caspase-8* null animals die at approximately embryonic day 10 and exhibit major proliferative defects of the ventricles, as indicated by the thinning of ventricular walls and defective trabeculae formation (Varfolomeev, Schuchmann et al. 1998; Kang, Ben-Moshe et al. 2004). *FADD -/-* and *cFLIP* animals all succumb to a strikingly similar phenotype seen in *caspase- 8* deleted mice, suggesting that non-apoptotic caspase function is modulated by the same group of molecules. Moreover caspase-3/-7 double null animals exhibit cardiomyocyte disarray and severe non-compaction (Lakhani, Masud et al. 2006). Importantly, however, the early lethality of these models have precluded any study or examination of caspase activity in cardiac hypertrophy.

OBJECTIVES AND HYPOTHESIS:

Lack of complete understanding of functions of caspase proteases in postnatal myocardium along with a multitude of evidence in the literature suggesting prominent roles outside of cell-death cascades prompted the question whether caspase activity may participate in the signaling events used to initiate and to sustain the hypertrophic response.

Objectives:

1.) To determine whether caspase proteases mediate events that give rise to morphological cell changes induced by hypertrophic stimuli *in vitro*.

2.) To determine whether caspase activity is implicated in the transcriptional control of prototypical markers of pathological cardiac hypertrophy.

CHAPTER 2. MATERIALS AND METHODS.

2.1 Primary Cardiomyocyte Culture.

Neonatal primary rat cardiomyocytes were isolated from the ventricles of 3-4 day old Sprague-Dawley rats (Charles River Canada, Montreal, QC) and were maintained in serum free medium of defined composition (DMEM/F12 1:1, Fatty Acid free, endotoxin free BSA (0.01 mg/mL), apotransferin, ITS Culture supplement, calcium chloride dihydrate and 100 U/mL penicillin/100 μg/mL streptomycin. Briefly, the ventricles from ten to thirty neonatal pups were excised and immediately placed in Joklik's Modified Eagles medium (Sigma-Aldrich, Oakville, Ontario) at room-temperature prior to digestion. Ventricles were minced into approximately 1 mm^3 pieces and were exposed to multiple rounds of enzymatic digestion (5X15 minutes) with Collagenase II (Worthington, New Jersey, USA) 220 U/mL with gentle agitation at 37°C. Following each round of digestion, cells contained within the supernatant were transferred to a 50 mL Falcon tube containing 5 mL of room-temperature fetal bovine serum to halt further collagenase digestion. Cells were then collected by centrifugation at 175 g for 5 minutes at room temperature and were resuspended in Dulbecco's Modified Eagle media (DMEM; Invitrogen Life Technologies, Carlsbad, CA, USA) supplemented with 10% FBS and 1X penicillin/streptomycin. Upon completion of enzymatic digestion, individual fractions were pooled and cells were collected by centrifugation and resuspended in 10% FBS in DMEM. The cell suspension was then filtered through 74 μm inserts (Costar Netwell, Corning Life Sciences, Lowell, Massachusetts) to remove any undigested ventricular tissue and debris. Subsequently, cells were differentially plated on uncoated cell culture dishes for two 30 min incubations at 37°C to remove fibroblasts. The resulting cardiomyocyte fraction was counted and plated on collagen-I pre-coated 6-well dishes (Becton Dickinson, Bedford, MA, USA) at the following concentrations: luciferase

assays - 350 x 10^5 cell/well, caspase assays - 250 x 10^5 cells/well, immunocytochemistry - 150 x 10^5 cell/well. Cardiomyocytes were allowed to recover in 10% FBS in DMEM for a period of 16-20 hours, at which time the media was replaced with serum-free media. Cardiomyocytes were serum-starved for 48 hours, with fresh SF media added every 12-24 hours prior to stimulation with either 100 μM phenylephrine (Sigma Aldrich, Oakville, Ontario) or 10% FBS (Invitrogen Life Technologies, Carlsbad, California). Adenoviral infections were carried out the day after the isolation at multiplicity of infections of 25 infectious particles per cell (MOI=25) followed by a 24-48 hour period to allow for transgene expression. Whenever used, caspase inhibitors were applied at final concentrations of 20 μM, Cyclosporin A was used at a concentration of 1 μg/mL, and rapamycin was used at concentration of 200 nM.

2.2. RNA Extraction, RT-PCR, and Real-Time PCR.

Total RNA was collected from primary cardiomyocytes using the Qiagen RNAeasy RNA purification kit following manufacturer's instructions (Qiagen, Mississauga, Ontario). Total RNA from adult hearts was purified using TRIzol Reageant (Invitrogen Life Technologies, Carlsbad, California). Briefly: hearts were excised from adult female Sprague-Dawley rats and snap frozen in liquid nitrogen in a total volume of 6 mL of TRIzol. The frozen hearts were individually homogenized for 5 X 30 second intervals using a mechanical homogenizer and were allowed to stand at room temperature for 5 minutes. 1.2 mL of chloroform (Fisher Scientific, Ottawa, Ontario) was added to each sample followed by centrifugation at 4°C for 25 minutes at 4,000 rpm in the table-top centrifuge. The top aqueous layer was removed and RNA was precipitated by adding an equal volume of ice-cold iso-propanol followed by an overnight incubation at -80°C. The next day samples were washed in 80%

Ethanol (made up in 1mg/mL DEPC-treated dH_2O) and Resuspended in RNAse-free water (Qiagen, Mississauga, Ontario) at concentrations of 1 μg/μL.1 microgram of RNA isolated either from each individual heart or a well of a collagen-coated 6-well culture dish was reverse transcribed into its complementary DNA (cDNA) using Superscript II Reverse Transcriptase, according to manufacturer's instructions (Invitrogen Life Technologies, Carlsbad, California). cDNA samples were then subjected either to PCR using Taq.DNA polymerase and were visualized on 2% TAE Agarose Gels. Alternatively, cDNA was used as a template in quantitative PCR reactions using SybrGreen Dye (SuperArray Biosciences, Frederick, Maryland). Integrity of the cDNA template as well as the presence of equal amounts in different samples was confirmed using rat GAPDH expression, which was also used as an internal calibrator in the qPCR reactions. To control for genomic DNA contamination, an aliquot of total RNA pool has been subjected to PCR amplification. Primer 3™ (Massachusetts Institute of Technology) software was used for design of all the primers listed in Table 1.

Table 2. List of primers used for amplification of indicated transcripts in Reverse Transcription PCR or quantitative Real-Time PCR.

Gene	Primer Sequences
Caspase-1	F-TATGGAAAAGGCACGAGACC R-CGCCACCTTCTTTGTTCAGT
Caspase-2	F-CCAGCAGCTCTATGTTCC R-CCAGCAGCTCTACCTGTTCC
Caspase-3	F-GGACCTGTGGACCTGAAAAA R-GCATGCCATATCATCGTCAG
Caspase-4	F-GCACATCTGGAGTTGCTTCA R-TGGTGGTGAAAGAGGAGCTT
Caspase-6	F-CTTCTGGCACCTAGCACTCC R-TGGCTCAGGAAGACACACAG
Caspase-7	F-TACTGTGAGCATGGCCTCTG R- CAACAGCCACCTTTTCCAAT
Caspase-8	F-TGAAGGAGCTGCTTTTCCAT R-ATCAAGCAGGCTCGAGTTGT
Caspase-9	F-AAGACCATGGCTTTGAGGTG R-CAGGAACCGCTCTTCTTGTC
Caspase-11	F-TGGTGGTGAAAGAGGAGCTT R-GCACATCTGGAGTTGCTTCA
Caspase-12	F-GGTTCTCAGCTTTGCTCAGG R-GCTGCCAAGAGAACACATGA
Caspase-14	F-GATGCCCTAGAACGCATGTT R-TGAGTACCACGAAGGCACAG
ANF	F-GGGGGTAGGATTGACAGGAT R-CTCCAGGAGGGTATTCACCA
BNP	F- GACGGGCTGAGGTTGTTTTA R- ACTGTGGCAAGTTTGTGCTG
GAPDH	F- GGCATTGCTCTCAATGACAA R- TGTGAGGGAGATGCTCAGTG

Quantification and processing of quantitative Real-Time PCR data was carried out as outlined in appendices section (See Appendix D for calculations used).

2.3 Recombinant Adenovirus Production and Titration.

Full-length p35 cDNA encoded by *Californica Autographica* baculovirus was provided by Dr. Kazuhiro Sakamaki (Institute for Virus Research, Kyoto University, Kyoto, Japan) in pIRES2 eGFP Vector (Clonetech, Mountain View, California). The 1.7kb DNA fragment including the p35 coding sequence, internal ribosome entry site from the virus, and eGFP was digested out of this plasmid using EcoRI and NotI, gel purified using Illustra DNA purification Kit according to manufacturer's instructions,

and sub-cloned into pShuttleCMV (Stratagene, San Diego, California) using blunt-end cloning. The sequence was confirmed by appropriate diagnostic restriction enzyme digests and sequence analyses (StemCore Core Facility, Ottawa, ON, Canada). P35_IRES_eGFP fragment was inserted into E5/E3 deficient adenoviral genome by recombination in *RecA* + BJ5183 *E.coli* strain. PShuttleCMV_p35_IRES_eGFP was linearized using *PmeI*, gel purified, using Illustra DNA purification Kit (Chemicon, Temecula, California) and electroporated into *E.coli BJ5183* cells stably transformed with adenoviral genome. Proper recombinants were identified by *PacI* diagnostic digests. Selected clones were re-transformed into DH5α *E.coli* strain and sufficient quantities of DNA were purified using cesium chloride gradient ultracentrifugation. Primary virus stock was generated by linearizing the plasmid encoding E-1 deficient recombinant adenoviral genome using *PacI* and transfecting 4 μg into sub-confluent HEK293 cells using Lipofectamine 2000 as outlined in **section 2.7**. Following, 14 days of viral packaging, cells were harvested and frozen overnight at -80°C in 10% v/v sucrose. Primary stock was further amplified by subsequent re-infections of HEK293 cells. Concentrated high titer virus was purified using cesium chloride banding and virus titration was determined by using Absorbance at A260 (see appendix II for details).

2.4 Immunocytochemistry and morphometric analysis.

Neonatal rat ventricular cardiomyocytes isolated as described above and cultured on collagen I coated 20 mm cover slips (Becton Dickinson, Bedford, Massachusetts) were washed twice in ice cold PBS and fixed in 3.8% paraformaldehyde for 15 min on ice. Permeabilization was carried out using 0.3% Triton X-100. Blocking was performed using 5% BSA. To identify cardiomyocytes within the potential mixed cell population sarcomeric α-actinin antibody was used (EA53, Sigma Aldrich,

Oakville, Ontario) at concentrations 1:800. For ANF detection, rabbit polyclonal anti-ANF antibody was used (Peninsula Laboratories, San Marcos, California) at a dilution of 1:400. Incubations with primary antibodies were carried out at 4°C overnight in 5% BSA, 001% TritonX-100. Secondary antibodies used included goat-anti mouse Alexa Fluor 594 and goat-anti rabbit Alexa Fluor 488 (both from Invitrogen Life Technologies, Carlsbad). Hoechst 3554 (Sigma-Aldrich, Oakville, Ontario) was used to counterstain the nuclei before mounting cover-slips on microscope slides in DAKO mounting media (DakoCytomation, Denmark). Quantification of cell areas was carried out using CellProfiler™ . Briefly, 5 random fields of view per replicate of each treatment were taken at 20X magnification using Olympus Inverted Microscope. The greyscale images were processed using the pipeline settings described in Appendix F.

2.5 TUNEL Assays.

Terminal deoxy-ribonucleotidyl dUTP-biotin Nick End Labeling (TUNEL) assay was carried out using ApopTag™ In Situ Fluorescein detection kit according to manufacturer's instructions (Millipore, Temecula, California). Briefly, cardiomyocytes were isolated as described in section 2.1 and cultured in specially formulated serum-free media on collagen coated coverslips for 48 hours before being stimulated with 100 μM phenylephrine or 10% FBS. At various time-points cells were fixed in 1% paraformaldehyde in PBS and stored at 4°C for 24 hours in PBS. Cells were then permeabilized by 5-minute incubation at -20°C in ethanol: acetic acid 2:1. Equilibration buffer (supplied) was then applied for 30 seconds. Working strength TdT Enzyme was then incubated in the tissue culture incubator for 60 min for 37°C. Stop buffer was applied for 30 seconds and the cells were incubated with monoclonal anti-digoxigenin antibody (Roche Diagnostics, Palo Alto, California) for 60 min at

room temperature. Following 5 washes with PBS (2 min per wash), secondary antibody (goat anti-mouse Alexa Fluor 594) was added for 60 min at room temperature. The total number of nuclei was counted and those positive for the Alexa594 signal were considered TUNEL positive. The ratio of TUNEL positive to total nuclei was calculated in three separate fields of view per replicate and repeated for a total for three independent isolations of cardiomyocytes.

2.6 Caspase Activity Assays.

Cells were washed twice in ice cold phosphate buffered saline (PBS) and lysed in modified RIPA Buffer (for composition see **section 2.5**). Lysates were cleared by the centrifugation and protein concentrations have been determined by Bradford Assay (Biorad, Mississauga, Ontario) using BSA as a standard. 15 μg of protein from each sample in duplicate was placed in a well of a COSTAR 4596 transparent 96-well plate containing a corresponding volume of caspase activity buffer (20 mM piperazine-N, N'-bis (2-ethanesulfonic acid)(PIPES), 100 mM NaCl, 10 mM dithiothreitol (DTT), 1 mM EDTA, 0.1% (w/v) 3-[(3-cholamidopropyl)-dimethyl-ammonio]-l-propanesulfonate (CHAPS), 10% (w/v) sucrose, pH 7.2. Fluorescent substrates (DEVD.AMC, IETD.AMC, VEID.AMC (Biomol International, Plymouth Meeting, Pennsylvania) were dissolved in DMSO and added to each sample at concentrations of 10 μM. Fluorescence was recorded in the fluorometer operating in the kinetic mode for 2 hours at 37°C using excitation at 380 nm and emission and 460 nm. The highest value in the linear range was used to assign caspase activity.

2.7 Western Blot Analysis.

Cells were rinsed twice in cold phosphate buffered saline (PBS), and lysed in modified RIPA buffer (50 mM Tris-HCl, 1% NP-40, 150 mM NaCl, and 1mM EDTA) containing phosphatase inhibitors (100 mM sodium fluoride (NaF) and 1 mM

Na_3VO_4. (sodium orthovanadate)) and protease inhibitors (Aprotinin, Leupeptin, phenylmethylsulfonyl fluoride, and Pepstatin; all at10 μg/mL). Lysates were cleared by centrifugation and protein concentrations were determined by Bradford Assay (BioRad, Mississauga, Ontario) using BSA as a standard. 15-30 μg of protein in each sample was heated at 95°C with Laemmli buffer (0.31 M Tris-HCl, pH 6.8, 0.35M SDS, 0.025% bromophenol blue, 25% β-mercaptoethanol) and separated by 10% SDS-polyacrylamide gel electrophoresis. Proteins were then transferred to an Immobilon-P membrane (Millipore Corp, Bedford, MA, USA) at 8 V for 150 minutes. Membranes were blocked at room temperature for 1 hour in blocking buffer (either in 5% non-fat dry skim milk in TBS/0.1% Tween-20 (0.1% TBS-T) or in 5% BSA in 0.1% TBS/-T) and incubated with the appropriate primary antibodies overnight at 4°C. The following day, membranes were subjected to several washes in 0.1% TBS-T and were incubated for 1 hour at room temperature with the corresponding secondary antibodies conjugated to horseradish peroxidase diluted in the blocking buffer. Chemiluminescence was then performed using the ECL™ Western Blotting Detection Reagents, according to the manufacturer's directions (Amersham Pharmacia Biotech Inc., Pescataway, New Jersey).

2.8 Luciferase Reporter Assays.

The following plasmids were transfected using Lipofectamine 2000 as outlined in **section 2.9**: 2.5 μg of the ANF promoter encompassing -700 to +35 bp relative to the transcription start site placed upstream of firefly luciferase (see Figure 2-1A), 2.5 μg of the BNP promoter encompassing -114 bp to +3 relative to the transcription start site and placed immediately upstream of firefly luciferase (Fig 2-1B, both kindly provided by Dr. Mona Nemer, IRCM, Montreal, Quebec), 2.0 μg of each 3XMEF2 (3 tandem cis-regulatory sequences found within muscle creatine kinase enhancer

[CCATGTAAGG] & placed upstream of the TATA box [TATATAAT] and followed by the firefly luciferase) and 2.0 μg of 3 X Nf -κB (containing 3 tandem sequences found with Interleukin-4 promoter [CCGGGAATTT] placed immediately upstream of the TATA box [TATATAAT] followed by the firefly luciferase) (Clonetech Technologies, Mountain View, CA, USA). 16 to 20 hours post.transfection, media was changed to a serum free condition, and hypertrophic agonists were applied as outlined in **section 2.1.** 20-24 hours later luciferase activity was assayed using Dual Luciferase Kit (Promega, Madison, Wisconsin) according to manufacturer's instructions. Briefly, cells were lysed in passive lysis buffer (provided with the kit), centrifuged at 4°C for 30 sec at 14,000 rpm, and 20 μL of the supernatant from each sample was used for recording the luciferase activity using the Lumistar Optima Luminometer (BMG Labtech, Durham, North Carolina) using a pump speed of 260 μL/S to dispense 100 μL of each reagent per well to a flat-bottom Corning-COSTAR 96-well luminometer plate (Corning Life Sciences, Lowell, Massachusetts).

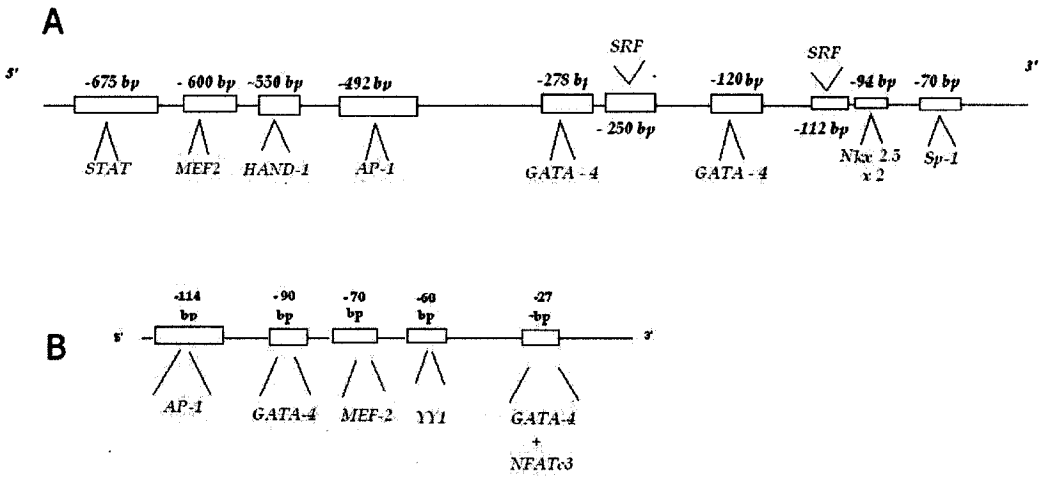


Figure 2-1. Schematic representation of ANF and BNP promoters illustrating numerous cis-regulatory elements that have been shown to be functionally relevant for the induction of both genes during cardiac hypertrophy.

2.9 Cell Culture, transient transfections, and viral infections.

Murine skeletal muscle myoblasts (C2C12), H9C2 cells, and Human Embryonic Kidney 293 (HEK293) cells (American Type Culture Collection, Manassas, VA, USA) were all cultured at 37^0C in the presence of 5% CO_2 in DMEM supplemented with 10% FBS and 100 U/mL penicillin/100 μg/mL streptomycin (growth media). Transient transfections of C2C12 and HEK293 cells were performed when cells reached ~ 90-100% confluence using Lipofectamine 2000 (Invitrogen Life Technologies, Carlsbad, CA, USA) according to manufacturer's instructions in Opti-MEM (Invitrogen Life Technologies, Carlsbad, CA, USA). 4-6 hours post-transfection media was changed to growth media. Adenoviral infections of H9C2 cells were carried out in growth media at a multiplicity of infection of 25 viral particles per cell (MOI=25) and fresh media was applied 16 hours post-infection. 24-48 hours were allowed for transgene expression to take place as monitored by the GFP expression. Cells were then used for caspase activity assays (see Section 2.4) or for western blot analysis (see section 2.5).

2.10 Statistical Analysis.

The analysis used to determine the statistical significance between the treatments was carried out using two-tailed, unpaired Student's T-test. If the calculated p-value was equal to or was found to be below 0.05, the data were said to be statistically significant. Error bars represent the standard error of the mean (S.E.M) based on three independent experimental trials each consisting of three replicates. MS Excel™ was used to carry out the calculations.

CHAPTER 3. RESULTS.

3.1 Expression analysis of caspase family members in primary cardiomyocytes and in whole heart tissue.

Using RT-PCR, the expression levels of all caspase family members encoded by the rat genome at the transcript level both in primary cardiomyocyte cultures and in the whole hearts of 6 month old male and female Sprague-Dawley rats were assessed (Figure 3-1). High levels of *caspase-3* and *caspase-8* transcripts in both NRVM cultures and in whole adult heart were detected. Messages for executioner *caspases -6 and -7* were also present although at slightly lower levels. Caspase-4 transcript also showed high expression levels while caspase-1 transcript was expressed at lower levels. Interestingly, messages for less studied family members (namely: caspase-11 and -12) were found to be present at high levels in both the adult heart and in primary cultures. The effect of hypertrophic stimulation on expression of caspase genes was also tested: neither phenylephrine nor serum treatments affected the expression level of any caspase transcript (Figure 3-1).

This expression screen also identified caspase-14 as the only differentially expressed *caspase* gene in neonatal cardiomyocytes versus whole adult rat heart. It was also determined that the expression of caspase 14 is not induced following 48 hour treatment with either of the hypertrophic agonists used throughout the study. To determine whether a similar developmental expression pattern is found in the mouse, the presence of *caspase-14* transcripts in hearts isolated from mice at three developmental stages: E14, P7, and 2 months old was assessed. No transcript was detected in any of the samples (Supplementary Fig. 1). To investigate the possible basis for the differential expression between two closely related rodent species , the genomic regions extending 5 kilobases upstream of the annotated transcription start site of *caspase-14* gene were aligned (Supplementary Fig. 2). This region was not conserved between the species, indicating an evolutionary divergence between the

cis-regulatory sequences. (Appendix A, Supplemental Figure 1 and Supplemental Figure 2 (A)). However, unlike caspase 14 the promoter of the heart specific endocrine peptide ANF displayed a remarkable degree of conservation, consistent with the observed expression pattern in multiple species (Appendix A, Supplementary Figure 2, B).

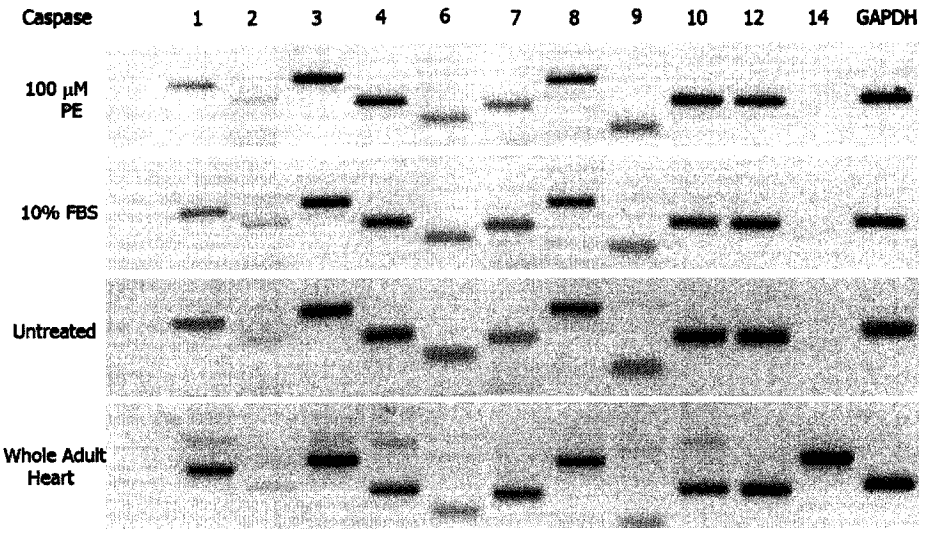


Figure 3-1. Expression of the various caspase protease transcripts as determined by RT-PCR in whole adult rat heart and in dissociated neonatal primary ventricular cardiomyocytes treated with indicated amounts of phenylephrine, fetal bovine serum, or control (untreated) (n=3).

3.2 Caspase Activity Assays and TUNEL assays.

It was next tested whether activity of selected family members may be present at any point during the hypertrophic response. To accomplish this, caspase activity assays were performed using a panel of commercially available artificial substrates, each with an increased reported affinity towards a specific family member. DEVD.AMC is a preferred substrate for executioner caspases-3, -6, and -7, while IETD.AMC and LEHD.AMC are known to be most actively cleaved by caspase-8 and -9 respectively. IETD-directed activity was the highest observed, increasing within the first two hours ($p<0.05$) following the stimulation with phenylephrine and remained elevated throughout the examined time points (Figure 3-2, b). DEVD-ase activity, could also be detected at most time points assayed, albeit at much lower levels, with a peak of activity 8 hours following FBS treatment (Figure 3-2, a). Measurable DEVD-ase activity could also be detected in the untreated cells. Caspase-9-like activity was not detectable in the conditions described (Figure 3-2, c).

It was also noted that high levels of VEID hydrolysis at various time points of an 18 hour hypertrophic stimulation. This motif is known to be most effectively cleaved by caspase-6. Observed VEID-ase activity was much higher than that determined for any other caspase substrate used (Supplemental Figure 4A). To verify a caspase 6 specific activity, caspase-6 was overexpressed in HEK293 cells the ability of this protease to cleave DEVD.AMC was examined (Supplemental figure 4B). As anticipated, over expressed caspase-6 was capable of robust DEVD.AMC cleavage (resulting in a fluorescence level half of that produced by VEID.AMC substrate). Importantly, when catalytically dead caspase-6 was over expressed, it also resulted high values of VEID.AMC fluorescence implying that either the stability of this substrate is compromised or that other proteases may cleave this substrate.

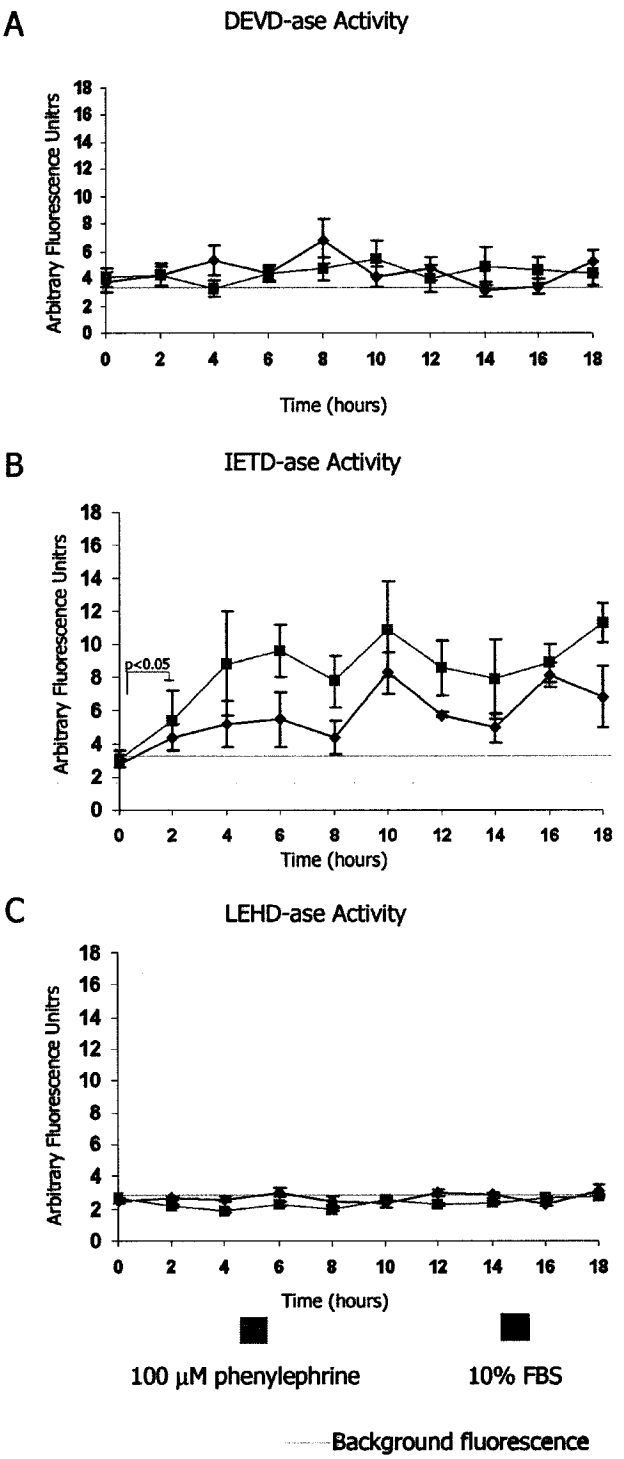


Figure 3-2. Changes in caspase activity towards selected artificial substrates a.) DEVD.AMC, b.) IETD.AMC, and c.) LEHD.AMC over the course of 18 hour stimulation of primary neonatal rat cardiomyocytes with indicated agonists (n=3).

Conceivably, observed caspase activity may emanate from the few cells undergoing apoptosis during cell culture, the activity peaks simply representing an increase in the number of dying cells. To assess the extent of programmed cell death occurring in our culture models we employed the TUNEL assay (Figure 3-3). The results of this assay demonstrated that in the absence of any hypertrophic agonists, about 10% of the cells in culture are TUNEL positive. An apparent increase in nuclei labeled by the TUNEL at 20 hour time point was found to be statistically significant at $p<0.05$ level (based on the unpaired, two-tailed Student's T-test) consistent with several studies reporting pro-apoptotic action of α-adrenergic agonists (Iwai-Kanai, Hasegawa et al. 1999; Pu, Ma et al. 2003).

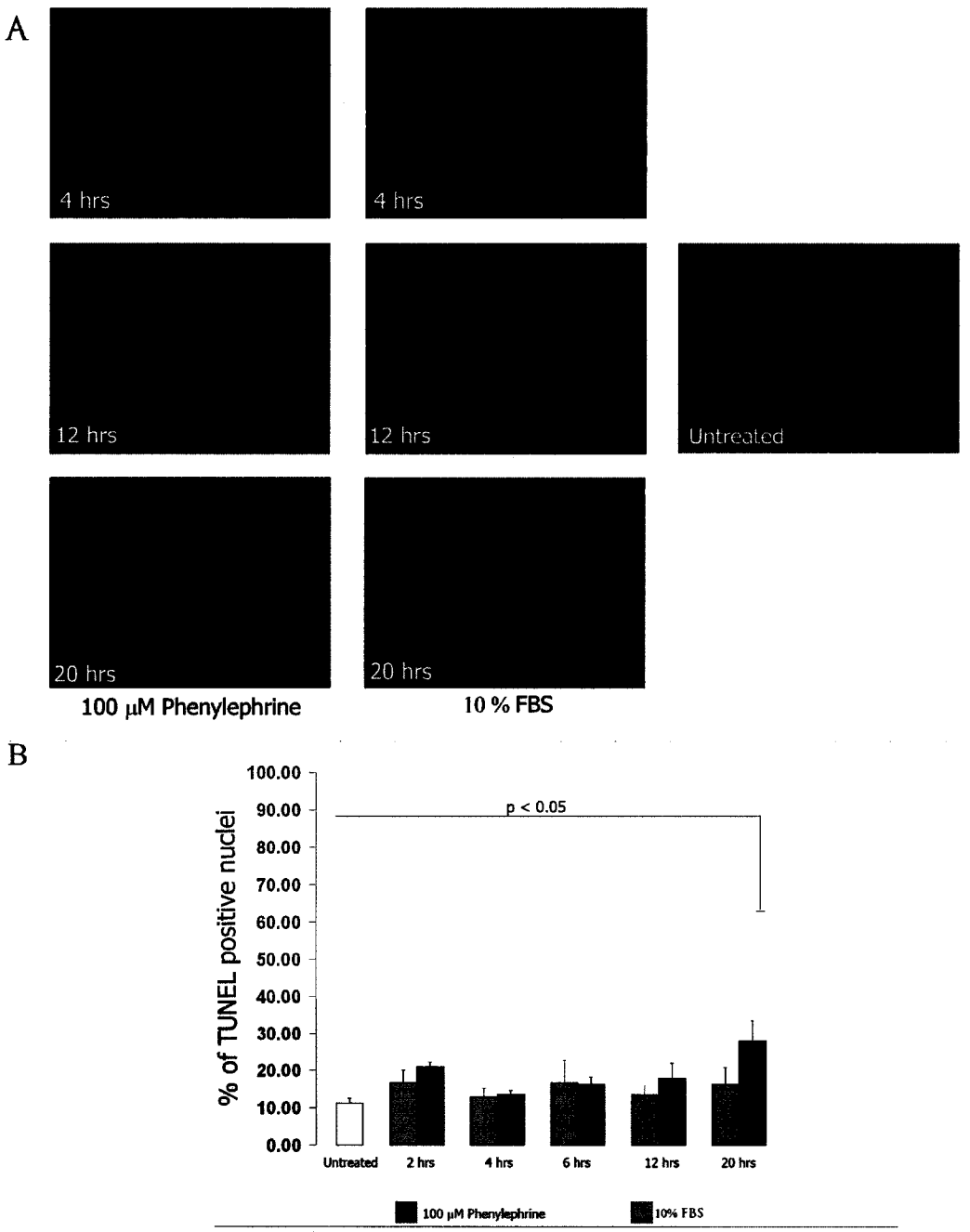


Figure 3-3. TUNEL assay used to determine the percentage of apoptotic cardiomyocytes following stimulation with hypertrophic agonists. A.) Fields of view at selected time points indicating TUNEL positive nuclei. B.) Quantification of TUNEL positive nuclei at indicated time points (n=3).

3.3 Effect of caspase inhibition on cell size increase.

Based on the variable nature of the caspase activity observed during pathological hypertrophy (Figure 3-2), a panel of commercially available pharmacological caspase inhibitors was utilized to assess whether the inhibition of this activity will influence the hypertrophic response as it manifests in an *in vitro* model, Inhibitor treatment in the absence of the hypertrophic agonists had no effect on cell area increase, while ~20% increase in cell areas was noted upon phenylephrine stimulation, and this number had increased to ~30% following FBS treatment (Figure 3-4, A). Regardless of which hypertrophic agonist was used, no apparent effect on the phenotype was observed as determined by the visual examination at high magnifications: cells treated with every type of inhibitor have re-assembled their sarcomeres and have undergone hypertrophic growth similarly to DMSO treated populations (Figure 3-4, A).

Cell areas at the end of the 48 hour period were quantified using automated cell processing software CellProfiler^{tm} (Lamprecht, Sabatini et al. 2007). Both phenylephrine and serum treatments resulted in statistically significant cell area increases ($P<0.001$). Consistent with visual observations, inclusion of caspase inhibitors did not prevent hypertrophic growth in response to either agonist. As additional controls, cardiomyocytes were treated with known antagonists of hypertrophic growth: pp2B (calcineurin) inhibitor Cyclosporin A and MTOR inhibitor Rapamycin. Consistent with previously published results (Molkentin, Lu et al. 1998; McMullen, Sherwood et al. 2004), both antagonists were effective at inhibiting hypertrophic growth (Figure 3-4). No such inhibition could be seen with serum however; possibly due to multiple redundant pathways being activated by this agonist.

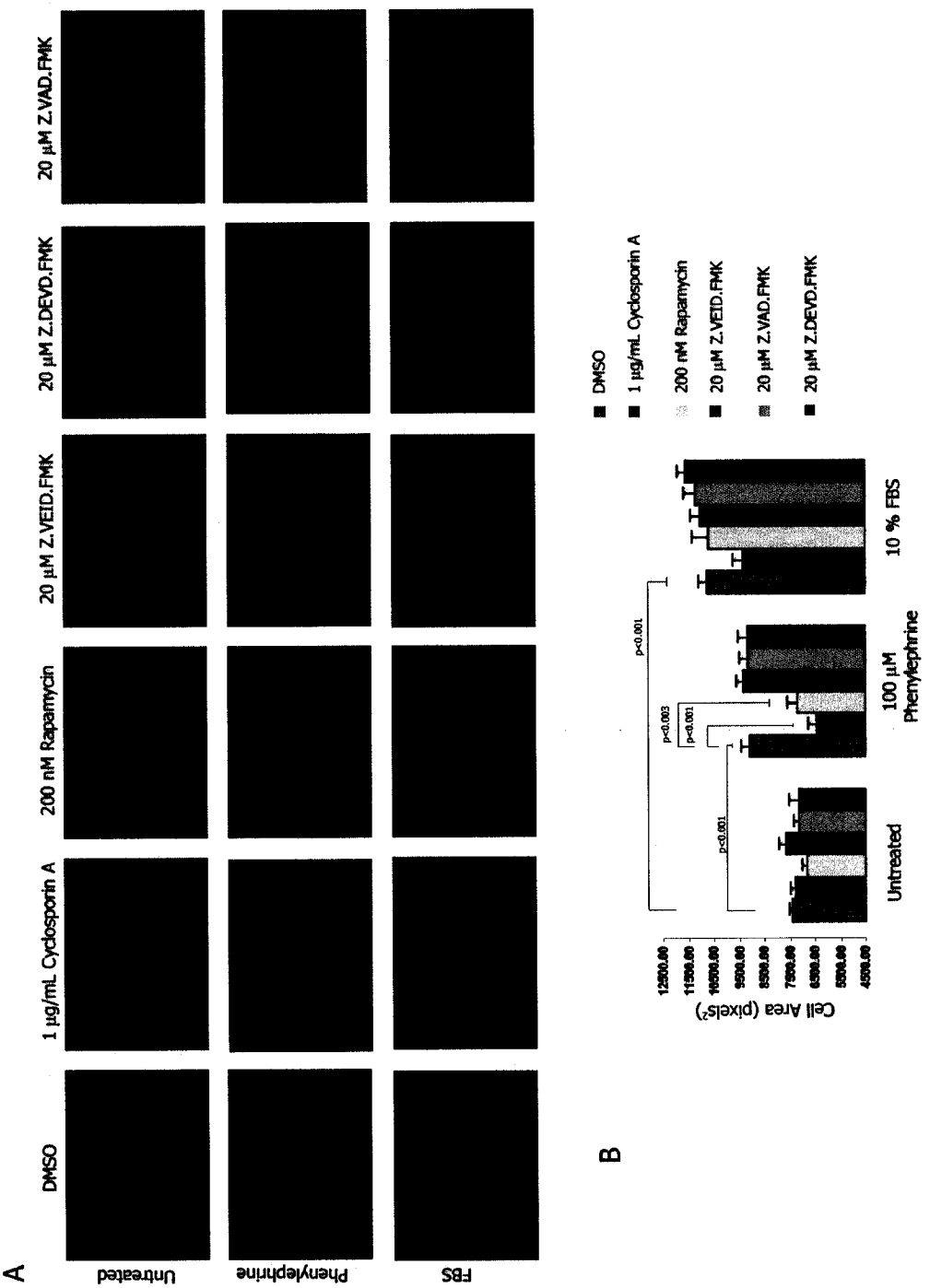


Figure 3-4. Pharmacological caspase inhibitors do not alter PE or serum-induced cell area increase of primary neonatal cardiomyocytes following 48 hours of stimulation (n=3).

The observed lack of effect on cell growth could simply be due to the inhibitor degradation or to inefficient cellular uptake. To overcome this issue, a highly potent irreversible biological pan-caspase inhibitor, p35 protein, encoded by *Autographa californica* baculovirus was utilized (Clem, Fechheimer et al. 1991; Fisher, Cruz et al. 1999; Lu, Min et al. 2006), sees Appendix B, Supplemental Figure 3 and Supplemental Table 1 for mechanism and additional information).

p35 acts as a competitive inhibitor: it itself becomes a substrate for a number of mammalian caspases and locks these enzymes in inactive state preventing any further catalysis. Catalytic cysteine of a caspase carries out a nucleophilic attack on Aspartate 87 found within the solvent protruding loop of p35. This results in formation of a covalent thioester bond linking p35 to a caspase (See Supplemental figure 3 for a detailed mechanism). Under normal conditions this intermediate would be quickly hydrolyzed by an incoming water molecule, making caspase available for the next proteolytic attack; however, separation of the remaining portion of the p35 following caspase attack causes conformational change, which culminates in the insertion of cysteine 2 residue of p35 into the active site of caspase forming a hydrogen bond with a histidine of the catalytic dyad. As a result, the imidazole ring of the histidine rotates about 90^0 and in this conformation, histidine is presumed to be incapable of forming the nucleophilic water molecule essential for peptide bond hydrolysis to occur (Lu, Min et al. 2006) (Appendix B, Supplementary Figure S2). Dissociation constants of p35 towards recombinantly expressed mammalian caspases are in the nM range (Supplementary Table 1, Los Walczak, 2002). Therefore p35 would block any endogenous intracellular caspase activity in a persistent and robust manner.

To this end, a recombinant adenovirus encoding p35 was generated for efficient delivery into primary cardiomyocytes and the effect of its over-expression on cell size in response to hypertrophic stimuli was assessed (Figure 3-4). Cardiomyocytes infected with p35 virus have responded to hypertrophic treatments similar to cells infected with a virus bearing GFP based on sarcomere re-assembly and apparent growth in volume. Quantification of cell areas confirmed that over expression of p35 is not influencing the increase in cell area that occurs as a result of either phenylephrine or serum treatments (Figure 2-3a; the effect of increasing viral titers has been investigated; but only MOI of 25 is shown). Caspase-inhibitory properties of p35 were confirmed using several functional assays under apoptotic conditions in various cell types including primary cardiomyocytes (Supplementary Figure 3). Based on these results, caspase activity does not influence the molecular mechanisms that control physical growth of the neonatal cardiomyocyte.

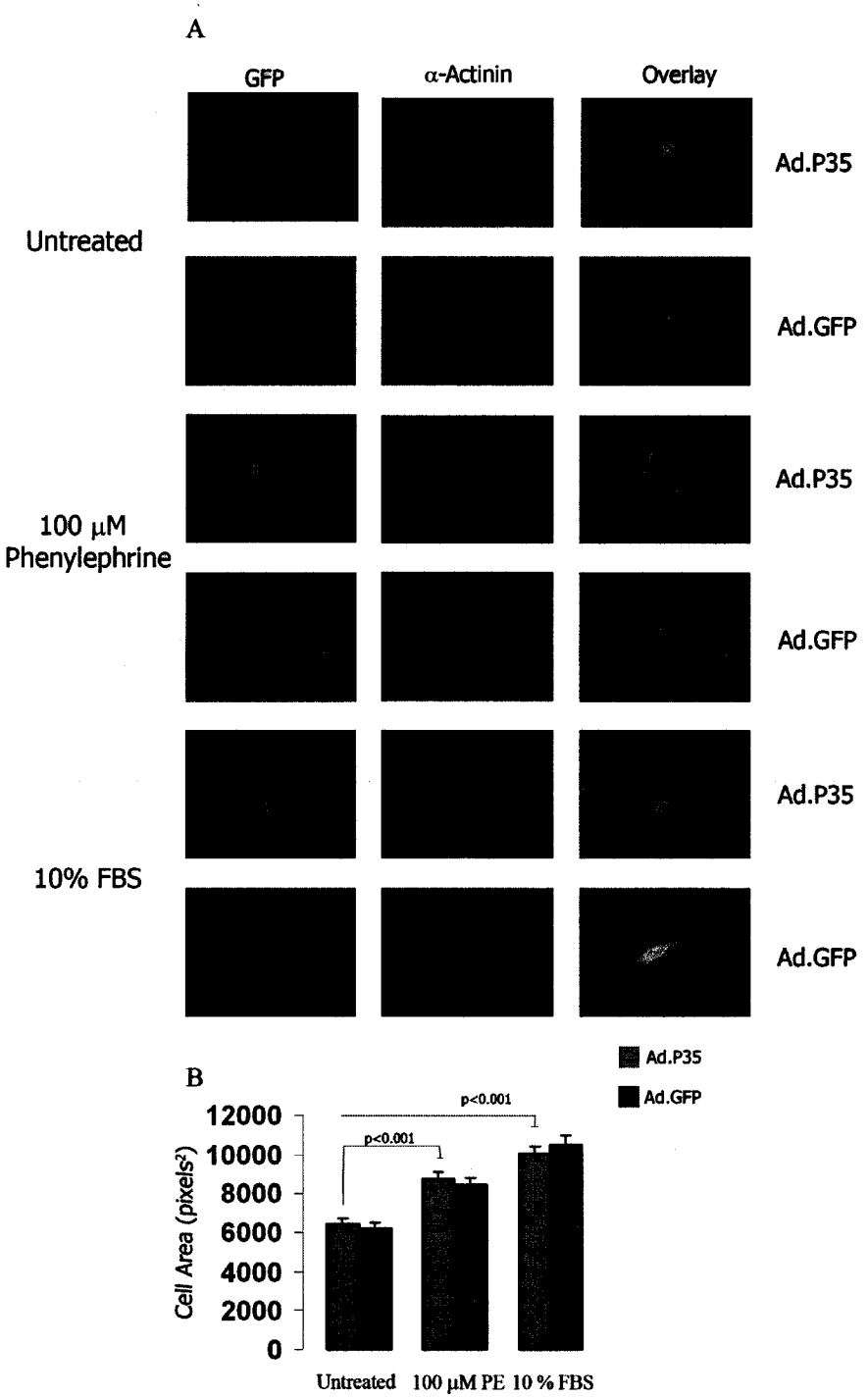


Figure 3-5. p35 over expression does not alter PE or serum-induced cell area increase of primary neonatal cardiomyocytes following 48 hours of stimulation (n=3).

3.4 Effect of caspase inhibition on ANF expression.

Next, the possibility of potential caspase involvement in the events mediating a switch to the fetal genetic program that is known to occur during pathological hypertrophy was examined. Expression of ANF is a standard indicator of pathological hypertrophic gene expression: its expression is greatly increased by most if not all hypertrophic agonists and represents a widely used gauge of the fetal transcriptional program. The number of ANF positive cardiomyocytes increased from 10% in the untreated condition to 45% of all cells following phenylephrine treatment, and treatment with serum further increased this number to 60 % (Figure 3-5). When the agonists were used in the presence of pharmacological inhibitors, the number of cells exhibiting ANF expression did not change. This effect was also tested using the p35 inhibitor and. as with the chemical inhibitors, no change in the numbers of ANF positive cells were noted compared to control cells (Figure 3-6). These experiments suggest that pathways giving rise to ANF expression do so independently of caspase enzymatic function,

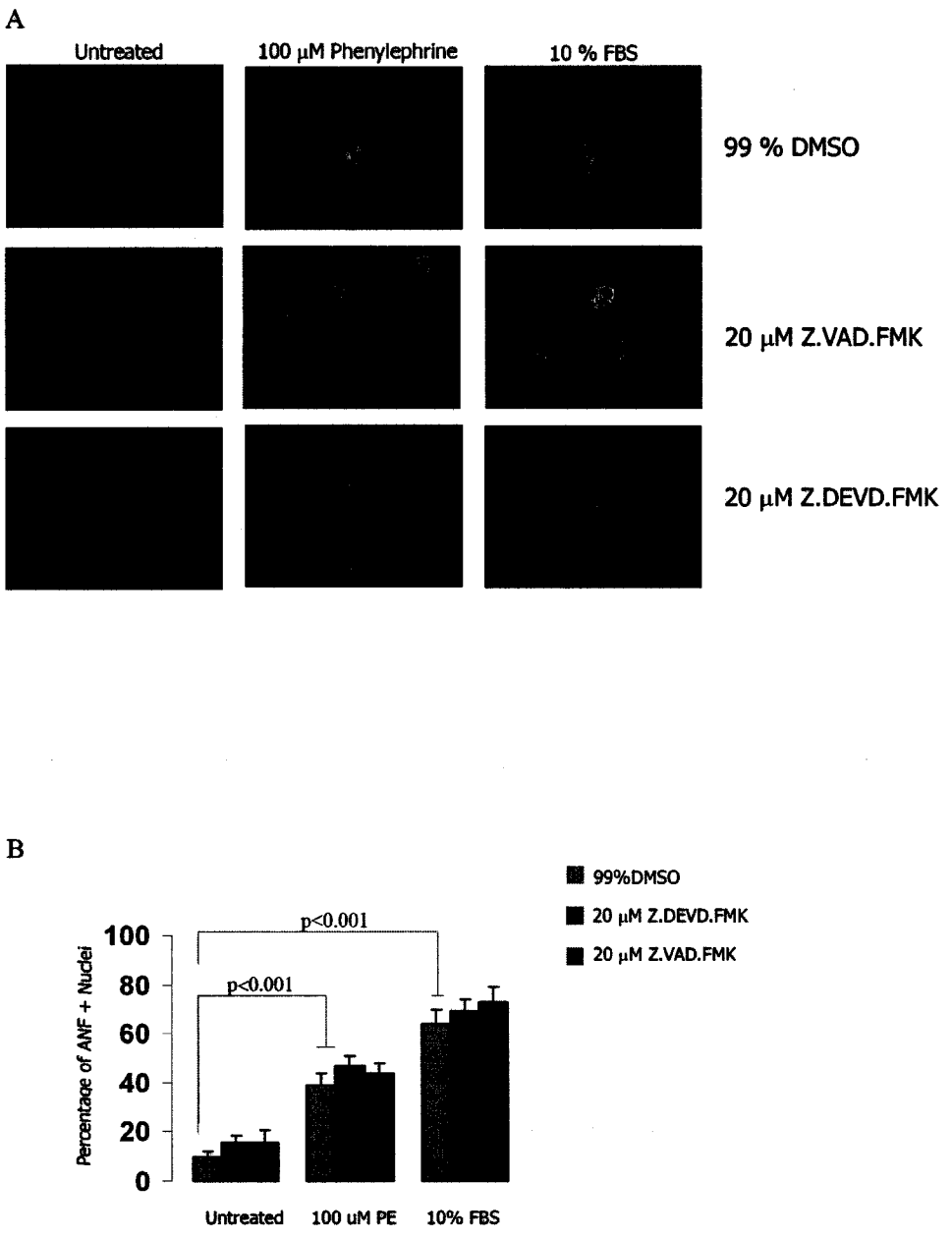


Figure 3-6. Pharmacological caspase inhibitors do not prevent an increase in ANF expression as determined by A.) Immunocytochemistry and confirmed by quantification of ANF positive cardiomyocytes following 48 hour stimulation with indicated hypertrophic agonists (n=3).

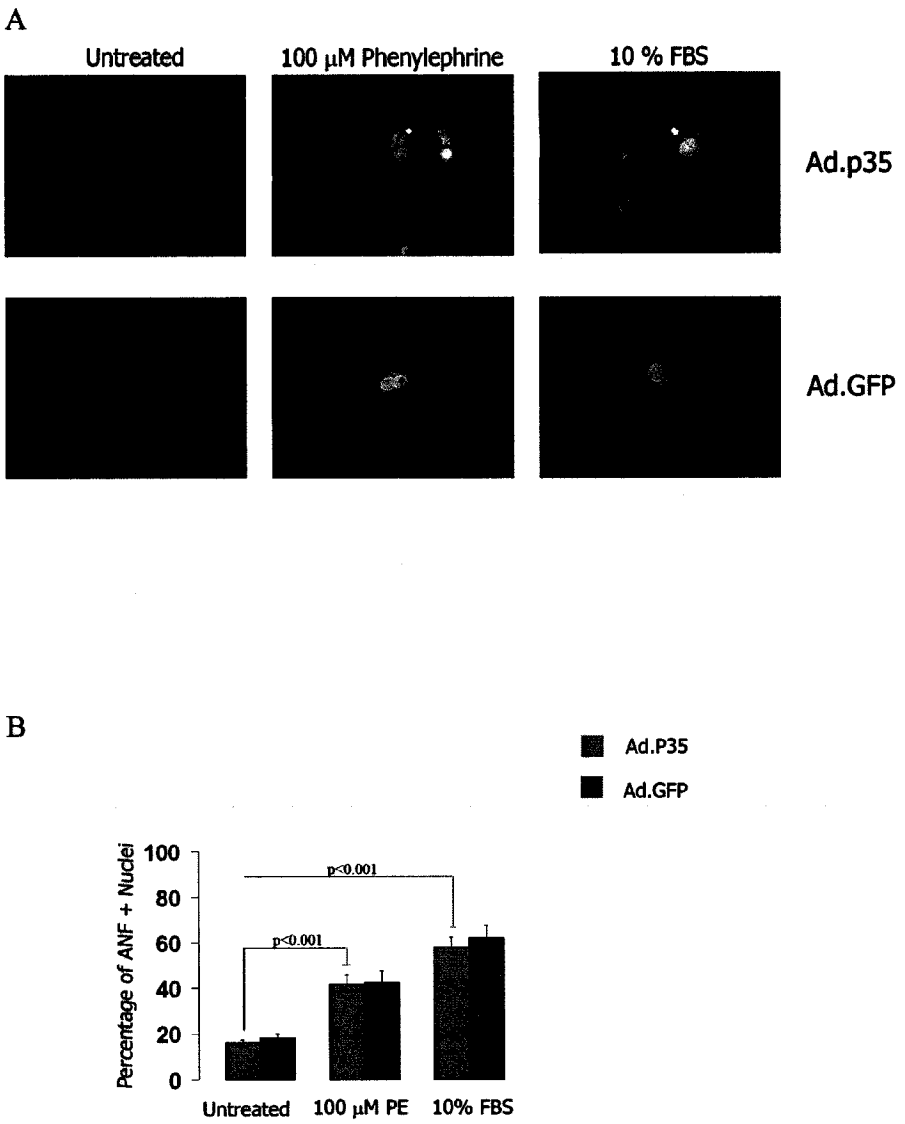


Figure 3-7. Over expression of p35 does not influence an increase in ANF expression as determined by A.) immunocytochemistry and confirmed by B.) quantification of ANF positive cardiomyocytes following 48 hour stimulation with indicated hypertrophic agonists (n=3).

3.5 Quantitative expression of ANF and BNP expression.

In order to determine whether subtle effects on the expression of the prototypical hypertrophic marker ANF may be present quantitative real-time PCR was used to examine the expression levels. In response to phenylephrine stimulation, vehicle treated cells, exhibited about 7-fold up regulation of ANF transcript whereas a 10-fold increase in expression was noted with inclusion of Z.VAD.FMK (Figure 3-8, A). This apparent increase in the induction of the transcript was not found to be statistically significant based on three independent trials (using unpaired two-tailed Student's T-test) and most likely represents variations inherent to this experimental method. Serum-treated cardiomyocytes exhibited a 4-fold up regulation of ANF message as compared to the untreated cells whether they were stimulated in the presence of DMSO or 20 μM Z.VAD.FMK. Adenovirally expressed p35 was used as a complementary means of caspase inhibition (Supplementary Figure 3). No difference in ANF induction between GFP and p35 over-expressing cells was noted: ~8 fold increase and ~6 fold increase was seen with both viruses in response to phenylephrine and serum exposure respectively (Figure 3-8, B).

The expression of another hypertrophic marker BNP (b-type natriuretic peptide) was also examined. Similarly to the ANF, its expression is markedly up regulated in response to hypertrophic stimuli both *in vitro* and *in vivo* (Hall 2004). When phenylephrine was used to induce hypertrophy, BNP expression increased 4-fold when compared to the untreated cells. In the presence of pharmacological pan-caspase inhibitor Z.VAD.FMK, expression was unregulated 11-fold but as with ANF, no statistical significance was found to confirm the significance of this increase. P35 infected cardiomyocytes upregulated BNP transcript expression to the same extent as GFP-infected cells: 8-fold following phenylephrine treatment. Serum treatments

caused a modest two-fold increase in BNP transcript levels and this increase was not influenced either by Z.VAD.FMK) or p35 (Figure 3-8).

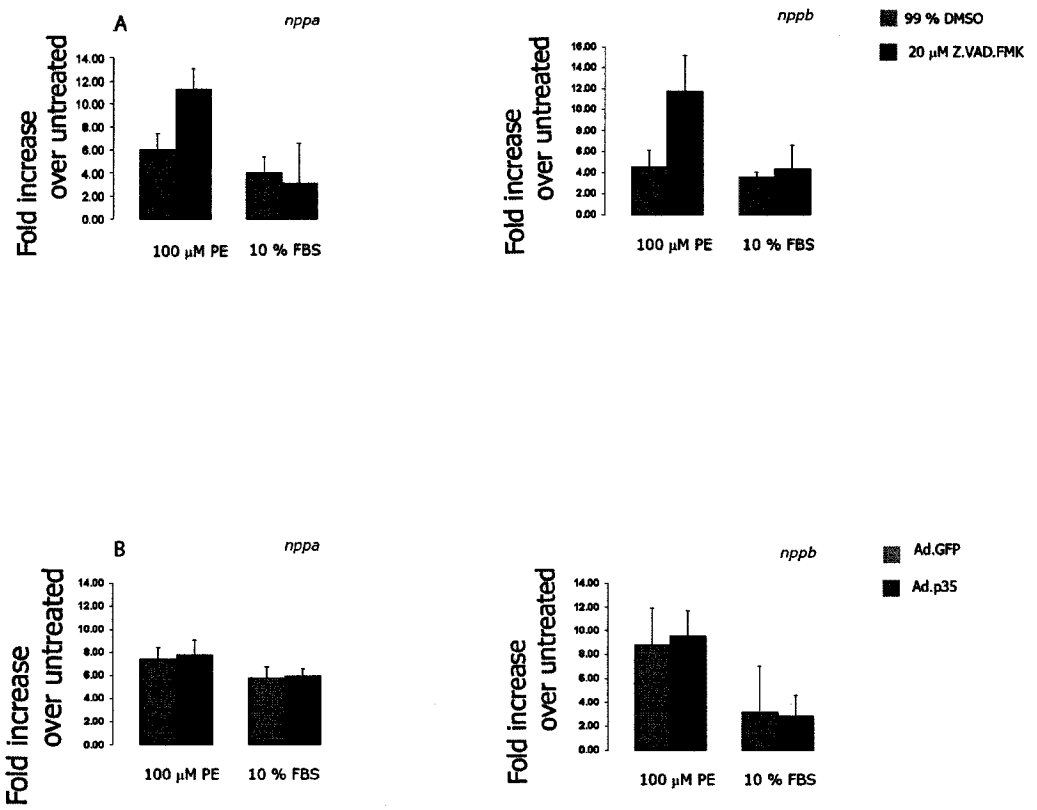


Figure 3-8. Expression of prototypical hypertrophic markers ANF and BNP is not repressed by the use of either A.) chemical pan.caspase inhibitor Z.VAD.FMK or B.) p35 over-expression (n=3).

3.6 ANF and BNP luciferase reporter assays.

As a complementary approach to the expression analysis, the effect of caspase inhibition on transactivation of ANF and BNP promoters was also examined. This was necessary for two reasons:

a.) due to up regulation of both transcripts in response to inhibitor treatments, it was important to check whether the promoters would similarly exhibit higher activity;

b.) induction of both promoters is a result of activation of numerous signaling cascades and given the remarkable degree of sensitivity of this reporter system, the assay provides an objective read out of the behaviour of various hypertrophic pathway(see Figure 2-1 for a detailed structure of each promoter).

In addition to using the pharmacological pan.caspase inhibitor Z.VAD.FMK and p35, caspase-3/-7 specific inhibitor Z.DEVD.FMK was included. To be able to attribute any possible effects directly to caspase enzymatic activity, a point mutation in p35 changing Aspartate 87 to Alanine (D87:A) was introduced. This substitution abolished the caspase recognition site rendering p35 incapable of any inhibitory action (see Supplementary Figure 3). The ANF promoter was induced to similar levels by both hypertrophic agonists and was not influenced by either of the chemical inhibitors (Figure 3-8). Cells transfected with the ANF luciferase reporter and either with wild-type p35 or p35 (D87: A) also produced the same levels of promoter induction with both hypertrophic agents used (Figure 3-9).

The -114 region of the BNP promoter was induced 3-fold in response to phenylephrine treatment and the presence of either Z.VAD.FMK or Z.DEVD.FMK did not suppress its activity. P35 co-transfections (Figure 3-9) did not have an effect on the ability of phenylephrine to transactivate this reporter (Figure 3-9). The BNP

promoter was activated in response to serum treatments to a similar extent, whether cardiomyocytes were pre-treated with vehicle or with the pharmacological inhibitors (Figure 3-10). Serum-stimulated cells co-transfected with either wild-type p35 or with p35 (D87: A) did not exhibit suppression of the activity of the BNP promoter following 24 hours of treatment (Figure 3-10).

In the absence of hypertrophic agonists, treatment with chemical inhibitors alone did not influence the behavior of either ANF or BNP reporters. Similarly, p35 co-transfection had no impact on the activity of either reporter (Figure 3-9 and Figure 3-10).

In summary, luciferase reporter assays convincingly showed that molecular pathways responsible for the induction of both ANF and BNP are not dependant on caspase enzymatic activity. This result further corroborates the trends observed in the quantitative real time PCR and immunocytochemistry experiments.

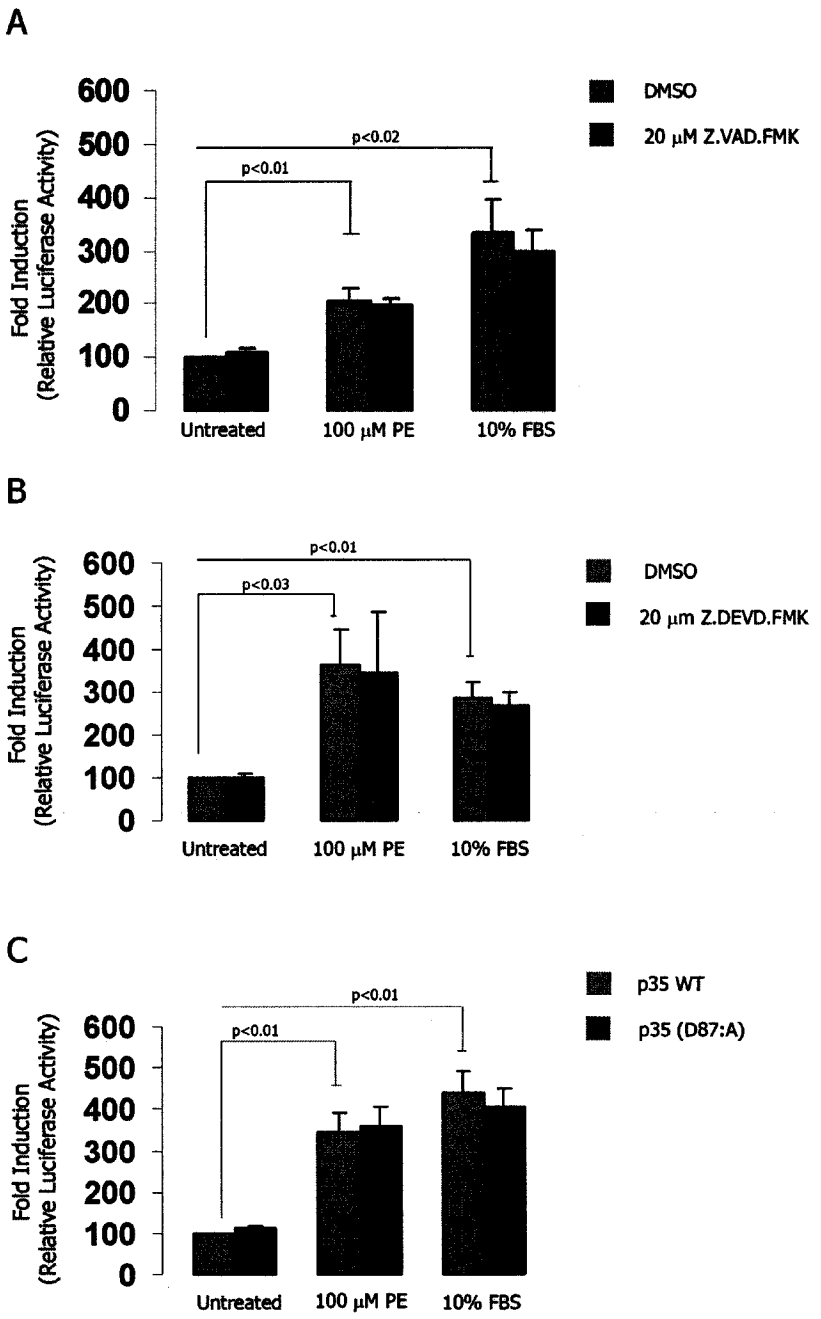


Figure 3-9. Transactivation of -700 bp ANF luciferase reporter is not significantly altered by the use of either A.) chemical pan.caspase inhibitor Z.VAD.FMK or B.) DEVD.FMK or by C.) p35 over expression (n=3).

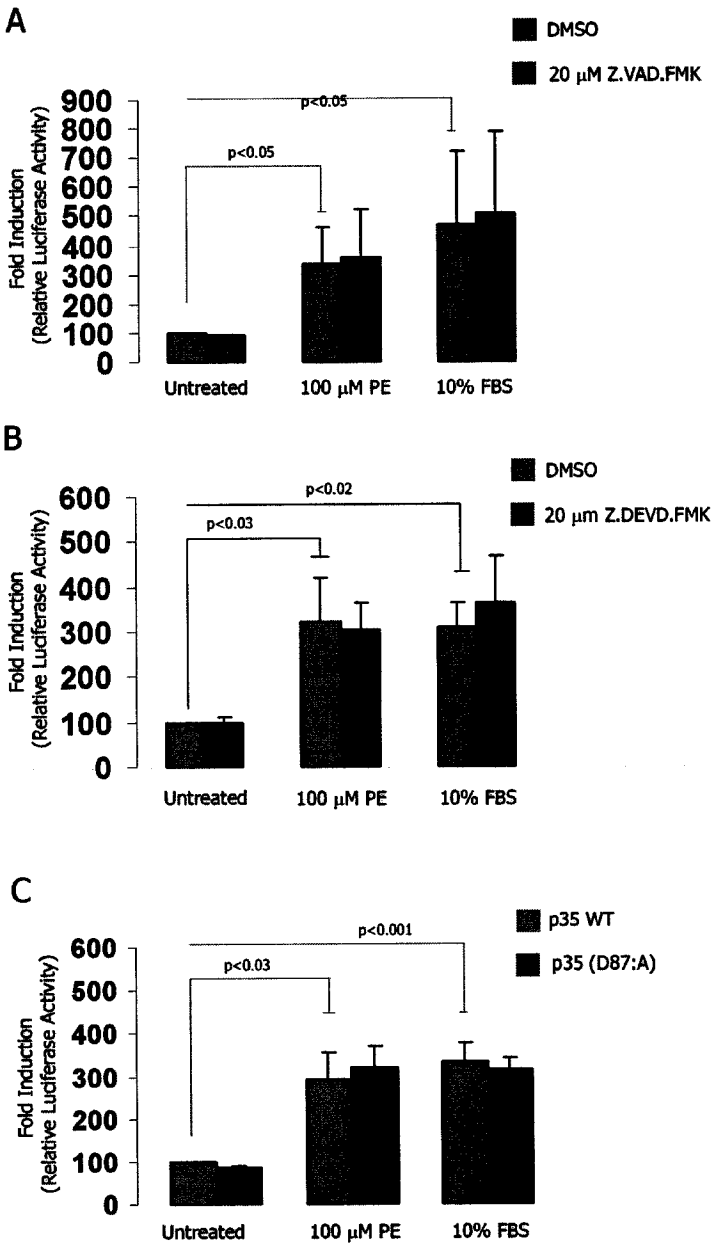


Figure 3-10. Transactivation of -114 bp BNP luciferase reporter is not significantly altered by the use of either A.) pan.caspase inhibitor Z.VAD.FMK or B.) DEVD.FMK or by C.) p35 over-expression (n=3).

3.7 Mef2 and NF-κB luciferase reporter assays.

The possibility that the caspase enzymatic activity (Figure 3-2) could be limited to a pathway(s) controlling activation of only a select transcription factor(s) response was also considered. As such, inhibiting caspase catalytic activity may not have an effect on the expression of prototypical hypertrophic markers examined for the following reasons:

a.) Given the number of transcription factors controlling expression of either Anf or Bnp, compromised function of a given regulator could be easily masked by a large set of functional regulators of hypertrophic growth;

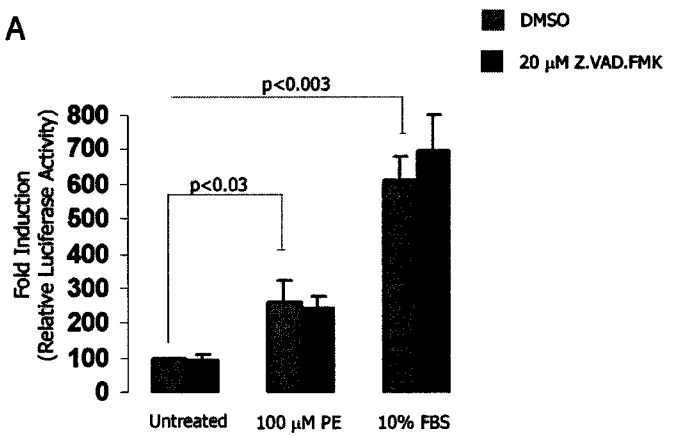
b.) Observed caspase activity may activate a transcriptional regulator that does not directly control the expression of either ANF or BNP genes;

To address this issue, luciferase-reporter assays were utilized to determine how caspase inhibition may influence the activity of two key transcription factors that contribute to the hypertrophic response: myocyte enhancer factor 2 (Mef 2), and nuclear factor kappa B family (NF-κB). Mef2 is a sensor of numerous stress-responsive pathways in the heart and is a central regulator of pathological hypertrophic growth. Moreover, Mef2 was shown to be a caspase substrate in neural tissues during apoptotic conditions (Okamoto, Li et al. 2002). Nf-*k*B is a prominent player in pathological cardiac hypertrophy and is known to be activated by caspase proteases in selected tissues (Lamkanfi, D'Hondt et al. 2005; Su, Bidere et al. 2005; Varfolomeev, Maecker et al. 2005; Lakhani, Masud et al. 2006).

Stimulation with phenylephrine resulted in a modest 2.5 fold increase in Mef2-driven luciferase activity, whereas exposure to serum for 24 hours, increased the Mef2 response approximately 6-fold. Treatment with pharmacological pan-caspase inhibitor Z.VAD.FMK did not result in significant attenuation the hypertrophic effect on the

MEF2 reporter (Figure 3-10 A). When the MEF2 luciferase reporter was co-transfected with wild-type p35, phenylephrine and serum treatments elicited the same response as when D87:A p35 was used for co-transfections (~2.5 and ~4.5 fold respectively) (Figure 3-10 B). These experiments demonstrated that Mef2 associated signaling pathways are not dependant on the catalytic activity of caspase proteases.

The Nf-κB response to caspase activity was also examined. When stimulated with phenylephrine, the nf-kb reporter was up regulated ~4.5 fold as compared to untreated cells. In the presence of chemical pan.caspase inhibitor, activation of the reporter was repressed by 33% (3.0 fold vs. 4.6 fold increase, $p<0.05$) although no significant inhibition was seen with serum. P35 co-transfection also impaired Nf-κB reporter transactivation (38%, 2.4-fold vs 3.9 fold, $p<0.01$) relative to the co-transfection with p35 mutant incapable of caspase inhibition. Serum treatments had no significant effect on NF-κB reporter activity irrespective of the caspase activity status. These results suggested that proper activation of Nf-κB in neonatal cardiomyocytes is at least partially dependant on caspase enzymatic activity.



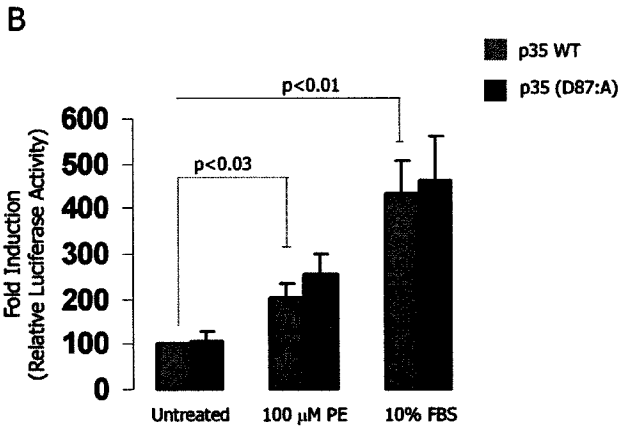
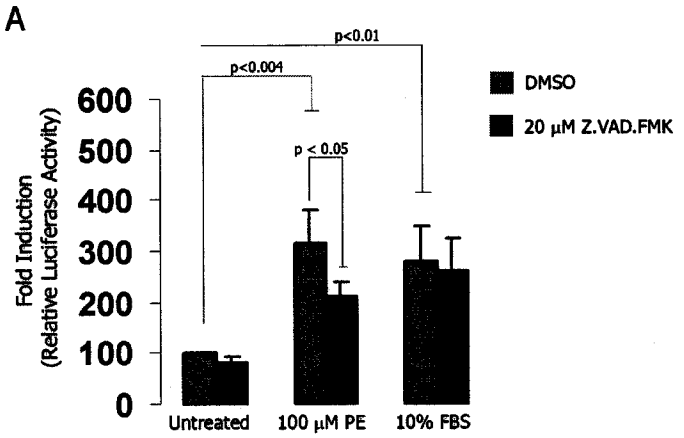


Figure 3-11. Transactivation of 4 X Mef2 luciferase reporters is not significantly altered by the use of either A.) chemical pan.caspase inhibitor Z.VAD.FMK or B.) p35 over-expression (n=3).



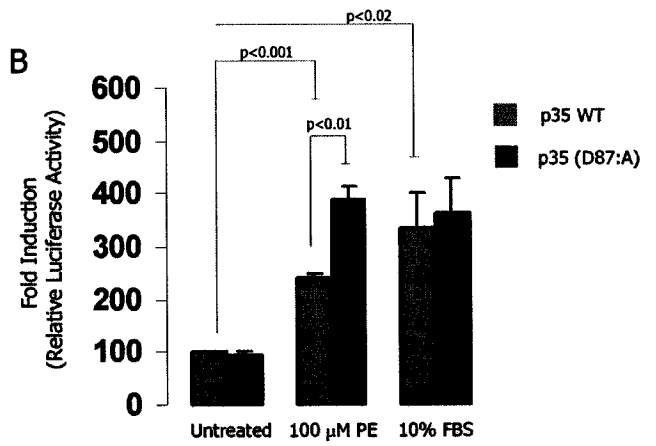


Figure 3-12. Transactivation of 3 x Nf–κB luciferase reporter is partially repressed by A.) chemical pan.caspase inhibitor Z.VAD.FMK B.) Derepression of caspase activity by the addition of a mutant p35 leads to upregulation of nf-kb reporter activity (n=3).

CHAPTER 4. DISCUSSION.

4.1 Caspases: heart-specific functions.

While a significant body of evidence points to the critical developmental functions of caspases and underscores their importance for the maturation of various cell types, little is known as to whether these molecules perform death-unrelated roles in terminally differentiated tissues. Targeted inhibition of caspases has been suggested to hold therapeutic potential for the treatment of heart disease (Yaoita, Ogawa et al. 1998), yet a non death role in this tissue would significantly complicate any blockade of caspase function To expand our understanding of caspase function in the post. natal cardiomyocyte compartment, the hypothesis that some family members may contribute to signal transduction cascades that give rise to pathological cardiac hypertrophy was tested. To this end, two independent criteria commonly used to assess this response as it unfolds *in vitro*: a physical increase in the overall cell area and induction of prototypical hypertrophic markers were examined.

4.2 Expression of caspase family members in neonatal cardiomyocytes and in whole heart tissue.

An expression screen encompassing all caspases known to be found in the rat genome was undertaken. This approach has revealed that pro-inflammatory caspases are present at high levels both in the neonatal ventricular cardiomyocyte cultures and in the whole adult heart (namely caspases-4, caspases-11, and caspases-12). However, reports describing the molecular functions of these caspases remain unkown. Interestingly, caspase-11 has been implicated in the cytoskeletal rearrangement events in leukocytes, independent of its enzymatic activity (Li, Brieher et al. 2007). The loss of function experiments utilized in this study were based on the premise that enzymatic activity is essential for non-apoptotic caspase function, whether caspases-11 may perform a similar function in cardiomyocytes remains an open question. The

expression screen also identified *caspase-14* to be present only in the whole rat adult heart but not in neonatal cardiomyocytes. However it is not clear whether this transcript is expressed specifically in cardiomyocytes or by other cell types found in the adult heart.

It was also established that no single *caspase* gene undergoes detectable changes in expression as a result of the hypertrophic stimulation. This finding aligns closely with previous reports which describe the differential expression between a healthy and a hypertrophic murine heart (Kim, Porreca et al. 2007). Nevertheless, this aforementioned study has identified caspase-14 as one of the transcripts that experiences minor down regulation in the mouse heart undergoing hypertrophic cardiomyopathy (Kim, Porreca et al. 2007). In the present study, no caspase-14 message could be detected in mouse heart tissues at any of the three developmental stages examined (Supplemental Figure 1A). This finding is also partially corroborated by previous investigations, which reported complete absence of caspase-14 transcript from all adult mouse tissues with the exception of skin epidermis(Eckhart, Ban et al. 2000; Eckhart, Declercq et al. 2000). Expression of this gene in different tissues between closely related rodent species can be explained by the divergence of regulatory sequence controlling the transcription of *caspase-14* (Supplemental Figure 1B).

4.3 Implication of caspase activity on the course of the hypertrophic adaptation.

Having established high abundance of transcripts for both *caspase-3* and *caspase-8* and slightly lower for those encoded by *caspases-6 and -7*, fluorogenic substrates were used to examine caspase activity profiles over the course of the 20 hour hypertrophic response. Modest increase in DEVD-ase activity was noted at several time points, indicative of the presence of catalytically mature executioner

caspases (-3, -6,-7). At the same time, use of a TUNEL assay revealed the presence of fragmented DNA in 10% to 20% percent of the cardiomyocyte population. There appears to be a disconnect between higher than expected number of fragmented nuclei and the low level of caspase-3 activity. As such the TUNEL procedure may have labeled nuclei of cells that are undergoing death in a caspase independent manner. Finally, the TUNEL positive nuclei may simply represent cells that are dying as the result of the isolation process.

An increase in the IETD-ase activity following 2 hours of stimulation with both agonists (although more pronounced with phenylephrine than with serum) was also noted and this activity remains elevated throughout the time points assayed. Although the increase in activity levels appear marginal, they are comparable to those reported in the literature for the other cell systems where caspases have been shown to function under non apoptotic conditions (Fernando, Kelly et al. 2002; Sordet, Rebe et al. 2002; Fernando, Brunette et al. 2005; Rebe, Cathelin et al. 2007; Koenig, Russell et al. 2008). Due to lack of the unique selectivity of artificial caspase substrates, it is not possible to assign a specific caspase as solely responsible for the observed cleavage (McStay, Salvesen et al. 2008). However, it is tempting to propose caspase-8 involvement in this instance as the only other family members capable of hydrolyzing this tetra peptide (caspase-3 and -6) (McStay, Salvesen et al. 2008) failed to cleave their much preferred DEVD.AMC substrate (Figure 3-2 A).

The presence of caspase-8 enzymatic activity during hypertrophic stimulation is interesting for two reasons: 1.) this caspase protease has been shown to influence the development of the murine heart and 2.) receptors associated with the extrinsic apoptotic pathway are known to play a prominent role in the cardiomyocyte growth. *Lpr* mice, which harbour a natural point mutation in the Fas receptor exhibit blunted

hypertrophic growth in response to arterial banding (Badorff, Ruetten et al. 2002). Moreover, stimulation of neonatal cardiomyocytes with Fas ligand results in hypertrophic growth and not in apoptosis, an observation that further underscores the importance of the Fas signaling axis for a proper hypertrophic response (Badorff, Seeger et al. 2005). TNF-α and its receptors also appear to drive cardiomyocyte hypertrophy as indicated by studies both in isolated adult cells (Yokoyama, Nakano et al. 1997) and in transgenic animals (Dibbs, Diwan et al. 2003). However, none of the aforementioned studies have examined the involvement of caspase-8 in the signaling cascades initiated by the "death" receptors in the heart.

Activity assays have also established that no active caspase-9 is present over the 18 hour time course of the hypertrophic stimulation. This finding is consistent with numerous studies that indicate silencing of genes implicated in the intrinsic apoptotic pathways in several fully differentiated tissues such as liver, skeletal muscle, brain, and retinal neurons (Burgess, Svensson et al. 1999; Yakovlev, Ota et al. 2001; Potts, Vaughn et al. 2005; Donovan, Doonan et al. 2006). Myocardium is not an exception in this list and the block to caspase activation appears to lie at the level of the apoptosome assembly. As a consequence, microinjection of purified cytochrome c is not sufficient to trigger apoptotic events in post natal cardiomyocytes (Potts, Vaughn et al. 2005). In addition, cardiomyocytes isolated from neonatal rats seem to be resistant to staurosporine treatment unless the key caspase- 9 activating protein apaf-1 is exogenously over-expressed (Sanchis, Mayorga et al. 2003). Furthermore, a study employing rat heart extract suggests that apoptosome assembly in the cardiomyocytes is also inhibited by high physiological ATP levels (Samali, O'Mahoney et al. 2007). Given our complete lack of understanding of the mechanisms controlling caspase activation under physiological, non cell-death conditions, it is

difficult to assess the consequences of faulty apoptosome assembly on the non-apoptotic caspase behaviour.

4.4 Effect of caspase inhibition on hypertrophic growth of isolated cardiomyocytes.

Many of the intracellular pathways that sense hypertrophic stimuli and stimulate the nucleus to engage in the hypertrophic remodeling have been deciphered and well characterized in the past fifteen years. Transcriptional circuitry underlying re-induction of embryonic genes has also received a significant amount of attention and the intricate interplay between various epigenetic regulators is emerging. Much less is known regarding the mechanisms that govern physical increase of the cell itself. Clearly, cardiomyocytes must increase protein synthesis to accomplish construction of new sarcomeres, which are added either in series or in parallel to the existing scaffolds (Nicol, Frey et al. 2001). A growing cardiomyocyte is also in need of major building blocks of cell membranes such as fatty acids and sterols (Porstmann, Santos et al. 2008); hence, pathways responsible for the synthesis of these macromolecules must somehow be hyper activated. The possibility that caspase enzymatic activities observed (Figure 3-2) may be involved in the regulation of processes that result in hypertrophic cell growth was therefore investigated. Using commercial caspase inhibitors directed against various family members as well as a potent broad range biological caspase inhibitor, it is demonstrated that caspase activity does not impact cardiomyocyte hypertrophy. Chemical pan.caspase inhibitors have been tested previously with no demonstrated effect (Pu, Ma et al. 2003). By targeting known regulators of cardiomyocyte growth such as large serine/threonine kinase mTOR and PP2B phosphatase (calcineurin) this investigation also demonstrates that cardiomyocyte growth is independent of caspase activity.

4.5 Hypertrophic marker induction in the presence of caspase inhibitors.

The end point of all pathologic hypertrophic signaling cascades irrespective of the stimulus is manifested in the induction of the natriuretic peptide expression. Functionally, these peptide hormones serve as attenuators of maladaptive hypertrophic growth but also serve as powerful predictors of the cellular behaviour of numerous upstream pathways responsible for activating their expression. In several biological systems, caspases were shown to influence various physiological processes via precise and controlled proteolysis of regulatory proteins (Fernando, Kelly et al. 2002; Cathelin, Rebe et al. 2006; Fujita, Crane et al. 2008). This investigation tests the hypothesis that these proteases may be utilized in a similar manner by the cardiomyocyte to add an additional level of control to caspase activation .To explore this possibility hypertrophic events were induced in isolated neonatal cardiomyocytes in the presence of caspase inhibitors. Using several expression analysis techniques with differing levels of sensitivity (immunocytochemistry and quantitative Real Time PCR), it is concluded that natriuretic peptide expression is not dependant on caspase function. This finding is further corroborated by the observation that behaviour of native promoters of both genes are not altered by caspase inhibition (Figures 3-9 and 3-10).

4.6 Influence of caspase activity on the behaviour of isolated transcription factors

The "Fetal gene" program characteristic of myocardial hypertrophy is driven by numerous developmentally important transcription factors (Oka, Xu et al. 2007). As such one of the objectives of this study was to investigate whether inhibition of caspase activity influences the behavior of two regulators of hypertrophic gene expression: MEF2 and Nf-κB.

Many Ca^{2+}-dependant signaling pathways in the hypertrophic cardiomyocyte converge on Mef2 transcription factors primarily through p38 directed phosphorylation, and potentially through ERk5, and PkC delta (Kolodziejczyk, Wang et al. 1999; Ornatsky, Cox et al. 1999; Liu, Cavanaugh et al. 2003). It was therefore asked whether activation of MEf2 dependent gene expression was dependant on caspase enzymatic activity. Experiments utilizing transient transfections of an artificial luciferase reporter containing tandem MEF2 consensus sequences, demonstrated that caspase activity does not influence the pathways that impinge on this family of regulators.

A subset of transcriptional modulators depend on proper MEF2 function for their recruitment to target promoters: MEF2is known to form physical interactions with GATA4, and dHAND transcription factors, increasing their affinity to corresponding sequences on selected cardiomyocyte-specific genes including ANF and BNP (Morin, Charron et al. 2000; Morin, Pozzulo et al. 2005). Therefore,, if caspase inhibition influenced MEF2 function, one may anticipate compromised response to the Anf and Bnp reporters. It is concluded that enzymatic function of caspase proteases is not part of the pathways, which activate MEF2 towards hypertrophic gene expression.

Unlike MEF2, precise mechanisms of how Nf-kB factors promote cardiomyocyte hypertrophy are much less clear. A few studies have shown that nf-kb nuclear/cytosolic shuttling during hypertrophy is mediated by canonical IkBa-dependant degradation (Purcell, Tang et al. 2001; Gupta, Purcell et al. 2002). Furthermore, caspases have been shown to modulate Nf-κB functions under non-apoptotic conditions (Lamkanfi, Declercq et al. 2006) in several other cell types. The possibility of whether similar mechanisms may be operating in the neonatal

cardiomyocytes was therefore tested. Loss of function experiments, utilizing both chemical and biological inhibitors, demonstrated that caspase activity was necessary for proper Nf-κB responsive luciferase reporter activation in response to phenylephrine stimulation. Failure to observe the same trend with serum exposure may be explained by the number of cytokines found in this mixture, which may activate parallel pathways that alter the transcriptional activity of various Nf-κB family members. Neither the ANF nor BNP promoter contains canonical Nf-κB binding sites. In addition, natriuretic peptide expression is not known to be controlled by Nf-κB factors (Temsah and Nemer 2005), an observation that may explain why the inhibitors did not influence activities of natriuretic peptide promoters (Figure 3-9 and Figure 3-10).

The mechanistic basis of how caspase inhibition alters Nf-κB transcriptional activity remains unknown. Several reports have identified p65 and p50 Nf-κB sub.units as direct substrates for caspase-3 (Ravi, Bedi et al. 1998; Kang, Lee et al. 2001) with cleavage of these proteins resulting in their complete inactivation. Clearly, these results do not account for the observations in the cardiomyocytes reported in the current investigation. The effect observed could be due to the inhibition of additional nf-kb inflammatory proteins, whose function is necessary for the maturation of cytokines that might be used to sustain hypertrophy *in vitro*. By inhibiting their action, a sufficient quantity of the mature cytokine is not produced, and hence proper stimulation of Nf-kB is jeopardized. In addition, the probability that the concatemer sequence taken from IL-4 promoter and used in the reporter assays is not recognized by other transcription factors that may be activated during hypertrophic stimulation cannot be completely ruled out. It would therefore be interesting to determine whether nuclear shuttling of p65/RelA (the principal effector

of Ikk-dependant canonical Nf-κB signaling) is at all influenced by caspase inhibition. Serum is known to contain various cytokines which can activate numerous signaling pathways capable of stimulating Nf-κb-responsive reporter. This likely explains why caspase inhibition is by-passed with this hypertrophic stimulant.

4.7 Conclusions and future research directions

In summary, the results presented above demonstrate that caspase enzymatic activity is not involved in the molecular events underlying prototypical hypertrophic response such as physical cell increase or expression of natriuretic peptides. The experiments were based on the premise that under non-apoptotic conditions, cardiomyocytes maintain/activate a small cytosolic pool of a fully mature protease. However, processing of pro-caspase zymogens is not at all required for the non-apoptotic caspase action as indicated by the numerous studies mostly focused on caspase-8 (Su, Bidere et al. 2005; Kang, Oh et al. 2008). Consistent with these findings, fully processed (p20/p10) caspase-8 tetramer is rarely captured in the cytosol under non-apoptotic conditions. Unlike caspases-3, -6, and -7, which exhibit no enzymatic activity in their unprocessed form, caspase-8 zymogen is much less efficiently constrained and is known to possess at least 1 % of the activity of a mature enzyme (Stennicke and Salvesen 1999; Timmer and Salvesen 2007). The active site architecture of an unprocessed monomer must be quite different from the mature enzyme which results in the reported altered substrate specificity (Chang, Xing et al. 2003). These observations raise the concern that the inhibitors used in this study may not target the catalytic cysteine residue of caspase found in the partially processed monomeric confirmation. Several recent studies confirm that enzymatic activity of both fully and partially processed caspase-8 molecules can in fact be effectively blocked with at least chemical inhibitors (Su, Bidere et al. 2005; Koenig, Russell et al.

2008). Recently, it was also shown that caspase-8 undergoes spatial restriction via the recruitment to membrane lipid rafts exclusively under non-apoptotic conditions (Su, Bidere et al. 2005; Koenig, Russell et al. 2008). This same study convincingly demonstrates that pharmacological inhibitors can successfully interfere with caspase catalytic properties within these membrane regions. Caspase inhibition strategies used to accomplish the objectives of this investigation therefore also address the possibility that partially processed, lipid-raft localized caspase-8 may be utilized for hypertrophic signaling.

In several instances caspases have been shown to influence signal transduction independent of their enzymatic activity (Chaudhary, Eby et al. 2000; Kreuz, Siegmund et al. 2004; Jun, Chung et al. 2005). In these cases, they act as scaffolds aiding in the assembly of selected signaling complexes. Experimental approaches used in this study do not take this possibility into consideration. Perhaps, by using RNA interference for depletion of individual family members it would be possible to confirm or to refute this possibility.

It is also presently unclear, which receptor(s) may be used by the cardiomyocytes for the activation of Nf-κB pathway. In lymphocytes, where Nf-kB factors have been exhaustively studied, stimulation of T-cell receptor, Toll-like receptors, TRAIL receptor (DR4) , TNFα receptors, and Fas (CD95) receptors have been shown to induce Nf-κB signaling (Perkins 2007). Caspase enzymatic activity is absolutely essential for the proper activation of Nf-kB in these cell types: human patients with caspase-8 deficiency, exhibit combined immunodeficiency due to defective activation of Nf-κB in T-cells, B-cells, and in natural killer cells (Chun, Zheng et al. 2002; Su, Bidere et al. 2005). As mentioned above, exposure of cardiomyocytes to supraphysiological levels of both Fas and TNFα lead to

cardiomyocyte hypertrophy and not to apoptosis. It will be interesting to determine whether Nf-kB factors a.) undergo activation once these receptors are agonized and b.) whether the activation is dependant on caspase enzymatic activity. It is worth mentioning that human patients with inactivating mutations of the *caspase-8* gene, do not present congenital or otherwise manifest myocardial defects. Furthermore, caspase-10, a closely related family member, does not prevent the combined immunodeficiency in these patients suggesting that functional compensation in the heart does not explain the lack of phenotype. The identity of the exact Nf-κB transcription factor mediating the effect observed is not readily apparent from the data obtained in this study.

4.8 Advantages and disadvantages of an *in vitro* neonatal cardiomyocyte cell culture as a model for pathological myocardial hypertrophy.

For all the experiments outlined above, cultures of neonatal rat ventricular cardiomyocytes (NRVMs) were employed as an experimental model. Cardiomyocyte tissue culture has been widely used as a model for studying physiological and biochemical characteristics of the mammalian heart since its establishment by Harary and Farley in 1966 (Harary, McCarl et al. 1966). When explanted from the neonatal heart, these cells offer all the advantages of the controlled and manipulative environment of the tissue culture system while maintaining stable phenotypes *in vitro* and faithfully recapitulating most of the signaling pathways mediating hypertrophic responses *in vivo*. Hypertrophic events in the NRVM cultures can be easily replicated by a variety of approaches most popular of which include use of agonists of -α or -β adrenergic receptors, phorbol-ester-mediated stimulation of PKC, addition of serum to serum starved cultures, as well as stimulation of cytokine-activated gp-130 receptor (Wollert, Taga et al. 1996).

In this study, cardiomyocyte hypertrophy was achieved either by the stimulation with an α-adrenergic agonist phenylephrine (PE) or with 10% fetal bovine serum treatment (FBS). The rationale for using these agonists was based on the fact that each agonist uses very distinct mechanisms to promote hypertrophy, i.e. phenylephrine leads to increases in cytosolic Ca^{2+} through phospholipase C activation, while addition of serum to serum starved cardiomyocytes triggers a distinct subset of multiple signaling pathways that drive cell growth. The choice of these stimulants are likely to be more reflective of the natural *in vivo* response, where both neurohormonal and cytokine signals contribute to the hypertrophic phenotype.

Although used as the primary model for studying signaling pathways in pathological cardiac hypertrophy, isolated neonatal cardiomyocytes present several problems inherent to most cell culture systems. These limitations include absence of the extra cellular matrix, lack of interactions with other cell-types, and unnatural two dimensional architecture of the growth environment. In addition to these concerns, neonatal cardiomyocytes are typically distinct from the cell that is typically engaged in hypertrophy, the adult cardiomyocyte. For example, during the first week of perinatal life the vast majority of cardiomyocytes permanently loose their proliferative capacity, suppress fetal isoforms of contractile genes/proteins, as well as factors that control metabolism and calcium homeostasis. Therefore, neonatal cardiomyocytes may not be the most appropriate cell type to model typical pathologic cardiac hypertrophy. (Argentin, Ardati et al. 1994).

The inherenet limitations of neonatal cardiomyocytes may explain why the promoters used for the reporter assays in the current study (See Figure 3-9 through Figure 3-11) demonstrate baseline activity. For example, pathways, which are normally not operational in young and adult cardiomyocytes still persist in the

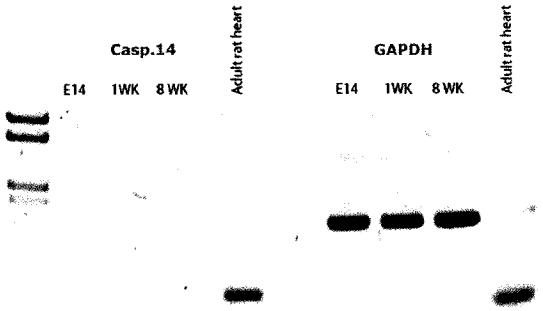
cardiomyocytes isolated from neonatal rats. Failure to properly suppress *anf* expression also accounts for the fact that at least 10% of the cardiomyocytes are positive for the ANF stain (Figure 3-6 and Figure 3-7) and express detectable levels of both ANF and BNP transcripts (Figure 3-8) under the untreated conditions. Isolation procedure itself exerts a sufficient degree of stress to trigger key stress sensing pathways leading to the activation of the hypertrophic gene expression in a subset of cells.

To circumvent these shortcomings, cardiomyocytes isolated from adult hearts may provide less ambiguous and more biologically relevant results. However, adult cardiomyocytes are extremely sensitive to the changes in calcium concentrations making their successful isolation and maintenance a significant technical challenge. Perhaps, a transgenic model that allows for the manipulation of caspase function *in vivo,* would ultimately serve as the most practical and as the most informative complementary approach to the *in vitro* neonatal cardiomyocyte model used for this study.

APPENDICES

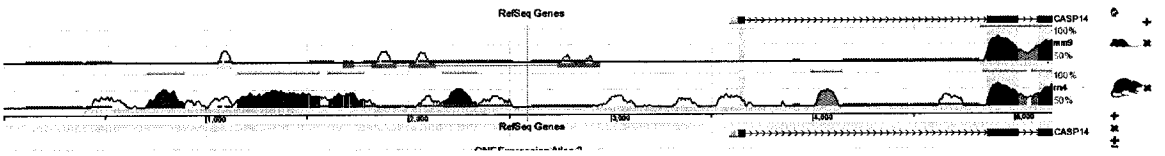
Appendix A. Caspase-14 expression analysis during murine heart development.

Supplemental Figure 1. RT-PCR showing the absence of caspase-14 transcript throughout developmental stages assayed in the mouse heart.

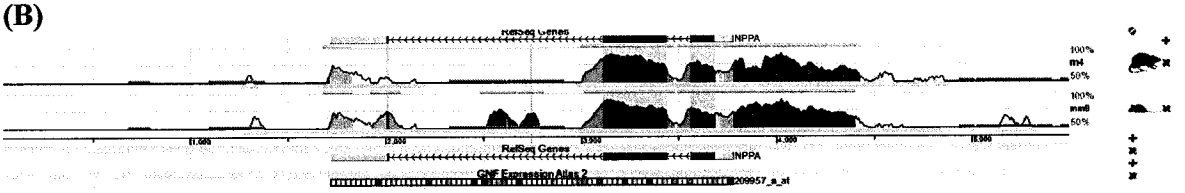


Supplemental Figure 2. Alignment of ~5 kb of cis-regulatory regions upstream of transcription start site of *caspase-14* gene (i) showing no conservation between a mouse and a rat. and (ii) *nppa* (anf gene).

(A)

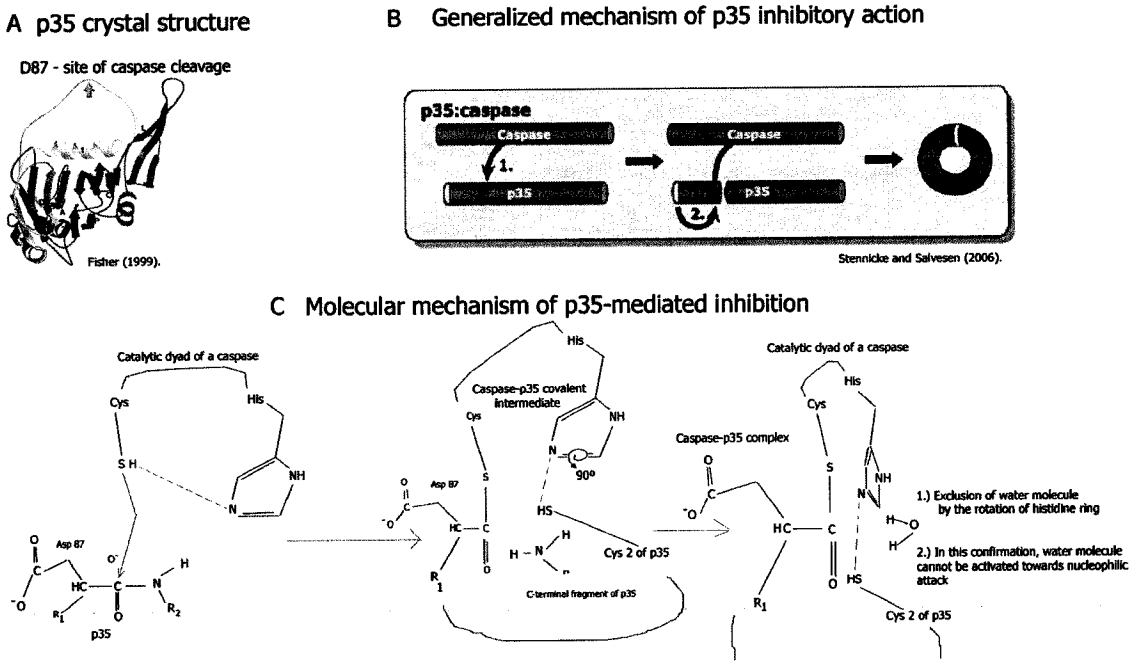


(B)



Appendix B.Properties of caspase inhibitory protein from baculovirus ***Autographica californica nucleopolyhedrovirus.***

Supplemental Figure 3. Properties and mode of action of baculoviral p35 caspase inhibitory protein



Supplemental Table 1. Dissociation constants of p35 towards various mature (processed) mammalian caspases.

Caspase	K_i (nM)
Caspase-1	9
Caspase-2	Unknown
Caspase-3	0.1
Caspase-4	Unknown
Caspase-6	0.4
Caspase-7	2.0
Caspase-8	0.5
Caspase-9	Unknown
Caspase-10	7.0

Adapted from Los and Walczak, 2002.

Appendix C.
Calculations:
A.) Used to determine viral titer and multiplicity of infection:

$A_{260\ (1)} = 0.053$; $A_{260\ (2)} = 0.044$; $A_{260\ (3)} = 0.074$;

Average $A_{(260)} = 0.057$;

Total Viral Particles/mL = Absorbance @ A_{260} * 1.1 * 10^{12} * dilution factor (5) = 0.31 * 10^{12} vp/mL;

Infectious Particle Units = Total Viral Particles / 50 = 6.27 * 10^{9} ipu/mL;

Desired MOI => **50 infectious viral particles per cell** or 50 ipu/cell;

# of cells to be infected = 2.5 * 10^{5};

# of ipu needed = 2.5 * 10^{5} cells * 50 infectious particles per cell = 1.25 * 10^{7} infectious particles;

Volume to achieve this=2.5*10^{7} particles / 6.27 * 10^{9} ipu/mL=0.003987 =3.99 * 10^{-3} mL ~ ***4 μL***.

B.) Real-time data processing (comparative $\Delta\Delta C_t$ method of relative quantification):

Normalized to the internal housekeeping gene presumed not to change as a result of a treatment (so-called "reference").

All results are presented relative to the untreated cells (so called "calibrator").

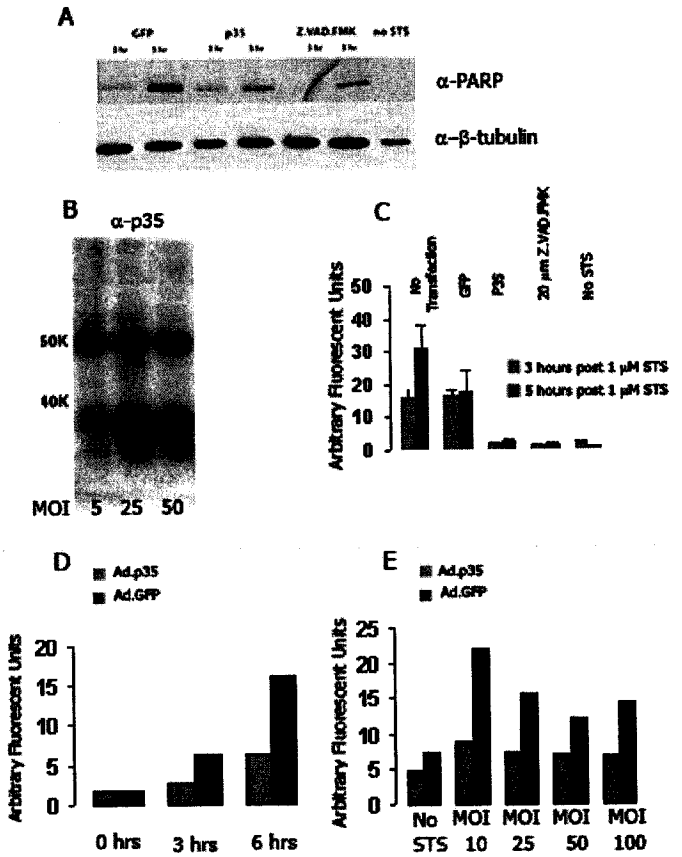
C_t – cycle threshold – cycle # at which fluorescence increases above manually set threshold in fluorescence intensity.

$1.8^{-\Delta\Delta C_t}$ - 1.8 is the efficiency of amplification in the linear phase of the reaction (i.e. 1.8 – fold increase in the numbers of amplicons generated with each subsequent cycle); the ideal theoretical value of 2 has been arbitrary assigned to 1.8 to account for a possibility in differences between primer efficiencies.

Sample	***ANF*** (average Ct based on triplicate rxns set up simultaneously).	***GAPDH*** Average Ct based on triplicate rxns set up simultaneously)	ΔC_t (ANF - GAPDH)	$\Delta\Delta CT$ (ΔC_t untreated-DCt treated)	**ANF relative to untreated** $1.8^{-\Delta\Delta Ct}$
Untreated	18.15	15.14	3.01	0	1
Phenylephrine	15.42	15.02	0.4	-2.61	6.11
10% FBS	14.98	15.75	-0.77	-3.78	13.74

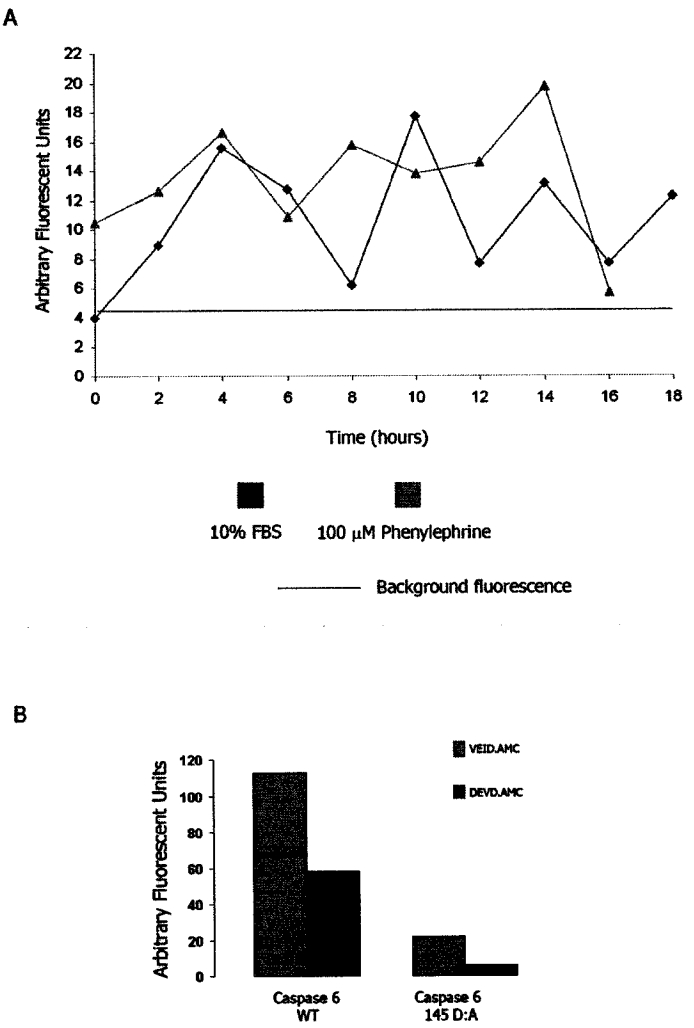
Appendix D.

Supplemental Figure 4. Expression and validation of caspase inhibitory properties of p35 under various apoptotic conditions:



A.) Transiently transfected p35 inhibits PARP cleavage in C2C12 cells following STS treatment. B.) P35 expression is detected on the western blot following infection of H9C2 cells using various quantities of adenovirus. C.) Transiently transfected p35 inhibits caspase -3/-7 activities in C2C12 cells following STS treatment. D.) Adenovirally expressed p35 inhibits caspase-3/-7 activities in H9C2 cells and in primary cardiomyocytes (E).

Appendix E. Supplemental Figure 5.

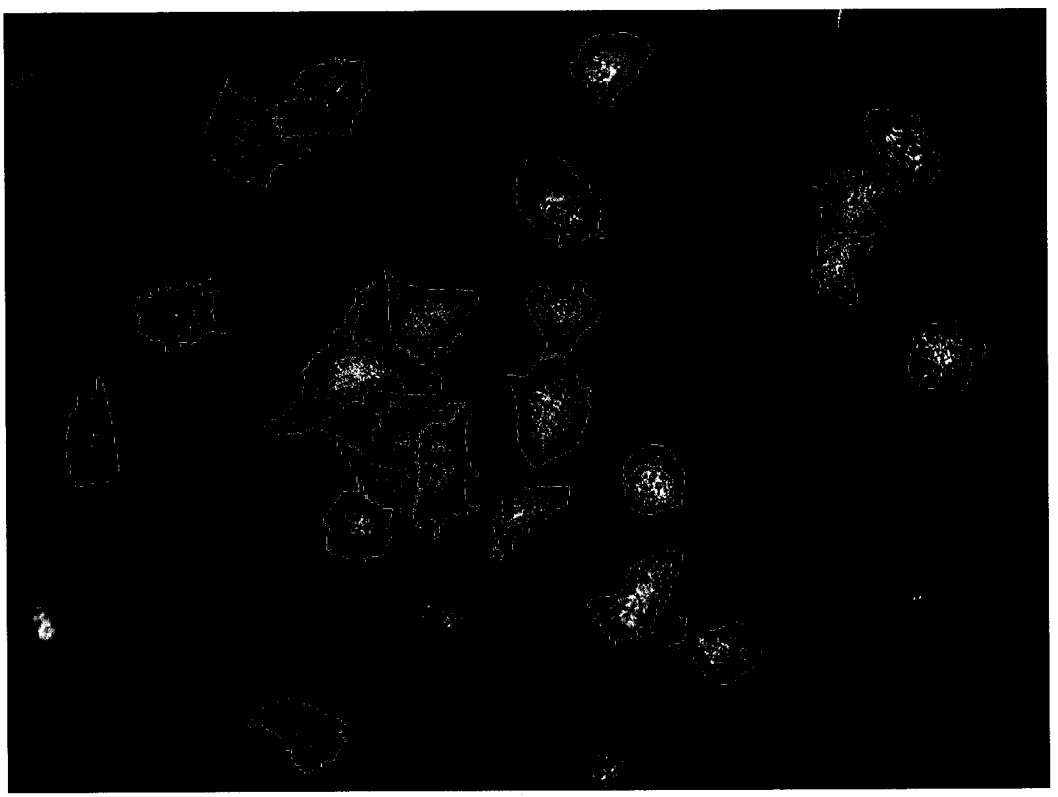


A.) VEID-ase activity determined in primary cardiomyocytes over 18 hours of stimulation with indicated hypertrophic agonists
B.) Fluorescent caspase activity assay demonstrating that wild type caspase-6 over expressed in HEK293 cells is capable of efficient DEVD.AMC cleavage.

Appendix F. Outline of the pipeline settings used for quantification of cardiomyocyte cell areas.

LoadImages
IdentifyPrimAutomatic
FindEdges
MeasureObjectIntensity
Measure ObjectAreaShape
OverlayOutlines
SaveImages
ExportToExcel

Supplemental Figure 6. Sample picture demonstrating how the software indentifies individual cells.



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