THE ROLE OF PROTEIN KINASE B/AKT
IN PATHOGENESIS OF COXSACKIEVIRUS B3 MYOCARDITIS

By

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ABSTRACT

Viral myocarditis is a major cause of sudden cardiac death in children and young adults. Among viruses, coxsackievirus B3 (CVB3), a small non-enveloped single-stranded RNA enterovirus in the Picornaviridae family, is the most prominent infective agent for myocarditis, causing myocardial injury that, at times leads to end-stage dilated cardiomyopathy. Recently, more consideration has been devoted to the role of signalling pathways in the pathogenesis of enteroviral myocarditis as a route for identifying new potential therapeutic targets. The role of signalling proteins including the extracellular signal-regulated kinase (ERK1/2 MAPK), stress-activated protein kinase c-Jun N-terminal kinase (JNK), and p38 mitogen-activated protein kinase (p38 MAPK) during CVB3 infection has been comprehensively studied by us and others. However, the potential regulatory function of other kinases such as the survival signalling molecule protein kinase B/Akt remains elusive. In the present study, we have investigated the potential regulatory function of the PI3K/Akt pathway in progression of CVB3-induced myocarditis.

Our findings demonstrate that CVB3 enhances Akt phosphorylation through a phosphatidylinositol-3 kinase (PI3K)-dependent mechanism. Inhibition of Akt significantly suppresses viral RNA expression and progeny release while increasing apoptosis of infected cells. Interestingly, inhibition of apoptotic events does not reverse the inhibitory effect of Akt inhibition on virus life replication, suggesting that the observed regulatory role of Akt is not dependent on the anti-apoptotic function of this protein kinase.
Virus-induced Akt phosphorylation in mouse cardiomyocytes and HeLa cells is also mediated through the integrin-linked kinase (ILK). Inhibition of ILK significantly reduces viral replication and progeny release and improves viability of infected cardiomyocytes. Over-expression of a constitutively active form of Akt protein within virus-infected cells has provided evidence that the protective effect of ILK inhibition is dependent on associated down-regulation of virus-induced Akt activation.

In the present study, we also investigated the role of the transcription factor nuclear factor kappa-B (NFkB) as a candidate downstream mediator of signals through the PI3K/Akt cascade in regulating CVB3 replication and virus-mediated cytopathic effects. Our findings suggest that activation of NFkB is an early event with an anti-apoptotic function. Inhibition of PI3K and Erk1/2 pathways significantly blocks virus-induced NFkB activation, proposing a major role for these kinases in regulation of host transcriptional events during CVB3 infection. Most importantly, short-term treatment of infected cells with tumor necrosis factor-α (TNF-α), a potent activator of NFkB, rescues infected cells, apparently through increasing the DNA-binding activity of NFkB. Long-term treatment with TNF-α, however, has been shown to be detrimental to the host cell, emphasizing the importance of the delicate balance between deleterious and protective effects of NFkB activation.

In summary, this dissertation presents the first report on the regulatory role of the PI3K/Akt pathway during the course of enteroviral infection, provides valuable information on molecular mechanisms underlying CVB3 pathogenesis, and offers new
insights to our effort in establishing an effective therapeutic approach to alleviate enteroviral myocarditis.
TABLE OF CONTENTS

ABSTRACT ii
TABLE OF CONTENTS v
LIST OF TABLES viii
LIST OF FIGURES ix
LIST OF ABBREVIATIONS xii
ACKNOWLEDGEMENT xvii
DEDICATION xviii

CHAPTER 1: INTRODUCTION TO COXSACKIEVIRUS B3 MYOCARDITIS

1-1 Overview of Myocarditis 2
1-2 Viral Myocarditis 5
1-3 Molecular Biology of Coxsackievirus B3 8
1-4 Experimental Murine Model of CVB3 Myocarditis 12
1-5 Mechanisms of CVB3-Induced Myocardial Injury 16
1-6 Death Signalling Pathways 21
1-7 Survival Signalling Pathways 28
1-8 Host Signalling Determinants of CVB3 Pathogenesis 38
1-9 References 41
1-10 Tables & Figures 75

CHAPTER 2: SUMMARY OF RESEARCH PROPOSAL, CENTRAL HYPOTHESIS, AND SPECIFIC AIMS
2-1 Background 89
2-2 Central Hypothesis 90
2-3 Main Objective 90
2-4 Specific Aims 90
2-5 Prospective 91
2-6 Figures 93

CHAPTER 3: PROTEIN KINASE B/AKT IN COXSACKIEVIRUS B3 INFECTION

3-1 Introduction 95
3-2 Materials & Methods 98
3-3 Results 103
3-4 Discussion 108
3-5 References 112
3-6 Figures 117

CHAPTER 4: INTEGRIN-LINKED KINASE AS A THERAPEUTIC TARGET FOR COXSACKIEVIRUS B3 MYOCARDITIS

4-1 Introduction 134
4-2 Materials & Methods 137
4-3 Results 142
4-4 Discussion 149
4-5 References 154
4-6 Figures 160
CHAPTER 5: NUCLEAR FACTOR KAPPA-B IN COXSACKIEVIRUS B3 INFECTION

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-1 Introduction</td>
<td>185</td>
</tr>
<tr>
<td>5-2 Materials &amp; Methods</td>
<td>188</td>
</tr>
<tr>
<td>5-3 Results</td>
<td>192</td>
</tr>
<tr>
<td>5-4 Discussion</td>
<td>196</td>
</tr>
<tr>
<td>5-5 References</td>
<td>200</td>
</tr>
<tr>
<td>5-6 Figures</td>
<td>205</td>
</tr>
</tbody>
</table>

CHAPTER 6: SUMMARY, CONCLUSION AND FUTURE DIRECTIONS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>6-1 Summary</td>
<td>219</td>
</tr>
<tr>
<td>6-2 Conclusion</td>
<td>220</td>
</tr>
<tr>
<td>6-3 Future Directions</td>
<td>221</td>
</tr>
<tr>
<td>6-4 References</td>
<td>225</td>
</tr>
<tr>
<td>6-5 Figures</td>
<td>227</td>
</tr>
</tbody>
</table>
LIST OF TABLES

CHAPTER 1

Table 1-1: Viruses reported in association with myocarditis 75
LIST OF FIGURES

CHAPTER 1

Figure 1-1: CVB3 genome, viral proteins, and their potential functions 76
Figure 1-2: CVB3 life cycle 78
Figure 1-3: Three distinct phases of viral myocarditis 80
Figure 1-4: Proposed model for immune-mediated cell injury in CVB3 myocarditis 81
Figure 1-5: CVB3-induced cell death 83
Figure 1-6: Cellular events in CVB3-infected cells 84
Figure 1-7: Integrin-linked kinase (ILK) signalling 85
Figure 1-8: Downstream targets of the PI3K/Akt pathway 86
Figure 1-9: Classical and alternative pathways of NFkB activation 87

CHAPTER 2

Figure 2-1: Schematic diagram of general hypothesis and specific aims 93

CHAPTER 3

Figure 3-1: CVB3 infection leads to phosphorylation and activation of Akt on both serine 473 and threonine 308 residues 117
Figure 3-2: Post-entry viral replication induces Akt phosphorylation via a PI3K-dependent mechanism 119
Figure 3-3: LY294002 blocks viral VP1 synthesis, viral RNA expression, and viral release in infected HeLa cells 121
Figure 3-4: Dominant-negative mutant of Akt1 blocks viral VP1 synthesis, Viral RNA expression, and viral release in infected HeLa cells

Figure 3-5: LY294002 and dominant-negative mutant of Akt1 induce apoptosis in CVB3-infected cells

Figure 3-6: Akt regulates CVB3 replication through a caspase-independent pathway

CHAPTER 4

Figure 4-1: CVB3 infection leads to Akt phosphorylation on Ser-473 and Thr-308 in mouse cardiomyocytes

Figure 4-2: CVB3 induces Akt phosphorylation via an ILK-dependent mechanism

Figure 4-3: ILK inhibition blocks CVB3 structural protein (VP1) synthesis and viral release in infected cardiomyocytes

Figure 4-4: ILK inhibition significantly decreases viral protein VP1 and viral RNA synthesis in infected HeLa cells

Figure 4-5: Specific ILK inhibitor QLT0267 markedly decreases CVB3-induced cytopathic effects in HL-1 mouse cardiomyocytes

Figure 4-6: ILK inhibition significantly decreases virus-induced cytopathic effects in infected HeLa cells

Figure 4-7: Adenoviral transfection of HL-1 mouse cardiomyocytes with a constitutively active form of Akt1 and GFP control

Figure 4-8: Constitutively active form of Akt1 increases viral protein synthesis and virus progeny release, and enhances cell death in infected cardiomyocytes

Figure 4-9: Constitutively active form of Akt1 reverses the protective effect of ILK inhibition in CVB3-infected HeLa cells

Figure 4-10: Beta-1 and beta-3 integrin subunits are not involved in CVB3 entry and replication in mouse cardiomyocytes and HeLa cells
CHAPTER 5

Figure 5-1: CVB3 induces translocation of NFkB p65 subunit into the nucleus of infected HeLa cells 205

Figure 5-2: CVB3 infection enhances DNA-binding activity of NFkB 207

Figure 5-3: Inhibition of NFkB activation in CVB3-induced HeLa cells significantly reduces the viability of infected cells 209

Figure 5-4: CVB3 infection results in phosphorylation of IKK-α but not IKK-β 210

Figure 5-5: Inhibition of PI3K and Erk1/2 pathways markedly reduces DNA-binding activity of NFkB 211

Figure 5-6: TNF-α treatment increases NFkB activity in CVB3-infected cells 213

Figure 5-7: TNF-α treatment does not affect CVB3 replication in HeLa cells 215

Figure 5-8: Short-term and early treatment of infected cells with TNF-α significantly restrains virus-induced cytopathic effects and augments host cell viability 216

CHAPTER 6

Figure 6-1: Synopsis of experimental findings, central hypothesis, and specific aims 227
### LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>AIF</td>
<td>Apoptosis-inducing factor</td>
</tr>
<tr>
<td>ANT</td>
<td>Adenine nucleotide translocator</td>
</tr>
<tr>
<td>Apaf-1</td>
<td>Apoptosis protease cytosol factor-1</td>
</tr>
<tr>
<td>APC</td>
<td>Antigen presenting cell</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic adenosine mono-phosphate</td>
</tr>
<tr>
<td>CAR</td>
<td>Coxsackievirus and adenovirus receptor</td>
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<tr>
<td>CHO</td>
<td>Chinese hamster ovary</td>
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<tr>
<td>Cyt c</td>
<td>Cytochrome c</td>
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<tr>
<td>CAD</td>
<td>Caspase-activated DNase</td>
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<td>cFLIP</td>
<td>Cellular FLIC inhibitory protein</td>
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<td>CNS</td>
<td>Central nervous system</td>
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<td>CPE</td>
<td>Cytopathic effect</td>
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<tr>
<td>CREB/ATF</td>
<td>cAMP response element binding protein/activating transcription factor</td>
</tr>
<tr>
<td>CVB3</td>
<td>Coxsackievirus B3</td>
</tr>
<tr>
<td>DAF</td>
<td>Decay accelerating factor (CD55)</td>
</tr>
<tr>
<td>DCM</td>
<td>Dilated cardiomyopathy</td>
</tr>
<tr>
<td>DFF</td>
<td>DNA fragmentation factor</td>
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<tr>
<td>DISC</td>
<td>Death inducing signalling complex (DISC)</td>
</tr>
<tr>
<td>DIABLO</td>
<td>Direct inhibitor of apoptosis-binding protein with low pI</td>
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<tr>
<td>EBV</td>
<td>Epstein-Barr virus</td>
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<td>EMCV</td>
<td>Encephalomyocarditis virus</td>
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<tr>
<td>EGF</td>
<td>Epidermal growth factor</td>
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<tr>
<td>eIF4G</td>
<td>Eukaryotic initiation factor 4γ</td>
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<td>EndoG</td>
<td>Endonuclease G</td>
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<tr>
<td>ERK</td>
<td>Extracellular signal-regulated kinase</td>
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<td>FADD</td>
<td>Fas associated death domain</td>
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<td>FAK</td>
<td>Focal adhesion kinase</td>
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<td>FKHR</td>
<td>Forkhead transcription factor</td>
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<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
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<tr>
<td>GM-CSF</td>
<td>Granulocyte/macrophage colony stimulating factor</td>
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<tr>
<td>GPI</td>
<td>Glycosyl-phosphatidylinositol</td>
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<tr>
<td>GSK3-β</td>
<td>Glycogen synthase kinase 3-beta</td>
</tr>
<tr>
<td>H3</td>
<td>Histon H3</td>
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<tr>
<td>HEK</td>
<td>Human epithelial kidney</td>
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<tr>
<td>HLA</td>
<td>Human leukocyte antigen</td>
</tr>
<tr>
<td>HSP</td>
<td>Heat shock protein</td>
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<tr>
<td>HTRA2</td>
<td>High temperature-requiring protein A2</td>
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<td>IAP</td>
<td>Inhibitor of apoptosis</td>
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<tr>
<td>ICAM</td>
<td>Intracellular adhesion molecule</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>Interferon gamma</td>
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<tr>
<td>ICAD</td>
<td>Inhibitor of caspase-activated deoxy-ribonuclease</td>
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<tr>
<td>IGF</td>
<td>Insulin-like growth factor</td>
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<td>Immunoglobulin class G</td>
</tr>
<tr>
<td>IgM</td>
<td>Immunoglobulin class M</td>
</tr>
<tr>
<td>IGTP</td>
<td>Interferon γ-inducible GTPase</td>
</tr>
<tr>
<td>IKB</td>
<td>Inhibitor of nuclear factor kappa B</td>
</tr>
<tr>
<td>IKK</td>
<td>Inhibitor of IkB kinase</td>
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</table>
IMS  Inter-membrane space
iNOS  Inducible nitric oxide synthase
IL   Interleukin
ILK  Integrin-linked kinase
ILKAP ILK-associated phosphatase
IRES  Internal ribosome entry site
ISH  In situ hybridization
JAK  Janus kinase
kb  Kilobase
kDa  Kilodalton
MAPK  Mitogen activated protein kinase
MHC  Major histocompatibility complex
ML-IAP  Melanoma inhibitor of apoptosis
MMP  Matrix metalloproteinase
MPT  Mitochondrial permeability transition
mRNA  messenger RNA
mTOR  Mammalian target of rapamycin
NFAT  Nuclear factor of activated T cell
NFkB  Nuclear factor kappa B
NK  Natural killer cell
NO  Nitric oxide
Oct-1  Octamer-binding transcription factor-1
ORF  Open reading frame
PARP  Poly ADP-ribose polymerase
PDGF  Platelet-derived growth factor
<table>
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<th>Full Name</th>
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<tbody>
<tr>
<td>PDK-1</td>
<td>Phosphatidylinositol-dependent kinase</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphatidylinositol-3 kinase</td>
</tr>
<tr>
<td>PINCH</td>
<td>Particularly interesting new cysteine-histidine rich protein</td>
</tr>
<tr>
<td>PKA</td>
<td>Protein kinase A</td>
</tr>
<tr>
<td>PKB</td>
<td>Protein kinase B</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein kinase C</td>
</tr>
<tr>
<td>PKG</td>
<td>Protein kinase G</td>
</tr>
<tr>
<td>PLCγ</td>
<td>Phospholipase gamma</td>
</tr>
<tr>
<td>PtdIns</td>
<td>Phosphatidylinositol</td>
</tr>
<tr>
<td>PTEN</td>
<td>Phosphatase and tensin homolog deleted on chromosome 10</td>
</tr>
<tr>
<td>RHD</td>
<td>Rel homology domain</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic Acid</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>RTK</td>
<td>Receptor tyrosine kinase</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse transcription polymerase chain reaction</td>
</tr>
<tr>
<td>SAPK</td>
<td>Stress-activated protein kinase</td>
</tr>
<tr>
<td>SCIC</td>
<td>Severe combined immunodeficiency</td>
</tr>
<tr>
<td>SCR</td>
<td>Short consensus repeat</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
</tr>
<tr>
<td>SH2</td>
<td>Src homology-2</td>
</tr>
<tr>
<td>SMAC</td>
<td>Second mitochondria-derived activator of caspases</td>
</tr>
<tr>
<td>TCR</td>
<td>T-cell receptor</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming growth factor beta</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll like receptor</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumor necrosis factor-alpha</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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</tr>
<tr>
<td>TNFR</td>
<td>Tumor necrosis factor receptor</td>
</tr>
<tr>
<td>TRADD</td>
<td>Tumor necrosis factor receptor associated death domain</td>
</tr>
<tr>
<td>TRAIL</td>
<td>TNF-α receptor apoptosis inducing ligand</td>
</tr>
<tr>
<td>UTR</td>
<td>Untranslated region</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
<tr>
<td>VP1</td>
<td>CVB3 capsid protein VP1</td>
</tr>
<tr>
<td>VPg</td>
<td>Viral protein g</td>
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</tbody>
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ACKNOWLEDGEMENTS

I am deeply thankful to have had the opportunity to work with Dr. Bruce McManus, my supervisor. Dr. McManus has been a great mentor, and his dedication and commitment to scientific excellence has guided me in my academic and scientific endeavors over the past few years, and will continue to inspire me through my future career.

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To my beloved husband, Hamid, and My wonderful son, Arash, who fill my life with love and joy

And to my parents, Pooran and Morteza Esfandiarei, and my brother Manouchehr, for their unconditional love
CHAPTER 1: INTRODUCTION TO COXSACKIEVIRUS B3 MYOCARDITIS

Based on the Manuscript:
Signalling events in coxsackievirus B3 myocarditis: The decision between life and death. Mitra Esfandiarei and Bruce McManus (manuscript in preparation).
1-1. OVERVIEW OF MYOCARDITIS

Myocarditis, by definition, is an inflammatory disease of the myocardium. Sobernheim [1] initially established the term in 1837. Thereafter, Feidler [2] used the term “isolated idiopathic interstitial myocarditis” to explain the disease process. In 1948, Burch and Ray [3] introduced the first clear description of clinical manifestations of acute and chronic forms of myocarditis based on histological studies on postmortem samples. Today, myocarditis is clinically defined as a cardiac disease associated with inflammation of the myocardium and necrosis and/or degeneration of adjacent myocyte in the absence of ischemic event [4-7].

i. Etiology

Various etiologies for myocarditis include toxic substances, allergic and hypersensitivity responses, immune diseases like rheumatic fever and systemic lupus erythematosus, cardiac transplant rejection, and infectious agents such as bacteria, viruses, protozoa, and fungi [8-10]. Myocarditis due to infectious agents has proven to be the most common form worldwide. In Central and South America, myocarditis is highly associated with Chagas disease caused by Trypanosoma Cruzi, whereas viruses are the primary infective agents in North American and developed countries [11-15].

ii. Epidemiology

The “true” incidence of myocarditis is not clear, largely due to asymptomatic, subclinical, and misdiagnosed cases. Early knowledge of the epidemiology of myocarditis came from postmortem studies. In late 1970s, introduction of
endomyocardial biopsy provided the clinicians and scientists with a useful diagnostic tool to assess the potential myocarditis in living patients [16]. Thereafter, the Dallas histological criteria were introduced to eradicate the problem of discrepancies in diagnostic evaluation of endomyocardial biopsies [4,5]. Yet, examination of endomyocardial biopsy samples from patients with new-onset congestive heart failure, has revealed a wide-ranging incidence of 0% to 67% for myocarditis [17-30]. The inconsistencies are most likely due to differences in host ethnic and genetic backgrounds, susceptibility to infection, virus epidemiology, sampling errors, diagnostic application of Dallas histological criteria, and the lack of unanimity among pathologists as to what constitutes myocarditis [31,32]. However, despite the low sensitivity, endomyocardial biopsy still represents the gold standard for diagnosis of myocarditis [9,33].

Early postmortem studies on autopsy series from victims of sudden accidental death reported a prevalence of 2.7-5% for myocarditis [34-36]. Later, a 10-year study based on 12,747 consecutive autopsies from victims of accidental death in Sweden, performed between 1975 and 1984, suggested a lower prevalence (1%) for myocarditis in the general population [37].

Analysis of four studies published between 1968 and 1975 has concluded that almost 52% of myocarditis occur among children and young adults (under 40 years), 14% in those aged 40 to 59 years and only 5% in patients over 60, indicating that susceptibility to viral myocarditis is likely age-related [38]. Other factors including gender, nutrition, and pregnancy may also contribute to susceptibility to viral myocarditis [11,39-42]. Young children, particularly infants under six months of age, are extremely sensitive to
enteroviral infections [11,43-45]. Myocarditis is the cause of approximately 20% of sudden unexpected death among young children [35,46-50]. Using molecular diagnostic techniques, the enteroviral sequences were isolated from 17-21% of heart biopsies taken from children with acute myocarditis [51-53].

iii. Diagnosis & therapy

Myocarditis is often asymptomatic and only around 10% of cases present with typical symptoms including chest pain, palpitation, ventricular dysfunction, and acute heart failure. Histological analysis according to the Dallas criteria is often inadequate in the diagnosis of myocarditis [54,55]. Thus, additional histoimmunological, serological, electrophysiological, and genetic analyses, as well as a detailed history and careful physical examination will allow a more definitive diagnosis [56,57].

The isolation of virus particles from adult heart tissue is highly uncommon. Enteroviruses circulate rapidly within the community, so the majority of the adults have had a prior exposure to these viruses. As a result, the isolation of virus particles from the peripheral tissues rather than the heart, and serological studies measuring the titer of circulating antibodies will confuse the diagnosis and are usually unrewarding. In recent years, advances in molecular techniques such as in situ hybridization (ISH) and reverse transcription polymerase chain reaction (RT-PCR) have facilitated the diagnosis of viral myocarditis and increased certainty of the histology-based diagnosis [11].

The therapy is mostly symptomatic, and is dependent on the etiology and presenting symptoms. The standard heart failure management should be considered in fulminant
myocarditis. When a patient presents with symptoms of end-stage DCM, supportive therapy and/or heart transplantation must be considered. The 5-year survival in biopsy-proven myocarditis/DCM is approximately 50% [29]. Currently, specific antiviral drugs for viral myocarditis are under development and evaluation. Antiviral drugs such as Isoxazoles (WIN 54954) and Pleconaril (VP 63843) are broad-spectrum capsid function inhibitors that block enterovirus uncoating, and have shown some protective effects [58-62]. Pleconaril, an experimental drug available on a compassionate basis, binds the hydrophobic pocket in viral capsid protein 1 (VP1), inhibiting enteroviral attachment and entry [50,58,63]. CVB3 Nancy strain is the only prototypic enterovirus that is resistant to Pleconaril [64]. Very recently, Schmidtke et al. [65] have shown that the amino acid substitution Ile-1092→Leu or Ile-1092→Met in the hydrophobic pocket in VP1 is responsible for observed resistance in laboratory Nancy strain.

In recent years, a few clinical trials using Pleconaril in children and adults have reported a decrease in severity and duration of symptoms in patients with enteroviral meningitis and upper respiratory tract infections [45,66,67]. Although immunosuppressive drugs such as prednisone have shown some promising effects, the result of an 18 year systemic review of the impact of immunosuppressive therapy on the outcome of acute myocarditis in children, has failed to prove any significant benefit [68]. As a result, the use of immunosuppressive therapy for treatment of acute and/or chronic viral myocarditis is still the crux of ongoing debates.

1-2. VIRAL MYOCARDITIS
Common clinical presentations of viral myocarditis vary from febrile illness with flu-like symptoms and/or gastrointestinal illness to ventricular dysfunction and congestive heart failure with a poor prognosis [9,11]. The acute form with multi-organ dysfunction is more common in neonates and young children, while adult cases usually present with a chronic form of disease, with only a few cases of acute myocarditis [11, 69]. Patients usually present with shortness of breath, chest pain, and ventricular arrhythmias. Almost one third of patients with viral myocarditis develop a chronic form of disease at times associated with viral persistence, progressing to end-stage dilated cardiomyopathy (DCM) and heart failure. The most compelling evidence for such persistence comes from studies showing the presence of viral RNA in the myocardium of patients with DCM [70-73].

i. Classification

Histopathological classification suggested for viral myocarditis includes [27,57] 1) **Fulminant myocarditis**, with the symptoms of left ventricular dysfunction and unexpected heart failure within 2-3 weeks of the onset of viral infection. Those who survive may have complete resolution of myocarditis and left ventricular abnormalities [74]; 2) **Sub-acute myocarditis**, with moderate left ventricular dysfunction and less distinct symptoms that is considered as a predisposing condition for dilated cardiomyopathy; 3) **Chronic active myocarditis**, with moderate left ventricular dysfunction and indistinct symptoms that is typically a precursor to restrictive cardiomyopathy. Endomyocardial biopsy may reveal an ongoing inflammation and active scar tissue (active or borderline myocarditis) [75]; and 4) **Chronic persistent myocarditis**, [520x41]
with normal left ventricular function and frequent palpitation or atypical chest pain as frequent symptoms. The persistence of inflammation is noticeable in biopsy examinations. On occasions, viral genome can be detected in biopsy samples using ISH and RT-PCR techniques [76,77].

ii. Etiology of viral myocarditis

More than twenty common viruses including adenoviruses, coxsackieviruses, influenza viruses, cytomegaloviruses, and human immunodeficiency viruses have been associated with myocarditis in humans [11,78-82]. However, coxsackieviruses are considered to be the prominent cause particularly in neonates and young children (Table 1-1) [11,83-86].

Coxsackieviruses are non-enveloped human enteroviruses in the family of Picornaviridae. They are omnipresent, circulating rapidly by fecal-oral transmission, and frequently causes small seasonal epidemics around the world [11,87,88]. There are two subgroups A and B with 23 known Coxsackie A serotypes causing mainly enteric diseases, and 6 known Coxsackie B serotypes (CVB1-6) associated with severe diseases in the heart, pancreas, and central nervous system (CNS) [89]. The first report of Coxsackie A infection in human goes back to 1948, when Dalldorf and Sickles [90] isolated an “unidentified, filterable agent” from the feces of children with febrile paralysis during an outbreak in the upstate New York town of Coxsackie. Shortly thereafter, the first Coxsackie B was isolated from patients with non-paralytic poliomyelitis and aseptic meningitis [91,92].
All six serotypes of CVB (CVB1-6) cause wide range diseases including febrile illness, aseptic meningitis, myocarditis, pancreatitis and presumably insulin-dependent (type-1) diabetes mellitus [11,38,93]. The serotypes CVB1-5 are more common in North America and Northern and Western Europe where CVB6 is rarely isolated [69]. The serotype CVB3 has been consistently reported as the prominent cause of myocarditis and has been implicated in approximately 20-25% of sporadic cases of acute onset or dilated cardiomyopathy [11,87,94,95]. De Jager and Van Creveld [96] were first to isolate Coxsackie B particles (CVB4) from the heart tissue of a neonate diagnosed with acute myocarditis. Recently, CVB2 and CVB4 sequences were also isolated from the hearts of patients with heart muscle disease (Keshan syndrome) in a selenium-deficient area in China [97]. In infants, all Coxsackie B serotypes can cause severe systemic disease, which is normally fatal [98,99]. The mortality rate for infants diagnosed with myocarditis is almost 30-50% and markedly increases when other organs are infected [44].

1-3. MOLECULAR BIOLOGY OF COXSACKIEVIRUS B3

In recent years, the development of experimental in vivo (mouse) and in vitro models of CVB3 infection has provided scientists with the opportunity to explore various genetic, cellular, and molecular aspects of CVB3 pathogenesis [100-102]. To gain a better understanding of the pathology of CVB3 infection, it is crucial to comprehend important characteristics of viral structure and life cycle.

i. CVB3 receptors
To initiate a productive infectious cycle, CVB3 binds to the decay-accelerating factor (DAF) as a primary attachment protein (co-receptor) and the coxsackievirus and adenovirus receptor (CAR) as an internalization receptor. DAF (CD55), a 70 kDa membrane glycosyl-phosphatidylinositol (GPI)-anchored protein, is widely expressed in human tissues; and normally protects cells from complement-mediated lysis by preventing the formation of C3 convertases [103]. DAF encompasses four contiguous short consensus repeats (SCR1-4) linked to a GPI-anchored C-terminal domain. It is suggested that binding to DAF protein facilitates the access of virus particles to CAR without causing any conformational changes in viral capsid proteins [104,105]. Through the GPI anchor, DAF also interacts with the Src-family protein tyrosine kinase, p561ck, which reportedly plays a critical role in CVB3 infection in T lymphocytes and virus-induced myocardial injury [106-108].

In 1997, CAR protein (46 kDa) was cloned and characterized by three independent groups using immuno-affinity purification [109], SDS-electrophoresis with $^{35}$S-labeled CVB3 [110], and expression cloning techniques [102]. CAR is a trans-membrane component associated with tight junctions in human epithelial cells; and has been shown to play a role as an adhesion molecule involved in neuro-network formation in developing mouse brain [111-113]. CAR protein comprises an extracellular domain composed of two immunoglobulin-like motifs D1 and D2, a trans-membrane helical domain, and a highly conserved cytoplasmic tail. The cytoplasmic domain encompasses a potential tyrosine phosphorylation as well as a palmitylation domain [114]. The cytoplasmic and trans-membrane domains of CAR are not required for virus binding, while the extracellular domain is necessary and sufficient for a productive coxsackievirus
and adenovirus infection [115]. Recently, Chung et al. [116] have reported that CVB3 enters HeLa cell via CAR-mediated internalization and clathrin-dependent endocytosis, and that intracellular trafficking of CVB3 is highly dependent on intact endosomal function. More in-depth studies are required to elucidate the actual mechanisms underlying CAR-mediated CVB3 entry.

Northern blot analyses of human tissue extracts have confirmed CAR mRNA expression in the heart, brain, pancreas, prostate, and testis, and a lower expression in liver, lung and intestine, which is consistent with the pattern of tissue susceptibility and clinical presentations in CVB3 myocarditis [102,117]. A significant decrease in CAR expression has been reported in mouse heart and brain with increasing age, indicating an age-related pattern of tissue tropism and host susceptibility for CVB3 [113].

In addition, nucleolin, a 100 kDa intracellular protein, has been shown to bind to all six serotypes of CVB3 on a virus overlay blot [118]. Further studies in Chinese hamster ovary (CHO) cells revealed that despite the functional virus-nucleolin interaction, these cells are non-permissive to CVB3 infection, casting uncertainty upon the proposed essential role for nucleolin in virus entry [119].

ii. Coxsackievirus B3 life cycle

CVB3 encompasses a 7.4 kb single strand positive sense RNA genome (Fig. 1-1). The single strand RNA comprises a large open reading frame (ORF) flanked on both 3' and 5' termini by untranslated regions (UTR). At the 5' end, a small virus-encoded protein VPg
(3B) replaces the usual eukaryotic 7-methyl guanosine cap structure [120,121]. Viral VPg protein is crucial for initiation of post-entry RNA and protein synthesis.

Following the attachment to CAR/DAF complex and virus uncoating, viral positive sense RNA enters the cytoplasm and serves as a template for translation of a large viral polyprotein of about 250 kDa via a cap-independent mechanism utilizing the internal ribosomal entry site (IRES) [122,123] (Fig. 1-2). The newly synthesized polyprotein is subsequently cleaved into three primary precursor molecules P1, P2, and P3 by virus-encoded proteases 2A\textsuperscript{pro} and 3C\textsuperscript{pro}. The P1 portion will be further cleaved by 3CD\textsuperscript{pro} to make the structural capsid proteins VP1, VP2, VP3, and VP4, while the P2 and P3 segments will make non-structural viral proteinases and polymerases (2A\textsuperscript{pro}, 2B, 2C, 3A, 3B/VPg, 3C\textsuperscript{pro}/3CD\textsuperscript{pro}, 3D\textsuperscript{pol}) [124-127].

Various functions have been reported for viral proteinase 2A. It cleaves viral polyprotein at the VP1-2A\textsuperscript{pro} junction [128,129], stimulates translation initiation on the cognate viral IRES [130,131], and cleaves a number of host cell proteins including the 220 kDa component of the eukaryotic initiation factor-4γ (eIF4G) and the poly(A)-binding protein (PABP), two crucial components of host cell mRNA translation and protein synthesis machinery [132-134]. Viral proteinase 2A further contributes to virus-induced myocardial injury through cleavage and disruption of cytoskeletal proteins dystrophin and dystrophin-associated glycoproteins α-sarcoglycan and β-dystroglycan in both \textit{in vitro} and \textit{in vivo} models [119,135,136]. Additional studies in endomyocardial biopsy specimens confirmed the presence of focal dystrophin and β-dystroglycan disruption in human myocarditis [137].
Viral proteinase 3C reportedly cleaves and inactivates cAMP response element binding protein (CREB/ATF), octamer-binding transcription factor (Oct-1), cellular TATA-binding protein, and the histone protein H3, leading to profound shutdown of host cell transcriptional and translational machinery [138-141].

Following the early translational event, viral 3D\textsuperscript{pol}, a virally encoded RNA-dependent RNA polymerase, utilizes the positive sense RNA as a template to construct negative strand RNA intermediates that are subsequently used for synthesis of multiple copies of positive RNA strands [142]. Viral 3D\textsuperscript{pol} initiates RNA synthesis by generating the protein primer VPg-pU-pU (uridylylation), that is required for the initiation of RNA synthesis at the 3' poly(A) region of viral genome [143-145].

A single negative-sense RNA can produce several copies of positive stands. Hence, at any given time, the ratio of positive to negative RNA strands is between 30:1 and 50:1 [146,147]. The positive RNA are packed and eventually released as progeny viruses to initiate new rounds of infection. The actual mechanism underlying virus release is not well understood. There is some evidence signifying that viral protein 2B increases the permeability of the plasma membrane and facilitates the release of progeny viruses [148,149]. Polioviruses carrying a 2B mutation exhibit defects in RNA amplification and viral release in cell culture [150].

1-4. EXPERIMENTAL MURINE MODEL OF CVB3 MYOCARDITIS

Much of our understanding of the pathophysiology and mechanisms contributing to the progression of human viral myocarditis is based on the studies in experimental murine
models. The mouse has become an excellent and well-defined model for viral myocarditis due to various characteristics such as the genetic similarity to human, easy and cost-efficient handling/breeding, availability of transgenic strains, and sensitivity to cardiotropic viruses. When inoculated into suckling and weanling mice, CVB3 replicates in the heart, pancreas, spleen, and brain causing clinical symptoms, which resemble human diseases [151-156]. In susceptible mice, following intraperitoneal injection of CVB3, three distinct pathological phases of disease have been characterized (Fig. 1-3).

During the “acute viremic” stage, a high titer of virus is present in the blood, spleen, pancreas and myocardium. Massive virus replication within 3 to 4 days post-infection leads to early virus-induced cardiomyocyte injury, focal necrotic myofibers, multi-vesicular vacuolation, and calcification in the absence of inflammatory infiltrates [157,158]. The expression of cytokines interleukin-1α (IL-1α), IL-1β, IL-6, IL-18, tumor necrosis factor-α (TNF-α), TNF-β, and interferon-γ (IFN-γ) are elevated prior to immune cells infiltration (up to day 5), indicating that these cytokines are likely released by cells intrinsic to the myocardium such as cardiomyocytes, endothelial cells, and fibroblasts in response to virus presence [159-161]. Neutralizing antibodies also appear around day 4 post-infection and play a critical role in limiting viral replication in the heart and other organs [162].

The “sub-acute phase” of disease starts with the release of progeny virus into the interstitium that instigates migration of natural killer (NK) cells and macrophages to the site of injury, followed by a considerable increase in pro-inflammatory cytokines and a second wave infiltration of immune cells including CD4+ and CD8+ T lymphocytes. At
this stage, extensive virus infection is associated with the development of multi-focal inflammatory lesions [163]. In both DBA/2 and BALB/c mice, virus is cleared by day 10 while the neutralizing antibody reaches the highest level on day 14 post-infection [164]. B-lymphocytes account for approximately 10-20% of infiltrating lymphocytes in infected myocardium [165]. The importance of neutralizing antibodies and the role of B-cell mediated immunity have been established by observation that prednisolone-induced suppression of neutralizing antibodies in encephalomyocarditis virus (ECMV)-infected BALB/c mouse can delay virus clearance in infected tissues [164].

NK cells, mainly activated by IL-2, eliminate virus-infected cardiomyocytes via the release of the cytotoxic molecule perforin in an attempt to limit virus dissemination [166,167]. The protective role of NK cells has been investigated in studies using NK-deficient mice, wherein increased viral titer and myocardial injury were observed in comparison with control group [168]. On the other hand, over-activation and/or long-term presence of NK cells may exacerbate myocardial damage due to excessive release of perforin within the myocardium. Perforin heterozygous (+/-) and homozygous (-/-) knock-out mice infected with EMCV and CVB3 have demonstrated a lower level of myocardial necrosis and lymphocyte infiltration on day 12 after viral inoculation [169,170].

On day 7 of sub-acute phase, increased expression of pro-inflammatory and regulatory cytokines IL-1β, IL-2, IL-10, IL-18, TNF-α, IFN-γ, and granulocyte/macrophage colony stimulating factor (GM-CSF) in the heart and plasma is associated with a vast infiltration of antigen-specific T-lymphocytes [159,161,165,171-173].
The cytotoxic T-lymphocytes target viral antigens presented on the surface of infected cardiomyocytes in association with major histocompatibility complex I (MHC-I) antigens [174]. IFN-γ and TNF-α participate in the process by 1) up-regulation of MHC-I antigens on the surface of infected cardiomyocytes, and by 2) providing the proper cell-cell contact between T-lymphocytes and infected myocytes via the up-regulation of intracellular adhesion molecule-1 (ICAM-1) on the surface of infected myocytes [175]. Seko et al. [176] reported an increased expression of human leukocyte antigen class I (HLA-I) and ICAM-1 associated with an enhanced infiltration of cytotoxic T-cells in the heart of patients with acute myocarditis and dilated cardiomyopathy.

The "Chronic phase" of myocarditis (day 15-90) starts with a complete clearance of progeny viruses from the blood and peripheral tissues. However, viral RNA and capsid protein VP1 reportedly persist in the heart, spleen and lymph nodes as shown in both human and experimental mouse model [71,74,171,177-180]. As the inflammatory response weakens, increased interstitial fibrosis and calcification, ventricular dilation, and cardiac hypertrophy become evident [63,163,173,181,182]. Alternatively, continual viral replication and immune cell infiltration within the heart may be responsible for the long-term tissue obliteration and progression of viral myocarditis to dilated cardiomyopathy and congestive heart failure [173,183-185]. Persistently infected cells are mainly localized within foci of chronic inflammatory lesions. A sustained expression of various cytokines has been reported during the chronic stage of viral myocarditis in humans [186,187] and murine models [188-190]. Expression of the regulatory cytokine IL-18, pro-inflammatory cytokine TNF-α, and immunosuppressive/fibrogenic cytokine tumor growth factor-β (TGF-β) in NMRI mouse heart on day 28 and 98 post-infection are
indications of chronic inflammatory infiltrates and ongoing wound-healing process within the myocardium [190].

1-5. MECHANISMS OF CVB3-INDUCED MYOCARDIAL INJURY

Despite the availability of a well-characterized experimental model and common acceptance of the fact that CVB3 causes myocarditis, the actual mechanism underlying pathogenesis remains controversial. Three suggested mechanisms include: (1) Excessive immune-mediated destruction of the myocardium by infiltrating immune cells targeting virus-infected cardiomyocytes, which frequently extends to the remote non-infected region, (2) Autoimmune-mediated destruction of cardiac cells by circulating auto-antibodies/auto-reactive immune cells or by immune-mediated obliteration of cardiomyocytes due to molecular mimicry between viral and host antigenic epitopes, and (3) Direct virus-induced cardiomyocyte injury.

i. Immune response-mediated myocardial injury

During the early course of infection, transient and self-limited release of cytokines initiates a protective and healing process within the heart. Mice treated with immunosuppressive agents or those with severe combined immunodeficiency (SCID) lacking functional B and T lymphocytes, present with more severe myocardial damage in response to CVB3 infection [158]. Furthermore, in 129/Sv mice homozygous for a disrupted form of inducible nitric oxide synthase (iNOS2, -/-), CVB3 replicates to a higher titer that is associated with more severe myocarditis [191].
However, sustained expression of pro-inflammatory cytokines along with persistent infiltration of sensitized immune cells within the heart may have detrimental effects, even in remote non-infected areas, contributing to remodeling of the myocardium. Thus, both innate and acquired immune responses, originally initiated as compensatory mechanisms to eradicate the insult while maintaining normal cardiac output, eventually decompensate and contribute to dilated cardiomyopathy and congestive heart failure (Fig. 1-4). The first evidence of such inappropriate and destructive responses was provided by Woodruff et al. [192], who demonstrated that depletion of T lymphocytes using irradiation or antithymocyte serum significantly decreased immune infiltrates and mortality rate in a CVB3 experimental model. In another study, the absence of both CD4+ and CD8+ T-cell sub-population in A/J mice has been shown to markedly reduce inflammatory infiltrates and mortality [185]. Interestingly, the absence of CD4+ or CD8+ cells alone had no significant effect on disease progression in this model [185].

ii. Autoimmune-mediated myocardial injury

Myocardial injury may originate from the adverse immune response against "self-antigens". Virus-mediated injury in infected cardiomyocytes probably leads to modification, exposure or release of antigenic epitopes that are usually secluded from the reactive immune system [193,194]. Early evidence for such deleterious effects was provided by the detection of auto-antibodies against cardiac myosin heavy chain, β-adrenergic receptor, laminin, and mitochondrial adenine nucleotide translocator (ANT) in the sera of patients with myocarditis and DCM [195-199]. The auto-antibodies against
ANT have been shown to disrupt cardiomyocyte energy metabolism in both *in vitro* and *in vivo* [200-202].

Furthermore, myocardial antigen-specific T-cells isolated from peripheral blood of myocarditis patients, induced heart disease in *SCID* mice [203]. In *SWR/OIa* mice, CVB3 infection increases expression of auto-antibodies (IgGs) against cardiac myosin heavy chain, tropomyosin, actin, desmin, heat shock protein 60 (HSP60), and HSP70 on day 7 post-infection [204,205]. Recently, using immunoelectron microscopy, Takata et al. [206] have shown that IgM auto-antibodies against cardiac actin and HSP60 are localized on the cardiomyocyte membrane after repetitive CVB3 infection.

There is also evidence to support the hypothesis of “antigenic mimicry” in viral myocarditis [207]. Antigenic mimicry between epitopes on cardiac muscle cells and virus particles may perpetuate a strong immune response from sensitized B and T lymphocytes. Antibodies against cardiac α-myosin and the cardiomyocyte sarcolemma have been detected in the heart and sera of myocarditis patients, and have been shown to cross-react with Coxsackie B viruses [208].

It is noteworthy that almost 5% of patients with ischemic heart disease are also positive for autoantibodies against cardiac tissue [195-199]. Thus, the relation between the autoantibodies and pathophysiology of viral myocarditis has proven elusive. Following a few promising reports on the use of immunosuppressive prednisolone to treat viral myocarditis, several studies have been conducted to evaluate the value of immunosuppressive therapy in human myocarditis [209-212]. However, two large randomized controlled trials, Myocarditis Treatment Trials [29,30,213], and the European
Study of Epidemiology and Treatment of Cardiac Inflammatory Disease [214,215] failed to show any significant beneficial effects for immunosuppressive drugs on primary endpoints, such as heart transplantation or death [216,217]. Furthermore, a meta-analysis of studies published from 1984 to 2003 based on a systematic review of the impact of immunosuppressive therapy in children with acute myocarditis, has reported that such therapy does not considerably improve the outcome of disease [68].

On the other hand, immunomodulation strategies of enhancing anti-viral immune response or targeting destructive effects of pro-inflammatory cytokines during the course of myocarditis have proven more promising. Recently, a single centre phase II clinical trail has revealed that administration of INF-γ in patients with enteroviral and adenoviral myocarditis or dilated cardiomyopathy, for a period of 24 weeks, significantly improved cardiac function in almost 60% of the study population [218].

The idea of the involvement of the immune system and auto-immunity in viral myocarditis is a continuing paradigm. Unquestionably, the observation that a reactive immune system may be involved in the severity of viral myocarditis highlights the importance of the balance between the protective and deleterious effects of the immune response. A more clear understanding of regulatory mechanisms responsible for sustaining such a critical balance will provide new insights into understanding the pathogenesis of CVB3 myocarditis in humans.

iii. Direct virus-induced myocardial injury
The hypothesis of direct virus-induced injury has been supported by numerous studies demonstrating that virus replication results in myocardial cell death during the early phase of myocarditis in both *in vitro* and *in vivo* [158,219-231]. In HeLa and mouse cardiomyocyte cell cultures, CVB3 induces direct cellular injury and irreversible cytopathic effects, which eventually lead to cell death [221,225,226,232,233]. Microscopic evaluation of tissues from mice shortly following infection (3-5 days) clearly indicates that cardiac myocytes are injured prior to immune cell infiltration of tissue, as determined by the distinctive cytopathic effects, coagulation necrosis, and contraction band necrosis [158]. In SCID mice, characterized by an absence of functional T- and B-lymphocytes, CVB3 infection has been shown to induce extensive myocardial injury [234]. Knowlton and colleagues [235] have demonstrated that cardiac specific expression of the replication-restricted full-length coxsackievirus B3 cDNA (CVB3Δ VP0) can cause myocardial damage and dilated cardiomyopathy independent of an immune response, suggesting that CVB3 proteins can directly cause cardiomyocyte injury. The same group have also shown that direct cleavage of the cytoskeletal protein dystrophin by CVB3 proteinase 2A leads to focal disruption of the dystrophin-glycoprotein complex and sarcolemmal integrity, which may contribute to the pathogenesis of CVB3-induced dilated cardiomyopathy [136,137,236].

Although, it is well accepted that the observed pathology in viral myocarditis is a result of co-operation and "team-work" between virus and host immune response at various stages of disease, there in no agreement on the concept as to which party should take a larger share of the blame. To better understand the interplay between virus and host cell,
one must comprehend intracellular events that influence viral and cellular functions and determine the fate of infected cells and disease pathogenesis and prognosis.

1-6. DEATH SIGNALLING PATHWAYS

Direct virus-induced damage is an important constituent of early myocardial obliteration and disease progression in viral myocarditis [234,237]. Within the myocardium, CVB3 infection causes multi-focal coagulative and/or contraction band necrosis even prior to inflammatory infiltrates [158,234,238]. The heart is an organ without external redundancy and regenerative capacity. Hence, the loss of contractile myocytes and excessive scar formation are the major foundations for tissue remodeling, which contributes to ventricular dysfunction and heart failure. The mechanisms underlying cardiomyocyte death/removal within the heart are not entirely understood. Apoptosis and necrosis are both considered potential mechanisms for cardiomyocyte loss during human ischemic and chronic cardiac diseases [239-242]. Cellular swelling and the loss of plasma membrane integrity followed by the release of cellular debris into surrounding tissue space characterize necrosis, a passive and unregulated form of cell death that can instigate a robust immune response [243-245]. Apoptosis, on the other hand, is an active, gene-regulated, and energy-driven process that eradicates infected and/or injured cells via an intrinsic suicide mechanism without altering cell membrane integrity, thereby, excluding the cellular contents from an aggressive response originated in the adjacent viable tissue [246].

The term "apoptosis" was first introduced by Kerr et al. in 1972 [247]. Thereafter, further studies showed that apoptosis has a fundamental function in embryonic development and
differentiation, cellular homeostasis, immune responses, and various human pathologies [248-250]. Cellular shrinkage, chromatin condensation, DNA fragmentation, membrane blebbing, and compartmentalization of the cell into apoptotic bodies are the hallmarks of apoptosis [247]. A defect in apoptosis is an important contributor to uncontrolled cellular proliferation and tumor growth in various malignancies, while disproportionate apoptosis, in response to a variety of stimuli, has been implicated in human pathological conditions such as ischemic brain/heart injuries, neurodegenerative diseases, autoimmunity, and infectious complications.

Caspases, a family of cysteine-dependent aspartate proteases (cysteine proteases that cleave the substrate after an aspartate residue), are major mediators of apoptosis. There are 14 distinct mammalian caspases, eleven of which are present in humans [251]. Caspases and their homologues are present in various species such as Drosophila melanogaster, C. elegans, and Saccharomyces cerevisiae, indicating a highly conserved function for these proteases through evolution [252-254]. In non-stimulated cells, all caspases are present as catalytically inactive pro-enzymes (zymogens) and must undergo proteolytic processing to exert their functions. In humans, at least seven caspases are involved in apoptotic responses and are divided into two major groups. Upstream “initiator” caspases including caspase 2, 8, 9, and 10 are activated through an autocleavage process in response to external or internal stimuli. Downstream “executioner” caspases including caspase 3, 6, and 7, are activated through the cleavage by initiator caspases. In mammalian cells, depending on the stimulation, caspases can be activated via either intrinsic or extrinsic pathways.
Our current understanding of extrinsic or death-receptor apoptotic pathway derives from numerous studies on cellular responses to death inducing factors Fas-ligand and TNF-α. Attachment of these ligands to their specific cell-surface receptors, Fas/CD95/Apo-1 and TNFR recruits the cytoplasmic adaptor molecules Fas associated death domain (FADD) and TNF-α receptor associated death domain (TRADD), respectively, to the cytoplasmic tails of the corresponding receptors to form the death inducing signalling complex (DISC). Pro-caspase 8 binding to DISC through its death domain leads to activation of caspase-8 and subsequent cleavage and activation of caspase-3.

The mitochondrion is the command center for the intrinsic apoptotic pathway [255]. In response to apoptotic stimuli, mitochondria sense the overwhelming cellular changes and, if necessary, commit injured cells to suicide by releasing death proteins that are normally retained within the inter-membrane space (IMS) or matrix [256]. Such proteins include cytochrome c (Cyt c), second mitochondria-derived activator of caspases/direct inhibitor of apoptosis (IAP)-binding protein with low pi (SMAC/DIABLO), apoptosis-inducing factor (AIF), endonuclease G (EndoG), and high temperature-requiring protein A2 (HTRA2) [257,258].

Cytochrome c is probably the most studied mitochondrial pro-apoptotic protein. Following release from mitochondria, cytochrome c binds to apoptosis protease cytosol factor-1 (Apaf-1), ATP/dATP, and pro-caspase-9 to form a caspase-activating complex, termed the apoptosome. Formation of apoptosome results in auto-cleavage of pro-caspase 9, followed by cleavage and activation of caspase-3 [179].
Activated caspases also cleave numerous cellular substrates, resulting in activation or inactivation of targeted proteins. Using various experimental models and apoptotic stimuli, a growing number of caspase substrates have been identified that include the poly (ADP-ribose) polymerase (PARP) [259,260], DNA-dependent protein kinase [261], inhibitor of NFκB (IκB-α) [262], Ras GTPase-activating protein [263], MEK kinase-1 [264], Raf-1 [263], Akt-1 [263], nuclear lamin A/C, focal adhesion kinase (FAK) [265], inhibitor of caspase-activated deoxyribonuclease (ICAD) [266,267], and DNA fragmentation factor (DFF) [268]. The majority of caspase substrates are involved in important cellular processes including cell cycle regulation, proliferation, DNA repair, and cell survival; and the loss or alteration of their functions contributes to the apoptotic phenotype and cellular death.

i. CVB3-induced cell death

CVB3-induced cardiomyocyte apoptosis is present in various murine models of viral myocarditis [61,169,230,231,269,270]. During the acute phase in BALB/c mice, viral proteins localize in apoptotic cells in the presence of a slight inflammatory infiltration [230,237]. Using biopsy tissues, cardiomyocyte apoptosis have been also reported in human myocarditis and in dilated cardiomyopathies associated with viral infection [271-273]. A recent postmortem study on 33 cases of fatal human myocarditis has shown a higher rate of cardiomyocyte apoptosis than that in control group [274].

In tissue culture, CVB3 causes profound functional and morphological alterations and cytopathic effects (CPE). Virus replication results in cellular shrinkage and rounding, as well as detachment of adherent cells from the culture dish. Virus-induced degenerative
changes occur in two distinct but related phases (Fig. 1-5). Following post-entry viral translation, several proteases are released within the cytosol targeting structural and functional proteins necessary for cellular homeostasis. Cleavage of host eIF-4G protein results in rapid and complete shutdown of cap-dependent RNA translation within infected cells. The remaining C-terminal domain of cleaved eIF-4G protein binds to viral RNA, facilitating IRES-dependent translation of viral polyprotein [275].

Some host mRNAs also contain IRES and undergo translation in a cap-independent manner [276]. One example is the up-regulation of cellular pro-apoptotic protein Apaf-1 following CVB3 infection, which is entirely reliant on IRES-dependent translation [277]. In recent years, several other cellular IRES-containing mRNAs including immunoglobulin heavy chain binding protein BiP, transcription factor c-myc, and translation initiation factor eIF-4G have been identified in eukaryotic cells, indentifying the mechanisms underlying the translation of these proteins in infected cells wherein the cap-dependent translation of most host mRNAs is entirely blocked [278,279].

Host cell transcription is also targeted by viral proteases. The 3C protease can block cellular transcription by cleavage of cellular RNA polymerases and transcription factors. Poliovirus 3D protein contains a nuclear localization signal (NLS) that targets the precursor protein 3CD to the nucleus [280]. In the nucleus, 3CD undergoes auto-cleavage processing, releasing a mature 3C protease that cleaves cellular transcription factors located in the nucleus [138-141]. Decreased host RNA transcription and protein translation results in an imbalance of protein synthesis and degradation, an event that is
thought to be responsible for observed cytopathic effects, myofibrillar disarray, and atrophic phenotype in viral myocarditis.

Viral proteases further contribute to morphological changes and cytopathic phenotype through cleavage of cytoskeletal proteins such as dystrophin and the dystrophin-associated glycoproteins α-sarcoglycan and β-dystroglycan in both experimental and human myocarditis [119,135-137]. Early studies in poliovirus infection have shown that inhibition of viral protein translation delays the onset of cytopathic changes in infected cells, supporting the hypothesis that certain virus-encoded proteins are cytotoxic [281]. Taken together, these findings emphasize the important role for virally encoded proteins in the development of degenerative morphology and cytopathic effects, even prior to caspase activation.

CVB3-induced cytopathic effects precede apoptotic events, which are characterized by cleavage of multiple members of the caspase family including caspases -2, -6, -7, -8, -9, and -3 [221,224]. Activated caspase-3, in turn, cleaves downstream substrates PARP, DFF, and nuclear lamin A/C [221]. PARP is a nuclear enzyme that binds to DNA and specifically detects and repairs DNA single strand breaks in order to maintain host genomic stability [282]. DFF or caspase-activated DNase (CAD) is a heterodimer nuclease composed of 40 and 45 kDa subunits [267,268]. DFF40 subunit has an endonuclease activity and is sequestered within the nucleus in a latent form by DFF45 subunit [266,268]. In infected cells, caspase-3 activation leads to the cleavage of PARP and DFF proteins, resulting in inhibition of DNA repair and initiation of oligonucleosomal DNA fragmentation, two major late events in virus-induced apoptosis.
As noted before, caspase-3 cleavage may occur through either extrinsic (receptor-mediated) or intrinsic (mitochondria-mediated) pathways. The release of mitochondrial cytochrome c has been demonstrated during CVB3 infection and it is considered the major upstream activator of caspase-9 and -3 in CVB3-infected cells [224] (Fig.1-6).

Mitochondrial membrane transition (MPT) and depolarization (ΔΨm) play a key role in apoptosis by regulating the release of cytochrome c and other death proteins [255]. Expression of the mitochondrial epitope 7A6, which is the early indication of altered MPT, has been detected in CVB3-infected HeLa cells [224,283,284]. In these cells, the general caspase inhibitor benzoyloxycarbonyl-Val-Ala-Asp-(OMe) fluoromethylketone (zVAD.fmk) blocks caspase cleavage without having any effect on mitochondrial expression of epitope 7A6, emphasizing that mitochondrial membrane alteration most likely precedes caspase activation in infected cells [224].

The Bcl-2 family of proteins is the major modulator of mitochondrial membrane permeability. The members of this family are either anti-apoptotic, such as Bcl-2 and Bcl-xL proteins, or pro-apoptotic, such as Bax, Bid, Bak, Bik, Puma, Noxa, and Bcl-Xs proteins [285,286]. Recent studies in CVB3 and poliovirus infections demonstrated that over-expression of the anti-apoptotic proteins Bcl-2 and Bcl-xL can delay mitochondrial cytochrome c release into the cytosol and subsequent apoptotic death [224,287]. More importantly, inhibition of virus-induced cytochrome c release and caspase cleavage by Bcl-2 over-expression or zVAD.fmk treatment, respectively, caused no measurable alteration in CVB3 replication and virus-induced cytopathic effects, while significantly blocking apoptotic morphology and virus progeny release [224]. These findings suggest
that apoptotic phenotypes and virus-induced cytopathic effects are probably two distinct phenomena.

Interestingly, a crosstalk and interplay between extrinsic and intrinsic apoptotic pathways has been reported, wherein activated caspase-8 cleaves the Bcl-2 protein Bid within the cytoplasm releasing a truncated form of Bid (tBid) [288]. tBid interacts with pro-apoptotic proteins Bak and Bax to induce mitochondrial permeability transition [289].

1-7. SURVIVAL SIGNALLLING PATHWAYS

Intracellular signal transduction is the foundation of cellular homeostasis that allows cells to monitor and detect intra and extracellular changes, and mount an appropriate response by utilizing the dynamic phosphorylation/dephosphorylation events [290 470]. Reversible phosphorylation of cellular proteins alters their conformations, stabilities, activities, localizations, and eventually their interactions with other proteins. Defects in such regulatory system are thought to be the cause or effect of various human diseases. Almost 30% of proteins encoded by the human genome carry covalently bound phosphate groups targeted by approximately 1000 protein kinases and 500 protein phosphatases [291].

Protein kinases control the function of downstream targets by adding a phosphate group to specific amino acid residues in their substrates, exploiting intracellular adenosine triphosphate (ATP) as the phosphate group donor. Protein phosphatases, on the other hand, regulate the extent of phosphorylation by removing previously added phosphate groups. Based upon the specific amino acid residues that they phosphorylate, protein kinases are divided in three major groups: Protein tyrosine kinases, protein
serine/threonine kinases, and protein kinases with dual specificity for tyrosine and serine/threonine residues.

As suggested by the name, protein tyrosine kinases (PTKs) phosphorylate tyrosine residues within their substrates, and are located either in the plasma membrane (receptor tyrosine kinases or RTKs) or within the cell (non-receptor protein tyrosine kinases). The RTKs consist of an extracellular ligand-binding domain, a single transmembrane domain, and a tyrosine kinase-containing cytoplasmic domain; and play a critical role in transmitting extracellular signals to intracellular space through receptor dimerization and subsequent trans-phosphorylation of tyrosine residues within their cytoplasmic tails [292,293]. Among RTKs are receptors for insulin-like growth factor (IGF), epidermal growth factor (EGF), vascular endothelial growth factor (VEGF), and platelet-derived growth factor (PDGF) [294-298]. Following the activation of RTKs, phosphorylated tyrosine residues act as a docking site to recruit and activate downstream signalling molecules that contain Src homology (SH2) or phosphotyrosine binding domains. Such downstream signalling molecules include non-receptor tyrosine Src kinase [299,300], Janus kinase (JAK) [301,302] and focal adhesion kinase (FAK) [303,304], phospholipase Cγ (PLCγ) [305], and the adaptor molecule phosphotidylinositol-3 kinase [306,307] that transduce the signals to downstream serine/threonine protein kinases.

Serine/threonine kinases phosphorylate either serine or threonine sites within the target substrate. Of importance are the members of AGC superfamily of protein kinases including cAMP-dependent protein kinase (PKA), cGMP-dependent protein kinase (PKG), protein kinase C (PKC), and protein kinase B (PKB/Akt) that regulate myriad
physiological processes including cellular metabolism, proliferation, migration, survival, and apoptosis [308-310].

i. **Protein kinase B/Akt: The central player of survival signalling**

Akt was initially identified by three independent groups based on its homology to protein kinase A [311] and protein kinase C [312] or as a cellular homolog to the transforming oncogene (v-Akt) of the Akt8 retrovirus [313]. To date, three isoforms of mammalian Akt have been identified: PKBa/Akt1 [314], PKBβ/Akt2 [315], and PKBγ/Akt3 [316]. All three isoforms share a highly conserved structure that consists of an N-terminal pleckstrin homology (PH) domain, a central kinase domain, and a C-terminal domain containing a hydrophobic motif.

Akt interacts with 3-phosphoinositide lipids in the inner side of plasma membrane through its pleckstrin homology (PH)-like domain. The binding process is crucial for recruitment of Akt to the plasma membrane. The central kinase domain that is highly conserved among AGC family contains a threonine residue (Thr-308/Akt1, Thr-309/Akt2, and Thr-305/Akt3) within the activation loop. The C-terminal regulatory domain contains a hydrophobic motif (F-X-X-F/Y-S/T-Y/F, where X is any amino acid). Phosphorylation of serine residue within the hydrophobic domain (Ser-473/Akt1, Ser-474/Akt2, and Ser-472/Akt3) is necessary for full Akt activation.

ii. **Upstream activators of Akt**

The phosphatidylinositol-3 kinases (PI3K), class 1A and 1B, are the major upstream activators of Akt. Activation of receptor tyrosine kinase or G protein-coupled receptors
leads to the recruitment and activation of the lipid kinase PI3K that, in turn, adds a phosphate group to 3'-OH position within the inositol ring of phospholipids located on the inner side of plasma membrane [317]. In the basal condition, phosphatidylinositol-4 phosphate [PtdIns(4)P] and phosphatidylinositol-4,5 phosphate [PtdIns(4,5)P_2] are concentrated in caveolae and lipid rafts. In response to various stimuli such as growth factors, activated PI3K produces a high concentration of phosphatidylinositol-3,4,5 phosphate [PtdIns(3,4,5)P_3] by adding a phosphate group to 3′-OH in PtdIns(4,5)P_2 [318-320]. Activated phospholipids bind to the Akt PH domain and recruit Akt in close proximity to the plasma membrane. Such events can be subverted by phosphatase and tensin homolog deleted on chromosome 10 (PTEN) that removes the 3′ phosphate group, leading to Akt detachment from membrane-localized phospholipids [321].

The upstream kinase responsible for Akt phosphorylation on Thr-308 in its catalytic domain, 3-phosphoinositide-dependent kinase-1 (PDK-1), has been thoroughly characterized [322,323]. PDK-1 is a 63 kDa serine/threonine kinase with primary structure similar to other AGC kinases. The C-terminal PH domain of PDK-1 binds to phosphatidylinositol-3 phosphate (PIP3), a process that places PDK-1 close to the membrane-recruited Akt [324-326]. Phosphorylation of Thr-308 only partially activates Akt, and the second phosphorylation on Ser-473 site within the regulatory domain is required for its full activation.

Three proposed mechanisms for Ser-473 phosphorylation are phosphorylation by PDK-1, auto-phosphorylation, or phosphorylation by a heterologous kinase (conveniently named PDK-2). Initially it was assumed that PDK-1 was the kinase responsible for Akt
phosphorylation on both threonine and serine sites. Although further studies using PDK-1 knockout embryonic stem cells have provided concrete evidence that PDK-1 does not phosphorylate Akt on its serine residue, there is evidence that PDK-1 may indirectly regulate this process [327,328]. Akt may undergo auto-phosphorylation at its hydrophobic motif. The auto-phosphorylation of Ser-473 seems to be tightly coupled to Thr-308 phosphorylation in the activation loop [329]. Conversely, other studies have shown that purified Akt phosphorylated at Thr-308 does not undergo auto-phosphorylation at Ser-473 [322,330].

The preferential and most supported hypothesis is the existence of a distinct kinase responsible for phosphorylation of Akt on serine. *In vitro* studies have identified several kinases that phosphorylate Akt on Ser-473. Those kinases include MAP kinase-activated protein kinase-2 (MK2) [331], p38 MAP kinase [332,333], PKC isoforms [334, 335], the double-stranded DNA-dependent protein kinase [336,337], and integrin-linked kinase (ILK) [338,339]. There are growing numbers of controversial findings from biochemical and genetic studies that support or contradict the involvement of each of the proposed kinases in Akt phosphorylation. Hence, the actual identity of the upstream kinase responsible for Ser-473 phosphorylation remains elusive and it seems to be highly reliant on cell environment and type of stimulation [340].

**Integrin-linked kinase (ILK)**

Since its discovery in 1996 [341], accumulating data have suggested a potential regulatory role for ILK in Akt activation in several *in vitro* and *in vivo* systems [339,342,343]. ILK is a ubiquitously expressed protein that interacts with the cytoplasmic
domain of β1 and β3 integrins [341]. Since then, numerous studies have shown that ILK has an essential role in cellular adhesion by connecting components of extracellular matrices and integrin receptors to the actin cytoskeleton, and in cellular signalling by transmitting the outside-in and inside-out signals to control cellular proliferation, contraction, migration, and survival [344,345] (Fig. 1-7).

The ILK protein comprises an N-terminal domain containing four ankyrin repeats, a conserved central PH-like domain, and a C-terminal catalytic domain. The N-terminal ankyrin repeats localizes ILK to focal adhesion plaques by binding to the adaptor molecule PINCH (particularly interesting new cysteine-histidine rich protein) [346] or the signalling molecule ILKAP (ILK-associated phosphatase) [347]. PINCH bridges ILK to growth factor receptors via the Src-homology domain-containing adaptor protein, NcK-2 [348]. Although the C-terminal catalytic domain of ILK has a high degree of similarity to other kinase domains, it lacks the conserved amino-acid triplets HRD and DFG in catalytic subdomains VI and VII, respectively [341,349]. The catalytic domain mediates ILK bindings to integrin subunits and to the actin-binding adaptor proteins α- and β-parvin [338,350,351]. The PH-like domain binds PtdIns(3,4,5)P3 and regulates the endogenous activity of ILK.

Inhibition of PI3K blocks ILK activation in response to fibronectin and insulin, while constitutive expression of the active form of PI3K significantly increases ILK activation [338]. Moreover, PtdIns(3,4,5)P3 enhances the kinase activity of purified ILK in vitro [338]. Over-expression of ILK leads to increased Akt phosphorylation on Ser-473 in vitro [338,343], while PTEN-null prostate carcinoma cells show a high level of ILK/Akt
activation, which can be suppressed by over-expression of wild-type PTEN or a dominant-negative mutant of ILK, or treatment of cells with highly potent cell permeable ILK inhibitors [342,352-355].

**Role of ILK in viral infections**

Despite the great attention devoted to investigating the role of ILK in various types of solid tumors, the possible connection between viral infection and ILK has not been considered. However, there is some evidence implicating a role for integrin receptors in viral infection. Rotavirus interacts with \( \alpha_v \beta_3 \) integrins following its binding to sialic acid in the plasma membrane, and this post-attachment interaction seems to be necessary for virus penetration [356]. In the hearts of patients with dilated cardiomyopathy, \( \alpha_v \beta_3 \) and \( \alpha_v \beta_5 \) integrins colocalize with human CAR [357]. In these hearts, CAR is highly expressed and localized to intercalated discs and the sarcolemma. The mechanisms underlying the dynamic regulation of CAR expression and redistribution in cardiomyocytes, and the role of integrin-mediated signalling in this process remain elusive. Recently, cDNA microarray and Northern blot analyses of extracellular matrix gene expression within the myocardium of mice (BALB/c) infected with CVB3 have shown a 2.4 fold increase in ILK mRNA expression as compared with the control group, pointing towards a potential role for ILK in disease progression [358]. Yet, the role and consequence of ILK up-regulation in viral myocarditis has not been investigated.

**iii. Downstream effectors of Akt**
Akt is considered the most critical mediator of cell survival. Constitutive activation of Akt is associated with various types of malignancies and metastatic growth, making it an attractive therapeutic target for cancer treatment. Conversely, inhibition of Akt activation leads to apoptosis and cell death. Akt exerts its anti-apoptotic function through activation or de-activation of a wide-range downstream signalling molecules that control various aspects of cellular homeostasis including cellular metabolism, proliferation, migration, growth, and apoptosis (Fig. 1-8).

**Anti-apoptotic targets**

BAD is an apoptotic member of the Bcl-2 family of proteins, and binds to Bcl-2 and Bcl-xL on the mitochondrial membrane, blocking their anti-apoptotic functions. Akt phosphorylates BAD on Ser-136 site enhancing its binding to 14-3-3 proteins and subsequent degradation by 26S proteasome. Akt can also block apoptosis by phosphorylation of caspase-9 [359]. The first hypothesis of Akt-mediated regulation of caspase-9 originated from the work by Cardone et al. [360] who demonstrated that PKB/Akt over-expression in 293T human epithelial kidney (HEK) cells leads to phosphorylation of caspase-9 on Ser-196 residue, resulting in inhibition of caspase-9 and -3 cleavage. However, further studies showed that the Akt phosphorylation site found in human caspase-9 is absent in mouse, rat, and monkey, suggesting that this regulatory function is not conserved in lower species [361,362]. Akt also regulates the FOXO family of Forkhead transcription factors, FKHR (FOXO1), FKHR-L1 (FOXO3a), and AFX (FOXO4). Phosphorylation by Akt results in retention and inactivation of these transcription factors within the cytoplasm leading to the down-regulation of FOXO-
regulated genes including the cyclin-dependent kinase inhibitor p27kip1 and Rb2 genes and up-regulation of cellular proliferation. Inhibition of FOXO transcription factors by Akt also results in a significant decrease in the expression of pro-apoptotic proteins Bim (thus far reported only in hematopoietic cells) and Fas-ligand, and subsequent suppression of apoptosis.

Glycogen synthase kinase3-beta

Akt phosphorylates glycogen synthase kinase 3β (GSK3-β) on a serine residue. GSK3-β is a ubiquitous serine/threonine kinase that controls myriad physiological processes ranging from cellular development and metabolism to cell cycle regulation and cell fate determination [363-365]. Several protein kinases including Akt, PKA, ILK, and p90 ribosomal S6 kinase (p90RSK) can phosphorylate Ser-9 near the amino-terminus of GSK3β, leading to inactivation of this protein kinase [207,366-369]. To date, several downstream targets have been identified for GSK3β including β-catenin, CREB, NFAT, c-Myc, eIF2B, cyclin D-1, and E-cadherin [365]. Some evidence points towards the view that the effect of Akt on cell survival is related to its regulation of cellular metabolism and that GSK3β may be involved in such regulation. Activated Akt promotes glucose utilization in stimulated cells; a process that has been shown to prevent Bax conformation change and subsequent apoptosis [370-372].

Nuclear factor kappa B (NFkB)

The NFkB family of transcription factors regulates the expression of more than 200 genes involved in inflammatory and stress responses, cell cycle progression, cell growth and
proliferation, and apoptosis [373,374]. NFkB is ubiquitously expressed in all cell types and highly conserved from Drosophila to man. Numerous studies have provided convincing evidence that Akt can regulate NFkB in most, if not all, biological systems [375-380]. To date, five members of the NFkB family has been identified that share a Rel homology domain (RHD), and include Rel-A (p65), Rel-B, c-Rel, NFkB1 (p50), and NFkB2 (p52). Under resting conditions, NFkB proteins are rendered inactive within the cytoplasm by the family of inhibitors of NFkB (IKBs) including IkB-α, IkB-β, IkB-γ (NEMO), IkB-ε, bcl-3, p105, and p100 [373,374].

Phosphorylation, ubiquitination, and degradation of IkB proteins lead to translocation of NFkB p50/p65 and p52/Rel-B heterodimers into the nucleus, wherein they bind to the enhancer region of various genes [374,385] (Fig. 1-9). It has been suggested that full activation of NFkB may require phosphorylation of S529 and S536 residues on the p65 subunit by Akt [332,386,387]. Inhibition of PI3K blocks NFkB activity in melanoma cells, without any inhibitory effect on IkBα phosphorylation, suggesting an IkB-independent mechanism for NFkB activation [388]. In another study, using LPS-treated macrophages, Tan et al. [389] have shown that over-expression of ILK enhances the promoter activity of iNOS and nitric oxide (NO) production through an Akt/NFkB-dependent mechanism, providing more evidence that Akt can regulate NFkB activation.

**NFkB, the delicate balance between life and death**

Various stimuli such as cytokines, double-stranded RNA, and bacterial and viral infections can activate NFkB [28,70,164,213,381,382]. Constitutive activation of NFkB
has been implicated in many types of malignancies including breast cancer, colon cancer, melanoma, and pancreatic cancer [381,383,384].

NFkB has been implicated in both apoptotic and anti-apoptotic pathways. In various mouse models, an individual knockout of NFkB subunits cause mild to severe immune-related diseases and developmental abnormalities due to increased apoptosis [390-393]. It is suggested that NFkB activation is required for normal functioning of the immune system by regulating the expression of various cytokines such as IL-2, IL-3, GM-CSF, and IFN-γ in T cells, TNF-α in macrophages, IL-6 in B cells, and IL-12 in antigen presenting cells (APCs) [394-397]. NFkB augments expression of anti-apoptotic proteins such as TNF receptor-associated factor 1 and 2 (TRAF-1 & -2), cellular inhibitor of apoptosis 1 and 2 (c-IAP-1 & -2), melanoma inhibitor of apoptosis (ML-IAP), Survivin, and Bcl-2 like proteins [393,398-402].

However, there are conditions in which NFkB can function as a pro-apoptotic transcription factor that are believed to be cell-type and stimulus specific. NFkB increases the expression of proteins such as Fas, p53, and c-Myc that enhance apoptosis in response to TNF-α treatment or UV-radiation [403-405]. In endothelial cells, oxidative stress induced by hypoxia stimulates apoptosis through NFkB activation [406]. The cross-talk between NFkB and mitochondria-mediated cell death has been shown by de Moissac et al. [407,408], who were able to demonstrate that over-expression of Bcl-2 in rat ventricular cardiomyocytes and human embryonic 293 cells prevents TNF-α-induced apoptosis in an NFkB-dependent fashion. These findings indicate that NFkB can function as a double-edged sword with both anti- or pro-apoptotic effects; and several factors
including the timing, duration, and the dose of stimuli as well as cell environment can alter the delicate balance between these opposing forces [384].

1-8. HOST SIGNALLINGS DETERMINANTS OF CVB3 PATHOGENESIS

Alteration in protein phosphorylation in CVB3–infected cells was first documented by Kandolf and his colleagues in 1997 [409]. CVB3 infection in HeLa cells resulted in tyrosine phosphorylation of two distinct proteins of 48 and 200 kDa around 4-5 hours post-infection. Further subcellular fractionation experiments revealed that tyrosine-phosphorylated proteins were localized to the cytosolic and membrane fractions of infected cells. Pre-treatment of infected cells with hebrimycin A, a specific inhibitor of Src-like protein tyrosine kinases, blocked CVB3-induced tyrosine phosphorylation and significantly reduced viral replication, indicating that tyrosine phosphorylation events were necessary for effective virus replication [409,410]. In CVB3-infected HeLa cells, Sam68, a cellular adaptor protein and a target for Src-like tyrosine kinases during mitosis, is associated with p21ras GTPase-activating protein RasGAP through its SH2 and SH3 domains [411-414]. CVB3 protease 3CDpro induces RasGAP cleavage around 6 hours post-infection, an event that cannot be prevented by the general caspase inhibitor zVAD.fm.k. Cleavage of RasGAP results in phosphorylation of the MAPK Erk-1/2 on T202 and T204, respectively, through a Raf-1/MEK-1 dependent pathway [414]. Early transient and late sustained activation of Erk1/2 in CVB3 infection have been shown by numerous studies in both in vitro and in vivo [227,415-418]. It is suggested that the early phosphorylation of Erk1/2 is a response to virus-receptor binding, whereas the late phosphorylation is dependent on viral replication [415,417]. Inhibition of Erk1/2 by
specific inhibitors significantly blocks virus replication [415,417]. However, the actual mechanism underlying such regulatory effect remains uncovered.

Very recently, phosphorylation of the stress-activated protein kinases (SAPKs) JNK1/2 and p38 MAPK has been shown to increase in CVB3-infected HeLa cells, apparently independent of caspase cleavage or virus-induced production of reactive oxygen species [419]. Although inhibition of these protein kinases did not affect CVB3 viral protein synthesis, inhibition of p38 MAPK but not of JNK1/2 resulted in significant reduction of viral progeny release, caspase-3 cleavage and host cell death [419]. These findings highlight the importance of host signaling events in the pathogenesis of CVB3 and most importantly in the severity of infection and the fate of infected host.
REFERENCES:


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Table 1. Viruses reported in association with myocarditis

<table>
<thead>
<tr>
<th>Viruses</th>
<th>England &amp; Wales #</th>
<th>World *</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1990-1993</td>
<td>1975-1985</td>
</tr>
<tr>
<td>Coxsackie B</td>
<td>147</td>
<td>665</td>
</tr>
<tr>
<td>Influenza A</td>
<td>42</td>
<td>75</td>
</tr>
<tr>
<td>Influenza B</td>
<td>25</td>
<td>80</td>
</tr>
<tr>
<td>Enterovirus Untyped</td>
<td>14</td>
<td>——</td>
</tr>
<tr>
<td>Picornavirus Unspecified</td>
<td>—</td>
<td>20</td>
</tr>
<tr>
<td>Coxsackie A</td>
<td>—</td>
<td>61</td>
</tr>
<tr>
<td>Poliovirus</td>
<td>—</td>
<td>13</td>
</tr>
<tr>
<td>Echovirus</td>
<td>5</td>
<td>293</td>
</tr>
<tr>
<td>Adenovirus</td>
<td>7</td>
<td>47</td>
</tr>
<tr>
<td>Cytomegalovirus</td>
<td>5</td>
<td>77</td>
</tr>
<tr>
<td>Other Viruses</td>
<td>8</td>
<td>226</td>
</tr>
</tbody>
</table>

* Viruses detected per 1000 infections for world data; # from 368 myocarditis cases reported to the Communicable Disease Surveillance Centre (CDSC); data modified from references [79 & 88].
Figure 1-1

<table>
<thead>
<tr>
<th>Viral Protein</th>
<th>Proposed Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>VPG (3B)</td>
<td>Priming RNA synthesis</td>
</tr>
<tr>
<td>5'-UTR</td>
<td>Initiation of IRES-mediated protein synthesis</td>
</tr>
<tr>
<td>VP1-VP4</td>
<td>Viral capsid proteins</td>
</tr>
<tr>
<td>2A</td>
<td>Host protein shutoff, cleavage of cellular dystrophin</td>
</tr>
<tr>
<td>2B</td>
<td>Increased plasma membrane permeability and viral release, inhibition of cellular secretion</td>
</tr>
<tr>
<td>3A</td>
<td>Inhibition of cellular secretion</td>
</tr>
<tr>
<td>3C&lt;sup&gt;Pro&lt;/sup&gt;</td>
<td>Cleavage of cellular transcription factors</td>
</tr>
<tr>
<td>3D&lt;sup&gt;Poly&lt;/sup&gt;</td>
<td>RNA-dependent RNA polymerization, VPG uridylylation</td>
</tr>
</tbody>
</table>
CVB3 RNA encompasses a single and large open-reading frame (ORF) flanked on both 3’ and 5’ termini by untranslated regions (UTR). Upon entry, the RNA encodes a large viral polyprotein that is subsequently cleaved into three primary precursor molecules P1, P2, and P3 by virus-encoded proteases 2A<sup>pro</sup> and 3C<sup>pro</sup>. The P1 portion will be further cleaved by 3CD<sup>pro</sup> to make the structural capsid proteins VP1, VP2, VP3, and VP4, while the P2 and P3 segments will make non-structural viral proteinases and polymerases (2A<sup>pro</sup>, 2B, 2C, 3A, 3B/VPg, 3C<sup>pro</sup>/3CD<sup>pro</sup>, 3D<sup>pol</sup>). The proposed function for each viral protein has been also included.
Figure 1-2. Coxsackievirus B3 life cycle

Following the attachment to the coxsackievirus/adenovirus receptor (CAR) and decay-accelerating factor (DAF), the viral genome enters the cell and encodes a polyprotein that will be processed to viral structural and non-structural proteins. Virus positive strand RNA acts as a template for transcription of negative strands of viral RNA that will eventually make new positive strand RNAs with the assistance of virally encoded RNA-dependent RNA polymerase 3D\text{pol}. The newly synthesized RNA and capsid proteins come together to make new infectious particle, which will be released from the infected cell by lysis.
Figure 1-3. Three distinct phases of viral myocarditis

**Acute Myocarditis**
- Viremia
- Increased virus-titer in myocytes
- Acute myocyte damage
- Focal microcalcification
- Macrophage activation

Cytokine Expression
- IL-1, IL-2, TNF-α, IFN-γ

**Sub-acute Myocarditis**
- Virus clearing
- Infiltration of immune cells (T cells, B cells)
- Neutralizing antibodies
- NK cells → perforin
- Nitric oxide

**Chronic Myocarditis**
- Virus absence
- Viral RNA persistence
- Myocardial fibrosis
- Cardiac dilation
- Heart failure
Figure 1-4. Proposed model for immune-mediated cell injury in CVB3 myocarditis

Cardiac and viral antigens released by the host cell, can be recognized by antigen presenting cells (APCs). The activation of toll-like receptors (TLR) by viral antigens leads to NFkB activation and its translocation into the nucleus of activated cells, leading to subsequent increase in expression of pro-inflammatory cytokines. Released cytokines activate CD4\(^+\) and CD8\(^+\) T-lymphocytes as well as antibody-producing B-lymphocytes.

The CD4\(^+\) T-cells recognize viral antigens associated with major histocompatibility complex class II (MHC-II). Activated CD4\(^+\) cells stimulate and attract B-lymphocytes and CD8\(^+\) T-lymphocytes to the area of injury. CD8\(^+\) T-cells recognize viral antigens in association with MHC class I (MHC-I) [184, 185, 192].
Viral proteins contribute to cell death in infected cells. CVB3 protease 2A has a significant role mainly through cleavage of cellular translation initiation factor eIF4γ leading to inhibition of host protein synthesis. The 2A protease can also cleave cytoskeletal dystrophin causing morphological destruction and it may directly cleave pro-caspase-3 to induce apoptotic cell death. Viral replication leads to the release of mitochondrial cytochrome c and subsequent activation of Caspase-3 and PARP.
Figure 1-6. Cellular events in CVB3-infected cells

Schematic of the cellular events following CVB3 binding and entry. The details of main events included are discussed in the text.
Figure 1-7. Integrin-linked kinase (ILK) signalling

ILK binds integrin β₁ and β₃ through its C-terminus. Integrin-mediated activation of ILK results in recruitment of the components of focal adhesion plaque that initiate and support actin polymerization, an essential step in cellular motility and migration. Activation of PI3K also recruits ILK through its PH-like domain to close proximity to the plasma membrane, where it can regulate cellular survival via phosphorylation and activation of Akt and inactivation of GSK3-β. ILK also interacts with PINCH and ILKAP through its ankyrin repeat domain within the N-terminal domain. The interaction has been shown to regulate ILK kinase activity.
Akt becomes activated via cell membrane receptors such as receptor tyrosine kinases (RTKs). Binding of cognate ligands to their receptor leads to recruitment of PI3K to the plasma membrane and formation of PtdIns(3, 4, 5)P3 (PIP3), which in turn recruits Akt to the plasma membrane where it becomes phosphorylated by upstream serine/threonine kinases. Activated Akt phosphorylates a number of target proteins involved in a myriad of cellular functions.
Figure 1-9

The Classical Pathway

- Ras
  - Raf
  - MEK
  - Erk1/2
  - PI3K

The Alternative Pathway

- TRAFs
  - NIK
  - IKKa
  - IKKβ
  - IKKγ

1) Apoptosis
   - Fas, Fas-L, TRAIL, C-myc, p53, TNF-α

2) Survival
   - Cyclin-D1, Survivin, TRAF2, cIAPs, cFLIP, Bcl-2, Bcl-xL

3) Metastasis
   - ICAM-1, VCAM-1, MMPs, COX-2
Figure 1-9. Classical and alternative pathways of NFkB activation

The classical NFkB pathway is activated in response to a variety of inflammatory signals. The pathway involves the IKK heterotrimeric complex and downstream IkB-α phosphorylation and ubiquitination that results in activation and translocation of the NFkB heterodimer p65-p50 into the nucleus. The alternative pathway results in translocation of NFkB p52-Rel-B heterodimer into the nucleus. It is suggested that the alternative pathway has an essential role in the development of lymphoid organs.
CHAPTER 2: SUMMARY OF RESEARCH PROPOSAL, CENTRAL HYPOTHESIS, AND SPECIFIC AIMS

2-1. BACKGROUND

Coxsackievirus B3 (CVB3) causes severe cardiac and pancreatic disease and is the most prominent infective agent of heart muscle cells, directly injuring infected cardiac myocytes. At times, viral myocarditis leads to end-stage dilated cardiomyopathy, for which the only available treatment is organ transplantation. In response to infection, the host cell activates different anti-apoptotic and stress kinase signalling pathways in an attempt to maintain myocyte viability and inhibit viral replication. CVB3 terminates host cell transcription and translation machinery not only to hijack copious cellular resources but also to protect itself against early innate immune responses. In order to provide the requisite time to complete its replicative cycle, the virus may activate numerous "survival" pathways; when the adequate number of progeny viruses is produced, it may subsequently activate a chain of events leading to apoptotic cell death.

Indisputably, initiation of early innate and subsequent acquired immune response in viral myocarditis is highly dependent on the presentation of foreign antigenic epitopes to the host quiescent immune system. This process necessitates modification, release, or exposure of viral as well as myocardial antigens within the infected heart. As such, the early interaction between infectious virus and individual host is of importance, and determines the nature and potency of the host response, severity of injury, and disease prognosis, particularly during the fulminant course of viral myocarditis. This has compelled several groups including ours to direct much effort in understanding the
interaction between virus and host during the early phase of CVB3 myocarditis. The major focus of this dissertation is to scrutinize various aspects of the early interplay between the CVB3 life cycle and host survival signalling, and to determine how the balance between death and survival will determine the fate of infected cells and eventually the extent of myocardial injury. Our previous findings that CVB3 can exert both pro- and anti-apoptotic effects have prompted us to further investigate the role of protein kinase B/Akt as the central player of cell survival signalling during CVB3 infection.

2-2. CENTRAL HYPOTHESIS

Direct cardiac myocyte injury during coxsackievirus B3 infection is determined by virus-triggered cellular apoptotic signalling pathways that compromise host cell integrity. In virus-infected cells, the Akt pathway is invoked in an attempt to protect the host cell. The balance between pro-death and pro-life signals determines the likelihood of myocyte fate and virus-induced cytopathic effects. Manipulation of such balance in infected cells may establish a novel therapeutic approach for enteroviral myocarditis.

2-3. MAIN OBJECTIVE

The main objective of this study is to dissect and characterize signaling events, particularly activation of protein kinase B/Akt, during the course of CVB3 infection. This study also aims to investigate the potential regulatory role and the therapeutic value of the Akt pathway in enteroviral myocarditis (Fig. 2-1).

2-4. SPECIFIC AIMS
Aim 1- To determine whether Akt is activated following CVB3 infection in HeLa cells and mouse cardiomyocytes and to determine whether this activation is phosphatidylinositol-3 kinase (PI3K)-dependent.

Aim 2- To assess the potential regulatory role of Akt activation in CVB3 life cycle and virus-induced destructive morphology.

Aim 3- To determine whether integrin-linked kinase (ILK) is an upstream mediator of CVB3-induced Akt phosphorylation, and to characterize the consequence of ILK inhibition on virus replication and host cell viability in CVB3-infected HeLa cells and mouse cardiomyocytes.

Aim 4- To investigate whether CVB3 infection may lead to activation of the transcription factor nuclear factor kappa B (NFkB) and its translocation into the nucleus of infected cells, and to investigate the role of NFkB activation in virus life cycle and virus-induced cytopathic effects.

2-5. SIGNIFICANCE

The opportunity presented in the proposed study relates to the little-evaluated cellular mechanisms by which life of CVB3-infected cells can be sustained, probably at the cost of associated dysfunction, and contributes significantly to the development of new therapeutic strategies for CVB3-induced myocarditis. This work will also complement other efforts being made in the laboratory to understand pro-life and pro-death pathways in enterovirus-infected cells. Deciphering the mechanisms, by which the virus
commandeers host signalling may provide an opportunity for developing novel treatments for enteroviral myocarditis.
Figure 2-1. Schematic diagram of general hypothesis and specific aims
CHAPTER 3: PROTEIN KINASE B/AKT IN COXSACKIEVIRUS B3 INFECTION

Objective:

To characterize the dynamics of Akt activation during CVB3 infection and to investigate the potential regulatory role of Akt in CVB3 pathogenesis.

The candidate performed or supervised all experiments presented. The assistance of research technician Agripina Suarez in performing the in situ hybridization technique is greatly appreciated.

Based on the Manuscript:
INTRODUCTION

CVB3 is a small non-enveloped positive-strand RNA Enterovirus in the Picornaviridae family. CVB3 has been known as the most common infectant of the heart that directly injures and kills infected cardiac myocytes [1,2]. CVB3-induced myocarditis was known historically as an immune-mediated disease [3-5]. However, the most recently supported hypothesis is that direct CVB3-induced injury prior to immune cell infiltration is a very important determinant of disease progression [1,2]. With regard to virus-infected cells, the fate of infected cell and the severity of disease are greatly influenced by the balance between anti-apoptotic and pro-apoptotic pathways. Our laboratory has carried out extensive work on CVB3-triggered death signaling pathway in the context of interaction between this pathway and the virus life cycle. We have characterized apoptotic signaling, in particular the caspases, following CVB3 infection in HeLa cells [6]. Although the interaction between CVB3 and survival cascades has not been investigated in detail, there is mounting evidence to support the idea that a few other viral proteins can modulate such pathways. The X protein of hepatitis B virus (HBx) activates survival signaling cascades including phosphatidylinositol-3 kinase (PI3K)/Akt and stress-activated protein kinase resulting in progression of hepatocellular carcinoma [7,8]. It has also been demonstrated that the BHRF1 protein of Epstein-Barr virus (EBV) and immediate-early proteins IE1 and IE2 of human cytomegalovirus markedly inhibit apoptosis in infected cells [9,10]. However, viral products may have a dual action in infected host. A well-characterized example is simian virus 40 (SV40) large tumor (T) antigen, which may either block or induce apoptosis depending upon the cell environment [11,12].
The role of signaling proteins during CVB3 infection and the modulation of these proteins by virus are not very well understood. Recently, we reported that a biphasic activation of the extracellular signal-regulated kinase (ERK1/2) participates in the regulation of viral replication and virus-mediated cytopathic effects in infected HeLa cells [13]. However, little is known about the role of the other protein kinases during CVB3 infection.

Protein kinases play a critical regulatory role as the second messengers in various intracellular signaling pathways by phosphorylation of proteins that control nearly all features of cell life. One such protein kinase, protein kinase B/Akt, has been intensively studied during the last decade [14-17]. Akt, is a cytoplasmic serine/threonine kinase containing a pleckstrin homology (PH) domain at its amino terminus and acts as a key protein mediator for a wide range of cellular processes [18,19]. Three different isoforms of Akt have been so far characterized: Akt1, Akt2, and Akt3. A separate gene encodes each isoform, but they all carry a central PH-like domain, a catalytic domain and a putative regulatory domain. Several studies have shown that activation of Akt by various stimuli including growth factors, insulin, and hormones is mediated by phosphorylation of both serine 473 and threonine 308 residues through a PI3K-dependent mechanism [16,20]. The observation that cell stimulation with growth factors may trigger Akt translocation to the nucleus, alongside findings that Akt activates transcription factors such as the forkhead transcription factor (FKHR) and nuclear factor kappa B (NFkB), suggests that Akt may up- or down-regulate the expression of several genes involved in homeostasis [16,21-23]. The role of PI3K and its downstream effector Akt in regulating
cell survival and apoptosis in a number of viral infection models has been a major recent interest [24-26].

The possibility that the Akt pathway participates in the preservation of host cell survival during viral infection, and may play a role in providing a supportive milieu for virus replication compelled us to investigate the interaction between CVB3 and this pathway. In the present study, we have shown that Akt can be phosphorylated and activated following CVB3 infection through a PI3K-dependent mechanism. We have also shown that inhibition of Akt suppresses viral RNA synthesis, viral protein expression, and viral release in CVB3-infected cells by either using a specific inhibitor of the PI3K/Akt pathway or over-expressing a dominant negative mutant of Akt1.
3-2. MATERIALS & METHODS

i. Akt1 constructs

Transfection grade eukaryotic expression vectors (pUSEamp) encoding a dominant negative mutant Akt1 (expressing the neomycin resistance gene for selection of transfected cells) were purchased from Upstate Biotechnology, Inc. (Charlottesville, VA), and confirmed by restriction analysis. The dominant negative mutant of Akt1 construct (encoding an alanine instead of a serine and a threonine) and the empty vector control were used in stable transfection of HeLa cells.

ii. Cell culture and transfection

HeLa cells (HeLa S3) were obtained from the American Type Culture Collection (ATCC). Sub-confluent cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated newborn calf serum (Invitrogen, Burlington, ON) at 37°C, in a humidified incubator in 5% CO2. Penicillin G (100 μg/ml) and streptomycin (100 μg/ml) (Invitrogen) were added to all culture media. For stable transfection of HeLa cells, 2 μg of cDNA was introduced using LipofectAMINE 2000 Reagent (Invitrogen) according to the manufacturer's instructions. Thirty-six hours post-transfection, Geneticin (G418) (Sigma-Aldrich, Oakville, ON) was added as a selective marker at the final concentration of 400 μg/ml for selecting the transfected clones and at the final concentration of 200 μg/ml for maintenance of transfection during the course of experiments.

iii. Virus infection
CVB3 (Nancy strain) was a gift from Dr. Reinhard Kandolf and was propagated in HeLa cells and stored at -80°C. The titer of virus was determined routinely prior to each experiment. Sub-confluent wild type or transfected HeLa cells were serum-starved for 24 hours before the introduction of virus to eliminate the effect of serum growth factors. Cells were infected with either CVB3 at a multiplicity of infection (MOI) of 10 or DMEM (extracted from HeLa cell culture) for control group. Following a 1-hour incubation at 37°C, cells were replenished with fresh DMEM. For inhibitor experiments, pretreatment with inhibitor was performed for one hour prior to infection and fresh inhibitors at the specified concentrations were added following medium changes.

iv. UV-irradiation and inactivation of CVB3

To prepare UV-irradiated virus, one ml of diluted virus was transferred to a 1.5 ml tube and then irradiated in a UV Stratalinker 1800 (Stratagene, La Jolla, CA) for a total dose of 15 J/cm² for 10 min [27,28].

v. Antibodies and inhibitors

Rabbit polyclonal phospho-Akt antibodies (Ser-473 & Thr-308), rabbit polyclonal Akt antibody, rabbit polyclonal phospho-GSK3 α/β (Ser-21/Ser-9) antibody, and a specific PI3K inhibitor 2-morpholino-8-phenyl-4H-1-benzopyran-4-one (LY294002) were purchased from New England BioLabs (Ipswich, MA). Rabbit polyclonal antibody against viral protein VP1 was obtained from Denka Seiken Co., Ltd. (Chuo-Ku, Tokyo); mouse monoclonal caspase-3 antibody, mouse monoclonal β-actin antibody, anti-rabbit-IgG and anti-mouse-IgG conjugated with horseradish-peroxidase were purchased from
Santa Cruz Biotechnology (Santa Cruz, CA); mouse monoclonal antibody against PARP was obtained from BioMol Co. (Plymouth Meeting, PA). The general caspase inhibitor benzyloxy carbonyl-Val-Ala-Asp-fluoromethylketone (zVAD.fmk) was obtained from Clontech (Mountain View, CA).

vi. Western blot analysis

Cells either untreated or treated with different experimental reagents were washed twice with ice-cold PBS containing 5% phosphatase inhibitor (Active Motif Co, Carlsbad, CA) and kept on ice for 10 min in lysis buffer containing 50 mM pyrophosphate, 50 mM NaF, 50 mM NaCl, 5 mM EDTA, 5 mM EGTA, 100 µM Na3VO4, 10 mM HEPES, pH 7.4, 0.1% Triton X-100, 500 µM phenylmethylsulfonyl fluoride, 10 µg/ml leupeptin, and 1 mM PMSF. Cell lysates were then collected by scraping and centrifuged at 12000 X g for 10 min at 4°C. Protein concentration was determined by the Bradford assay. Twenty to 40 µg of extracted protein was fractionated by 10% SDS-polyacrylamide gels, electrophoretically transferred to nitrocellulose membranes (Amersham Biosciences Corp., Piscataway, NJ), and blocked with PBS containing 0.1% Tween-20 and 5% nonfat dry milk for 1 hour. Afterward, the membrane was incubated with specific primary antibody overnight at 4°C, followed by secondary antibody for 45 min at room temperature. The immunoblots were visualized with enhanced chemiluminescence detection system according to the manufacturer’s protocol (Amersham Biosciences Corp.). Densitometry analysis was performed by using the National Institutes of Health ImageJ software, version 1.27z. Density values for proteins were normalized to the level for control groups (arbitrarily set to 1.0 fold).
vii. **Viral RNA in-situ hybridization (ISH)**

Sub-confluent HeLa cells were grown and maintained on 2-chamber culture slides in serum-free conditions for 24 hours prior to CVB3 infection. Cells were infected with either DMEM or CVB3 (MOI=10). Following 1-hour incubation at 37°C, cells were washed with serum-free DMEM and replenished with fresh DMEM. Culture slides were then washed gently with PBS and fixed with 10% normal formalin buffer for 15 min, and subsequently washed with PBS and incubated with 70% ethanol for 2 min, and then air-dried at room temperature. Culture slides were then submitted for *in situ* hybridization assay to detect sense- and antisense-RNA of CVB3 as previously described [29].

viii. **Virus release plaque assay**

Wild type or transfected HeLa cells either untreated or treated with specific inhibitors were infected with CVB3 at the MOI of 10 for 1 hour. Cells were washed and replenished with serum-free DMEM for 9 hours. The supernatants were collected and kept at -80°C for viral replication assays. Plaque assays were carried out in HeLa cells in duplicate using standard procedure as described previously [29]. Agar overlays were fixed with Carnoy's fixative (25% acetic acid, 75% ethanol) at 3 days after infection. Cells were stained with 1% crystal violet dye to visualize plaques. Images of plates were obtained and the virus titer was calculated as plaque forming units per milliliter (pfu/ml).

ix. **Statistical analysis**
Two-way analysis of variance with multiple comparisons and paired Student's t-tests were performed. Values shown are the mean ± SD. A $p$ value of <0.005 was considered significant.
3-3. RESULTS

i. Coxsackievirus B3 induces Akt phosphorylation at both serine 473 and threonine 308 residues

In the present study, we initially determined whether coxsackievirus B3 infection of HeLa cells resulted in phosphorylation of Akt. Serum-starved HeLa cells were incubated with CVB3 or DMEM. DMEM treatment used as a control was extracted from HeLa cells in culture under identical conditions as those used for virus propagation to account for the potential effect of proteins within the media. Cell lysates were then collected at 10 and 30 min, and at 1, 3, 6, and 9 hours post-infection. As shown in Fig. 3-1, CVB3 induces Akt phosphorylation at both Ser-473 and Thr-308 residues at 6-9 hours following the infection. The phosphorylation of the Akt-regulated downstream target, glycogen synthase kinase-3 α/β, was determined as a measure of Akt kinase activity. Total Akt was measured to ensure equal protein loading.

ii. Coxsackievirus B3 stimulates Akt phosphorylation via a PI3K-dependent mechanism

To investigate whether Akt phosphorylation and activation upon CVB3 infection occurs through the PI3K pathway, HeLa cells were pretreated with increasing doses (5, 10, 25, and 50 µM) of the specific PI3K inhibitor LY294002 one hour prior to virus infection. LY294002 inhibits PI3K activity through a competitive inhibition of the ATP binding site located on the p110α subunit of PI3K [30,31]. Cell lysates were collected at 9 hours post-infection and subjected to Western blot analysis to detect the phosphorylation of Akt on
Ser-473 and Thr-308. Fig. 3-2A shows LY294002 inhibition of CVB3-induced Akt phosphorylation and activation in a dose-dependent manner. The observation that the phosphorylation of Akt was markedly decreased using a higher dose of LY294002 (50 μM), suggests that virus-induced Akt phosphorylation occurs through a PI3K-dependent mechanism.

iii. UV-irradiated CVB3 is unable to induce Akt phosphorylation and activation

To investigate the mechanism behind the activation of Akt, CVB3 was exposed to UV radiation for 10 min for a total dose of 15 J/cm². UV-irradiation blocks the virus’s capability for RNA transcription and protein synthesis, but has no affect on virus receptor attachment and subsequent entry into the host cell [32-34]. HeLa cells were infected with either wild-type CVB3 or UV-inactivated virus and cell lysates were collected at 7 hours post-infection and then subjected to Western blot analysis to detect the phosphorylation of Akt on the Ser-473 and Thr-308 sites. As shown in Fig. 3-2B, UV-inactivated CVB3 failed to induce Akt phosphorylation, implying that Akt phosphorylation is dependent on post-entry viral replication.

iv. LY294002 blocks viral RNA synthesis, viral protein VP1 expression and virus release in CVB3-infected HeLa cells

In order to investigate the interaction between the PI3K/Akt cascade and CVB3 replication and to understand whether the activation of this pathway plays a role in CVB3 replication, we examined the effect of the specific PI3K inhibitor LY294002 on different stages of the virus life cycle in infected cells. Sub-confluent HeLa cells were grown on
culture slides and pretreated with either inhibitor or DMSO prior to infection. The supernatants were collected at 9 hours post-infection for the plaque assay experiment to calculate the number of progeny virions released from infected cells. Concurrently, HeLa cells on glass slides were fixed and subjected to in situ hybridization to assess viral RNA expression. The expression of VP1 in the presence or absence of LY294002 was determined by Western blot. As shown in Fig. 3-3A, LY294002 blocked viral protein expression in a dose-dependent manner. LY294002 also significantly decreased viral RNA synthesis (Fig. 3-3B) and viral progeny release (Fig. 3-3C) as compared to control groups. These results suggest that PI3K contributes to the virus life cycle and is likely beneficial to virus replication.

v. Dominant negative mutant of Akt1 also decreases viral RNA synthesis, viral protein VP1 expression, and virus release in infected cells

To further study the contribution of Akt as a key downstream effector of PI3K to CVB3 replication, we utilized a different approach by using a dominant negative mutant of Akt1 that is incapable of being phosphorylated in response to different stimuli. The dominant negative Akt1 cDNA contains Myc-His tagged Akt1 (in pUSEamp) under the control of the CMV promoter and has substitution of methionine for lysine and serine and threonine for alanine. In this experiment, HeLa cells were stably transfected with either a dominant negative mutant of Akt1 or a vector control (pUSEamp) plasmid. Akt phosphorylation in the transfected cell line was evaluated prior to the experiment. As expected, in the presence of serum, the dominant negative Akt1 mutant significantly blocked Akt1 phosphorylation and activation in transfected HeLa cells as compared to control cells
(Fig. 3-4A). Subsequently, transfected HeLa cells were infected with CVB3 for 1 hour and viral protein expression, viral RNA synthesis, and viral progeny release were determined at 9 hours post-infection. Our observations showed that the dominant negative mutant of Akt1 blocked CVB3 replication including viral protein expression (Fig. 3-4B), viral RNA synthesis (Fig. 3-4C), and viral release from infected cells (Fig. 3-4D). These findings, together with our results from inhibitor-based experiments show that virus-induced PI3K/Akt activation augments CVB3 replication in HeLa cells.

vi. PI3K inhibitor and the dominant negative mutant of Akt1 enhance apoptosis in CVB3-infected HeLa cells through a caspase-dependent mechanism

To elucidate the role of Akt in regulating apoptosis in CVB3-infected cells, Akt activation was blocked by either a specific PI3K inhibitor or the dominant negative mutant of Akt1 construct. Apoptosis was assessed by measuring caspase-3 and PARP cleavage at 9 hours post-infection. The dominant negative mutant of Akt1 (dn-Akt1) and LY294002 both increased caspase-3 and PARP cleavage (Fig. 3-5A & 5B) and enhanced cytopathic effects (Fig. 3-5C) in CVB3-infected cells. Since we have already shown that inhibition of Akt results in a significant decline in CVB3 replication, these findings suggest that increased cytopathic effects in infected cells are due to the inhibition of anti-apoptotic activity of Akt by either LY294002 or by expression of a dominant negative form of Akt1. It is noteworthy that the pro-apoptotic effect of the PI3K-inhibitor or the dominant negative Akt1 construct in CVB3-infected cells is less so as compared to that observed in non-infected cells, suggesting that the virus-induced survival pathways may play a role in contravening apoptosis in an attempt to initially foster host cell survival and
viral replication. The pro-apoptotic effect of dominant negative Akt1 in infected HeLa cells could be reversed by adding the general caspase inhibitor zVAD.fmk. HeLa cells transfected with either dn-Akt1 or vector control plasmids were pretreated with zVAD.fmk (50 μM) one hour prior to infection. As shown in Fig. 3-6A, caspase inhibitor zVAD.fmk reversed the apoptotic effect of dominant negative Akt1 in CVB3-infected cells by blocking caspase-3 cleavage.

vii. Inhibition of viral protein expression by dominant negative Akt1 is not caspase-dependent

To clarify whether inhibition of viral protein expression by dominant negative Akt1 is due to acceleration of apoptosis in infected cells, we compared the expression of viral protein VP1 in dn-Akt1-transfected HeLa cells in the presence or absence of the general caspase inhibitor zVAD.fmk. As shown in Fig. 3-6B, inhibition of caspase-3 cleavage and apoptosis does not reverse the inhibitory effect of dn-Akt1 on viral protein expression. The observation that zVAD.fmk is capable of blocking apoptosis in infected cells (Fig. 3-6A) without having any effect on viral RNA translation and VP1 expression (Fig. 3-6B) indicates that the regulatory effect of Akt on virus replication is not a caspase-dependent process.
3-4. DISCUSSION

During viral infection, viruses encode highly competent products in order to optimize viral replication, produce huge numbers of progeny virions, and release infectious particles to launch a new round of infection. However, host cells activate pro-survival defenses aimed to preserve host cell viability in response to viral infection. Viruses may evolve strategies to block or delay such host defense mechanisms. How viruses interact with and manipulate host cell signaling pathways to augment their replication is currently the subject of intense study. Our laboratory has previously reported that the phosphorylation and activation of the ERK1/2 during CVB3 infection is required for viral replication. We have also shown that CVB3 activates ERK1/2 in a biphasic manner, in early transient and late sustained phosphorylation events. We have recently reported that interferon γ-inducible GTPase (IGTP) over-expression enhances host cell survival in CVB3-infected cells, likely by inducing the activation of Akt [35]. We also revealed that IGTP over-expression inhibits viral replication probably by its overwhelming negative effect on viral RNA translation. These finding provoked our group to further investigate the role of Akt activation in the course of CVB3 infection.

In the present study, we report that CVB3 enhances Akt phosphorylation on both serine 473 and threonine 308 residues resulting in the activation of this protein kinase. The activity of phosphorylated Akt was assessed by phosphorylation of a downstream target glycogen synthase kinase-3 α/β. Replication-deficient CVB3 (UV-irradiated), which is capable of receptor attachment and cell entry but not replication, fails to induce Akt phosphorylation indicating that virus attachment and entry are not sufficient for Akt
activation. It also supports the view that post-entry viral RNA synthesis and subsequent steps in viral replication and progeny production are required for Akt phosphorylation and activation in infected cells.

In the current study, we also investigated the regulatory role of PI3K in CVB3-induced Akt phosphorylation by using different dosage of the specific PI3K inhibitor LY294002. The results suggest that CVB3 can trigger Akt activation via a PI3K-dependent mechanism. It has previously been shown that PI3K is a major upstream activator of Akt through the production of phosphatidylinositol-3, 4, 5-triphosphate in response to growth factors and hormones [16,36]. Recent studies revealed that PI3K is commonly stimulated upon activation of membrane receptors that either couple to heterotrimeric guanosine triphosphate (GTP)-proteins or have tyrosine kinase activity [37-39]. Our observation that replication-deficient CVB3 cannot activate PI3K/Akt pathway supports the idea that activation of PI3K/Akt during CVB3 infection is not a receptor-dependent event and it is more likely this activation is induced directly or indirectly by viral products.

To increase our understanding of the interaction between Akt and virus replication, we also examined the effects of Akt activation in different checkpoints of virus life cycle including virus RNA transcription, viral RNA translation and protein expression, and viral release from infected host cells. We observed that inhibition of Akt phosphorylation resulted in a significant decrease in virus RNA transcription and translation as well as virus assembly and release. Although the regulatory mechanism by which Akt controls CVB3 replication is not well understood, there is mounting evidence indicating that viral proteins can be phosphorylated by various protein kinases. Several protein kinases
including ERK1/2 and protein kinase C (PKC) have been shown to enhance HIV-1 infectivity by direct phosphorylation of viral proteins Vif, Tat, Gag, and Nef [6,29,42-45]. Hepatitis B virus large envelope protein (L) can be phosphorylated by ERK-type mitogen-activated protein kinases [46]. There is also an indication that poliovirus-specific RNA-dependent RNA polymerase (3D\textsuperscript{pol}) can be phosphorylated at serine residues [47]. These findings support the postulation that Akt may regulate CVB3 replication by direct phosphorylation of viral products including viral polymerases.

The PI3K pathway including the downstream effector Akt also plays a pivotal role in cell survival and proliferation. It has been reported that the inhibition of this pathway results in a significant decrease in host cell viability in different cell environments [25,48-50]. It is presumable that CVB3 enhances Akt phosphorylation to increase the viability of infected host cells for viral replication to take place. Although the downstream effectors of Akt involved in cell survival and proliferation have been extensively studied in tumor models, the mechanism and significance of anti-apoptotic activity of Akt during CVB3 infection remains obscure.

We have found that Akt inhibition markedly enhances virus-induced cleavage of caspase-3 and PARP, indicating an anti-apoptotic role for Akt during CVB3 infection. Since we have already shown that inhibition of Akt results in a substantial decrease in virus replication, it is likely that increased apoptosis in infected cells is a direct consequence of the inhibition of anti-apoptotic activity of Akt, but not a result of virus replication. Treating dn-Akt1 cells with general caspase inhibitor zVAD.fmk blocked caspase-3 cleavage. Although zVAD.fmk blocked caspase-3 cleavage in infected cells, it did not
show any effect on viral protein expression. In other words, even by blocking apoptosis, we were unable to reverse the inhibitory effect of dn-Akt1 on viral protein expression and preserve VP1 production. This evidence strongly supports the view that Akt regulates viral replication through a mechanism that is not caspase-dependent.

In summary, our study illustrates an important role for the PI3K/Akt pathway during CVB3 infection in HeLa cells. The findings suggest that Akt activation facilitates a productive viral infection.
REFERENCES:


24. Dawson CW, Tramountanis G, Eliopoulos AG, Young LS: Epstein-Barr virus latent membrane protein 1 (LMP1) activates the phosphatidylinositol 3-kinase/Akt pathway to


Figure 3-1. CVB3 infection leads to Akt phosphorylation on both serine 473 and threonine 308 residues.

Following serum starvation for 24 hours, HeLa cells were either sham- or CVB3-infected (MOI=10) for one hour. Cell lysates were collected at specified times following infection and subjected to Western blot to detect the phosphorylation of Akt on both Ser-473 and Thr-308 sites. The activity of phospho-Akt was determined based on the phosphorylation of glycogen synthase kinase 3 α/β (GSK 3 α/β), a downstream substrate of Akt. Total Akt protein was measured to ensure equal protein loading. Protein density is expressed as the increase in the level of phosphorylated protein vs. non-infected control (*, **, ***; p < 0.005). The data shown are representative of quadruplicate experiments.
Figure 3-2

A)

P-Akt (Ser-473)
P-Akt (Thr-308)
P-GSK3 α
P-GSK3 β
Total Akt

LY294002 (μM)

Control CVB3 (9hr pi)

B)

7 (hr pi)

Control CVB3 UV-CVB3

P-Akt (Ser-473)
P-Akt (Thr-308)
Total Akt
Figure 3-2. Post-entry viral replication induces Akt phosphorylation via a PI3K-dependent mechanism.

A) HeLa cells were serum-starved for 24 hours. After being treated with LY294002 (50μg/ml) or vehicle (DMSO), cells were infected with either CVB3 (MOI=10) or DMEM for one hour. Total protein was extracted and analyzed by immunoblotting. Akt phosphorylation on both Ser-473 and Thr-308 residues was prevented by the PI3K inhibitor, indicating that Akt activation by CVB3 was mediated via a PI3K-dependent pathway. Total Akt protein was detected to ensure equal loading protein. Density values for phospho-proteins are expressed as fold change compared to non-treated CVB3-infected cells (*, **; ***, p < 0.005). The data are representative of two independent experiments. B) HeLa cells were infected with wild type or UV-irradiated CVB3, and cell lysates collected at 7 hours post-infection and subjected to Western blot to detect Akt phosphorylation on Ser-473 and Thr-308. Virus attachment to a specific receptor-coreceptor complex is not sufficient for Akt phosphorylation, suggesting that viral post-entry replication is required for Akt activation. Results are representatives of triplicate experiments.
Figure 3-3

A) Viral Protein VP1 Expression

B) Control CVB3 (9hr pi)

LY294002(µM)

VP1

β-Actin
Figure 3-3, Cont'd...

C)

P < 0.005

Log 10 CVB3 Titer (pfu/ml)

CVB3 (9hr pi)

LY294002(μM)

0 50

*
Figure 3-3. LY294002 blocks viral VP1 synthesis, viral RNA expression, and viral release in infected HeLa cells.

A) CVB3-infected HeLa cells were treated with either DMSO or different doses of LY294002 for 9 hours. Cell lysates were collected for Western blot to detect viral capsid protein VP1 expression. β-actin expression was measured to ensure of equal protein loading. As shown, LY294002 blocks VP1 synthesis in a dose-dependent manner. B) In situ hybridization of CVB3-infected HeLa cells at 9 hours post-infection using a digoxigenin-labelled viral strand-specific riboprobe (brightfield images on the upper row and fluorescent images on the lower row). Positive signals indicate sense (+) strand RNA of CVB3. Treatment of infected cells with 50 μM LY294002 significantly blocked viral RNA transcription and replication in infected cells. C) Viral release in LY294002-treated HeLa cells. Supernatants from culture slides (from B) were collected and assayed for infectious virus by the agar overlay plaque assay method. Comparison of infectious virus particles in the indicated conditions showed that the PI3K inhibitor decreased viral progeny release from infected host cells. The data represent three experiments where * indicates $P < 0.005$ (mean ± SE) as compared to non-treated infected cells.
Figure 3-4

A)

Vector | dn-Akt1 | Vector

- | + | +

10% Serum

P-Akt1

P-GSK3 α

P-GSK3 β

β-Actin

B)

Vector | dn-Akt1

- | + | - | +

CVB3 (9hr pi)

VP1

β-Actin
Figure 3-4. Dominant-negative mutant of Akt1 blocks viral VP1 synthesis, viral RNA expression, and viral release in infected HeLa cells.

A) Phospho-Akt1 level in stably transfected HeLa cells. Transfected HeLa cells were treated with either serum or PBS for 30 minutes and 40 μg of cell lysates were separated by SDS-PAGE and probed with anti-phospho-Akt and anti-phospho GSK3α/β antibodies. Akt1 phosphorylation and activity are markedly decreased in dn-Akt1 cells as compared to vector group treated with serum. B) Viral protein synthesis in HeLa cells over-expressing a dominant negative mutant of Akt1. The dominant negative mutant of Akt1 (dn-Akt1) decreases viral protein synthesis as compared to CVB3-infected vector group. C) *In situ* hybridization of stably transfected HeLa cells at 9 hours post-infection (brightfield images on the upper row and fluorescent images on the lower row). Dn-Akt1 significantly suppresses viral RNA replication. D) Virus release in dominant negative-transfected HeLa cells. Comparison of CVB3 infectious particles released in the culture media of slides from section “C” shows that dominant negative mutant of Akt1 also diminishes virus release from infected cells. The data represent four independent experiments where * indicates P< 0.005 as compared to CVB3-infected vector.
Figure 3-5

A)

Control CVB3 (9hr pi)

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<tr>
<th>LY294002(μM)</th>
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Cleavage of Caspase-3 and PARP

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<td>50</td>
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Cleaved Caspase-3
Cleaved PARP

* **
B)

**Figure 3-5, Cont’d...**

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**Graph:**

- **Caspase-3 & PARP Cleavages (Fold Change)**
  - Black bars: Cleaved Caspase-3
  - White bars: Cleaved PARP
  - Control: Vector and dN-Akt1
  - CVB3 (9hr pi): Vector and dN-Akt1
  - Significant difference marked with * (p < 0.05) and ** (p < 0.01).
Figure 3-5, Cont'd...

C)

Control  CVB3 (9hr pi)

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<td>50 LY294002(μM)</td>
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Control  CVB3 (9hr pi)

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<th>CVB3 (9hr pi)</th>
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<td>dn-Akt1</td>
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Images show the effects of CVB3 infection and treatment with LY294002 and expression of dn-Akt1 on cell morphology.
Figure 3-5. LY294002 and dominant-negative mutant of Akt1 induce apoptosis in CVB3-infected cells.

A) Cleavage of caspase-3 and PARP in LY294002-treated HeLa cells. Cells treated with LY294002 or DMSO were infected with either DMEM or virus and cell lysates subjected to Western blot analysis for caspase-3 and PARP cleavages. LY294002 increases caspase-3 and PARP cleavage in CVB3-infected cells. Results are representative of two different experiments. *, ** indicate p < 0.01 as compared to non-treated infected cells.

B) Cleavage of caspase-3 and PARP in HeLa cells transfected with the dominant-negative Akt1 construct. Dominant negative mutant of Akt1 enhances caspase-3 and PARP cleavages. Density values for caspase-3 and PARP cleavages are expressed as fold change as compared to CVB3-infected cells transfected with vector control (*, **; p < 0.005). The results shown are representative of two independent experiments.

C) Phase-contrast microscopic images of wild-type HeLa cells treated with LY294002 and transfected HeLa cells following CVB3 infection. As shown, both LY294002 and dn-Akt1 construct enhanced cytopathic effects and decreased host cell viability in infected HeLa cells as assessed by morphological changes.
Figure 3-6

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Cleaved Caspase-3

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VP1

β-Actin
Figure 3-6. Akt regulates CVB3 replication through a caspase-independent pathway.

A) Cleavage of caspase-3 and PARP in HeLa cells transfected with the dominant-negative Akt1 constructs is reversed by the multi-caspase inhibitor zVAD.fmk. Transfected HeLa cells were treated with zVAD.fmk (50 μM) starting 1 hour prior to infection. β-actin expression has been measured for equal protein loading. B) Inhibition of caspase-3 cleavage by zVAD.fmk does not preserve viral protein expression in infected HeLa cells, indicating that the regulatory effect of Akt on virus replication is not a caspase-dependent process. The data represent two independent experiments.
CHAPTER 4: INTEGRIN-LINKED KINASE (ILK) AS A THERAPEUTIC TARGET FOR COXSACKIEVIRUS B3 MYOCARDITIS

Objective:

To identify the potential upstream kinase responsible for CVB3-induced Akt phosphorylation in Serine 473 residue and to determine the role of ILK in virus replication and virus-induced cellular injury.

All the experimental designs and procedures have been performed or supervised by the candidate. The assistance of summer student Ansel Amaral in performing Western blot analysis and tissue culture, and laboratory technician Zhongshu Luo for in situ hybridization is much appreciated.

Based on the Manuscript:
A novel regulatory role for integrin-linked kinase (ILK) in modulation of coxsackievirus B3 replication and virus-induced cardiomyocyte injury. Mitra Esfandiarei, Agripina Suarez, Ansel Amaral, Maziar Rahmani, Xiaoning Si, Shoukat Dedhar, and Bruce M. McManus, (Submitted, Circulation Research, April 2006).
INTRODUCTION

Myocarditis, an inflammatory disease of heart muscle, is a major cause of unexpected and sudden cardiac death in people under age of 40 [1]. More than twenty viruses have been associated with myocarditis, causing mild to severe injury in the myocardium with ultimate manifestation of end-stage dilated cardiomyopathy and heart failure [1,2]. Serogroup B1-5 coxsackieviruses have been implicated in 25–40% cases of acute myocarditis and dilated cardiomyopathy in infants and young adolescents [3-5].

Coxsackievirus B3 (CVB3), a small non-enveloped single-stranded RNA enterovirus in Picornaviridae family, is the most prominent cause of human myocarditis. CVB3-induced myocarditis was known historically as an immune-mediated disease [6-8]. However, direct CVB3-induced injury during acute phase of disease and prior to target-organ immune cell infiltration has been shown to be a very important determinant of disease progression and prognosis [9-11].

At present, our knowledge of molecular factors involved in initiation and progression of CVB3-induced myocarditis is very limited. However, it is well founded that the fate of infected cells and severity of disease are related to the balance between multiple and contemporaneous pro-apoptotic and anti-apoptotic processes, both viral and host cell in origin. Our laboratory has carried out extensive studies on CVB3-triggered death signaling events. In both cell culture and experimental animal models, CVB3 infection leads to the release of mitochondrial cytochrome c, formation of the apoptosome complex, activation of caspase-9, and subsequent cleavage of executioner caspases including caspase-3, -6, and -7 in the cytoplasm of infected cells [12-15]. These events
invoked by the offending virus result in morphological features of apoptotic cell death and virus-induced cytopathic effects, including cell shrinkage, cytoplasmic and chromatin condensation, and DNA fragmentation with corresponding loss of homeostatic regulation of cellular processes. Host cell signaling may re-balance cellular homeostasis, block apoptotic cell death, and diminish viral progeny release. Of the host cell signals, the phosphotidylinositol-3 kinase/Akt (PI3K/Akt) pathway has been implicated in survival, metabolism, proliferation, and apoptosis [16,17].

Previously, we reported on phosphorylation and activation of Akt in CVB3-infected cells [18]. We have also shown that during the early phase of CVB3 infection, Akt regulates virus replication through a PI3K-dependent pathway, an event of importance to the progression of CVB3 infection [18]. However, much remains to be uncovered about the actual mechanisms underlying virus-induced Akt activation and the significance of this event in virus-induced cytopathic effects and cardiac cell death.

Full Akt activation requires phosphorylation of Thr-308 residue in the catalytic domain and Ser-473 residue in the C-terminal hydrophobic domain of this protein kinase [19]. The 3-phosphoinositide-dependent kinase 1 (PDK-1), the kinase responsible for Akt phosphorylation on Thr-308, has been identified and thoroughly investigated [20-22]. Several studies in PDK-1 knockout cells have emphasized the existence of a distinct Ser-473 kinase [23]. Cumulative evidence suggests that integrin-linked kinase (ILK) is the upstream kinase responsible for Akt phosphorylation on Ser-473 in vitro [24-28]. Conversely, genetic studies in mouse fibroblasts, C. elegans, and Drosophila revealed
that ILK kinase activity may not be required for complete Akt activation, casting uncertainty upon previous findings [29-32].

ILK is a serine-threonine protein kinase encompassing four ankyrin-like repeats at the N-terminus, a central pleckstrin homology (PH)-like domain and a catalytic domain at C-terminus [33], and plays a key role as an adaptor protein in extracellular matrix adhesion through interacting with cytoplasmic tails of β1 and β3 integrin subunits [26,34]. ILK also functions as a central effector protein kinase bridging integrins and growth factor receptors in the plasma membrane with downstream signaling molecules involved in cell cycle regulation, cell migration, cell survival and apoptosis [35,36].

In the present study, we investigated whether ILK is an upstream kinase mediating virus-induced Akt activation in our viral infection models. We also studied the effect of ILK inhibition on virus replication and progeny release as well as virus-induced cytopathic effects, all of which are important events in the pathogenesis of viral myocarditis.
4-2. MATERIALS & METHODS

i. Cell culture and virus infection

HeLa cells (HeLa S3) were obtained from the American Type Culture Collection (ATCC) and grown as previously described [18]. A mouse atrial cardiomyocyte cell line (HL-1) was established and provided by Dr. William Claycomb (Louisiana State University Health Sciences Center, New Orleans, Louisiana). HL-1 cells were grown in culture dishes pre-coated with 0.00125% fibronectin (Calbiochem, San Diego, CA) and 0.02% gelatin (Sigma-Aldrich, Oakville, ON), and in Claycomb Media™ (JHR Bioscience, Lenexa, KS) supplemented with 10% heat-inactivated fetal bovine serum, 100 μg/ml penicillin G (Invitrogen, Burlington, ON), 100 μg/ml streptomycin (Invitrogen), 2 mM L-glutamine (Sigma-Aldrich), and 0.1 mM norepinephrine (Sigma-Aldrich) to maintain the contractile phenotype of adult cardiomyocytes. All cell cultures were incubated at 37°C in a 5% CO₂ water-jacketed incubator.

CVB3 (Nancy strain) was a kind gift from Dr. Reinhard Kandolf (University Hospital Benjamin Franklin, Berlin, Germany). The titer of the virus was determined routinely prior to each experiment. Sub-confluent wild type or transfected HeLa or HL-1 cells were serum-starved for 24 hours prior to virus infection to eliminate the effect of serum growth factors. Cells were infected with either CVB3 at a multiplicity of infection (MOI) of 10 (HeLa cells) and 100 (HL-1 cells) or culture medium for control groups. Alternatively, for some experiments, cardiomyocytes were infected with virus at MOI of 10 to allow a longer infection cycle. Following a 1-hour incubation at 37°C, cells were replenished with fresh medium. For inhibitor experiments, pretreatment with the inhibitor was
performed for 1-2 hours prior to infection and, if needed, fresh inhibitors at the specified concentrations were added following medium changes, if needed.

ii. **Adenoviral Akt constructs**

Adenoviral constructs encoding constitutively active form of murine Akt1 tagged with the HA epitope (Ad-myr-Akt1) or control GFP (Ad-GFP) were kindly provided by Dr. Kenneth Walsh (Whitaker Cardiovascular Institute, Boston University School of Medicine, Boston, MA) and were described previously [37]. HeLa and HL-1 cells were infected with adenovirus particles at MOI of 25 and 100, respectively. Following overnight incubation at 37°C, cells were replenished with fresh serum-free medium. Fluorescence microscopy and Western blot analysis were used to assess transfection efficiency at 48 hours post-transfection.

iii. **ILK inhibitors and constructs**

HeLa and HL-1 cells were pre-treated with various doses of the specific ILK inhibitors KP392 and QLT0267 (QLT, Vancouver, BC) for 2 hours prior to infection. Inhibitor-containing medium was removed and replaced by serum-free medium during virus incubation to avoid any possible interference with virus binding. For transfection experiments, cells were transiently transfected with 2 μg of His-V5-tagged kinase-dead ILK (S343A), His-V5-tagged kinase-deficient ILK (E359K), and the control vector (pCDNA3.1) using Effectene reagent (Invitrogen) according to the manufacturer's instructions. Transfected cells were used for various experiments at 48 hours post-transfection.
iv. **ILK RNA inhibition (RNA interference)**

A 21-base pair double-stranded small interfering RNA molecule targeting the PH domain of ILK, and a control nonspecific siRNA were described previously [26]. HL-1 cells were transfected with various amounts of ILK siRNA using 6 µl of Lipofectamine™ Transfection Reagent (Invitrogen), followed by an overnight incubation at 37°C. At 24 hours post-transfection, cells were replenished with fresh medium and incubated for desired time prior to virus infection.

v. **Western blot analysis**

Cells either untreated or treated with different experimental reagents and constructs were washed twice with ice-cold PBS containing 5% phosphatase inhibitor (Active Motif Co., Carlsbad, CA) and cell lysates were collected as described previously [18]. Forty to 80 µg of extracted protein was fractionated by 9-10% SDS-polyacrylamide gels, electrophoretically transferred to nitrocellulose membranes (Amersham Biosciences Corp., Piscataway, NJ), and protein expression, phosphorylation and cleavage were detected using enhanced chemiluminescence system as previously described [18].

Rabbit polyclonal anti-phospho-Akt (Ser-473 & Thr-308), rabbit polyclonal anti-Akt, and rabbit polyclonal anti-phospho-GSK3β antibodies were purchased from Cell Signalling (Beverly, MA). Mouse monoclonal antibody against viral protein VP1 was obtained from Dako (Mississauga, ON); mouse monoclonal caspase-3, mouse monoclonal β-actin, mouse anti-ILK, HRP-conjugated anti-rabbit-IgG, and anti-mouse-IgG were purchased from Santa Cruz Biotechnology (Santa Cruz, CA); mouse anti-hemagglutinin (HA)
antibody was obtained from Biocompare company (San Francisco, CA). Mouse anti-human integrin $\alpha_v\beta_3$ monoclonal blocking antibody was from Chemicon International (Temecula, CA). The RGD cyclic peptide GRGDNP ($\alpha_v\beta_1$ blocker) was obtained from Calbiochem (Mississauga, ON). The RGD cyclic peptide XJ735 ($\alpha_v\beta_3$ blocker) was from Bachem (Torrance, CA).

vi. **Viral RNA in-situ hybridization (ISH)**

Sub-confluent cells were grown and maintained in serum-free conditions for 24 hours prior to each experiment, and were incubated with either CVB3 or control vehicle for 1 hour. At desired timepoints after infection, cells were washed gently with PBS and fixed with 10% formalin (15 minutes) and 70% ethanol (2 minutes). Culture slides were then submitted for the *in situ* hybridization assay using IsHyb *in situ* hybridization kit (Biochain Ins. Inc., Hayward, CA) according to the manufacturer’s protocol.

vii. **Virus release assay**

Wild type or transfected cardiomyocytes either untreated or treated with specific inhibitors were infected with virus and the supernatants were collected at desired timepoints following infection and kept at -80°C for viral release assay. An agar overlay plaque assay was carried out in HeLa cell cultures in duplicate using standard procedure and the virus titer was calculated as plaque forming units per milliliter (pfu/ml) as previously described [18,38].

viii. **Cell viability assay**
Sub-confluent cardiomyocytes were plated in 24-well culture plates, treated with either specific inhibitors or constructs, and then infected with CVB3. The CellTiter 96® AQquous Non-Radioactive Cell Proliferation Assay (MTS) was used to measure cell viability according to the manufacturer's protocol (Promega, Madison, WI). As a complementary approach, cell viability was assessed using the Live/Dead Double Staining kit (Oncogene Research Products, Boston, MA) according to the manufacturer's protocol. This kit utilizes Cyto-dye, a cell-permeable green fluorescent dye to stain live cell; and propidium iodide (PI), a cell non-permeable red fluorescent dye to stain dead cells.

ix. Statistical analysis

Two-way analysis of variance with multiple comparisons and paired Student's t-tests were performed. Values shown are the mean ± SD. A p value of <0.005 was considered significant.
4-3. RESULTS

i. CVB3 infection results in Akt phosphorylation on both Ser-473 and Thr-308 in HL-1 mouse cardiomyocytes

Previously, we reported on phosphorylation and activation of Akt following CVB3 infection in our well-established HeLa cell model [18]. To characterize the activation dynamics of Akt in virus-infected mouse cardiomyocytes, we infected HL-1 cells with CVB3 and collected the cellular extracts at various timepoints following infection. Cell lysates were subjected to SDS-electrophoresis and immunoblotting to detect Akt phosphorylation on both serine and threonine residues. CVB3 infection results in phosphorylation of Akt on both sites, with a phosphorylation peak around 4-5 hours post-infection (Fig. 4-1). As shown, Akt phosphorylation on both sites is concomitant with the appearance of viral protein VP1 expression in infected cardiomyocytes.

ii. CVB3-induced Akt phosphorylation in Ser-473 is ILK-dependent

As mentioned previously, full Akt activation depends on phosphorylation on both Ser-473 and Thr-308 sites. The identity of the upstream kinase responsible for serine phosphorylation is the centre of controversy. Growing evidence supports the notion that ILK is the upstream kinase responsible for Akt phosphorylation on serine residue [24,26-28]. However, there are reports indicating that ILK may not be required for Akt full activation [29-32], giving rise to the hypothesis that ILK-induced Akt activation highly depends on the cell environment as well as the type of cellular stimulus. To examine whether ILK is an upstream kinase regulator of CVB3-induced Akt phosphorylation,
mouse cardiomyocytes were pretreated with increasing doses of the specific small-molecule ILK inhibitors KP392 and QLT0267, and Akt phosphorylation on both residues was assessed. Inhibition of ILK significantly blocked CVB3-induced Ser-473 phosphorylation in infected cardiomyocytes, while having no effect on threonine phosphorylation, indicating that ILK is an upstream kinase mediating virus-induced Akt phosphorylation on Ser-473 in mouse cardiomyocytes (Fig. 4-2A). Similarly, transient over-expression of both kinase-dead (S343A) and kinase-deficient (E359K) mutants of ILK in infected HeLa cells resulted in considerable inhibition of virus-induced Ser-473 phosphorylation as compared to the control cells (Fig. 4-2B).

iii. Inhibition of ILK significantly blocks CVB3 replication.

As previously reported, blocking Akt phosphorylation by either specific inhibition of upstream PI3K or over-expression of the dominant negative mutant of Akt, leads to a significant decrease in virus replication in vitro [18]. To assess the effect of ILK inhibition on various phases of CVB3 life cycle, we measured viral protein VP1 expression, RNA synthesis, and progeny release from infected cells following ILK inhibition. The specific small-molecule ILK inhibitors KP392 and QLT0267 markedly diminished viral protein VP1 and virus release from infected mouse cardiomyocytes in a dose-dependent manner as measured by Western blot analysis and plaque assay, respectively (Fig. 4-3A & 3B). Likewise, inhibition of ILK expression by short interference RNA also markedly decreased virus protein expression. Treatment of mouse cardiomyocytes with 100 nM siRNA blocks ILK expression at 96 hours post-transfection (Fig. 4-3C).
Furthermore, in infected HeLa cells, ILK inhibition caused a substantial decline in VP1 expression (Fig. 4-4A) and viral RNA synthesis (Fig. 4-4B). As an additional assenting experiment, HeLa cells were also transfected with His-V5-tagged kinase-dead (S343A), kinase-deficient (E359K), or control pCDNA3.1 vectors for 48 hours. As shown in Fig. 4-4C, over-expression of both ILK inactive mutants substantially blocked virus protein expression. The observed phenomenon is associated with apparent down-regulation of virus-induced Akt phosphorylation in infected HeLa cells (Fig. 4-2B).

iv. ILK inhibition prevents CVB3-induced cytopathic effects and markedly enhances the viability of infected cells.

In virus-infected cells, excessive viral replication is associated with the activation of apoptotic pathways leading to the loss of cellular homeostasis and viability. Our previous findings have shown that the late virus-induced apoptosis/cytopathic effects and the secondary necrosis would facilitate viral progeny release from the infected cells, a process necessary for disease progression [13,15]. In a variety of experimental models, ILK inhibition has been shown to enhance cell cycle arrest and disrupts cell adhesion and migration leading to apoptosis [24,39].

Considering the observation that ILK inhibition markedly reduces virus replication, it was a matter of question whether under these circumstances, inhibition of ILK in virus-infected cells, would elevate cardiomyocyte apoptosis. To examine this hypothesis, we investigated the effect of ILK inhibition on virus-induced cytopathic effects and host cell viability in infected mouse cardiomyocytes. Morphological studies show a significant decline in virus-induced cytopathic effects in the presence of specific ILK inhibitor
QLT0267 in a dose-dependent mode (Fig. 4-5A). To further assess the viability of infected cells following ILK inhibition, we measured the mitochondrial metabolic function in CVB3-infected cardiomyocytes using an MTS viability assay. Our observations verified that ILK inhibition significantly enhanced the viability of infected cardiomyocytes (Fig. 4-5B). Fluorescent microscopy using the Double Live/Dead staining, moreover, confirmed that ILK inhibitor markedly blocked virus-induced cell death (Fig. 4-5C).

Additionally, experiments in HeLa cells using increasing doses of ILK inhibitors also corroborated our findings in mouse cardiomyocytes, signifying that ILK inhibition is beneficial to virus-infected host cells by preventing the detrimental cytopathic effects caused by CVB3 replication (Fig 4-6A). Notably, inhibition of ILK by either specific small-molecule inhibitors or by inactive ILK mutants had no effect on virus-induced caspase-3 cleavage in infected HeLa cells while significantly diminishing cellular cytopathic features (Fig. 4-6B) giving more credibility to the idea that a caspase-independent pathway is also involved in the process of destructive morphological changes caused by CVB3.

v. Constitutively active form of Akt1 subverts the protective effects of ILK inhibition in CVB3-infected cardiomyocytes

Previously, we provided evidence that Akt activation was required for a full productive virus replication [18]. Here, to investigate the potential causal relationship between the loss of ILK activation, the resulting Akt inhibition, and subsequent restriction of virus-induced cytopathic effects, mouse cardiomyocytes were transiently transfected with
adenoviral vectors expressing either the constitutively active form of Akt1 (Ad-myr-Akt1) or GFP (Ad-GFP). Transfection efficiency was evaluated by careful screening of Akt phosphorylation, GSK3-beta phosphorylation (an indication for Akt activity), and HA-tagged protein expression at 48 hours post-transfection in a serum-starved condition (Fig. 4-7A). To exclude any potential cytotoxic effect caused by adenoviral infection and to evaluate GFP expression (transfection efficiency) in mouse cardiomyocytes, transfected cells were observed at 48 hours post-transfection using brightfield and fluorescent microscopy techniques (Fig. 4-7B).

In this part of the study, to increase the sensitivity of the assessment, cardiomyocytes transfected with either Ad-myr-Akt1 or Ad-GFP were treated with a low dose of the ILK inhibitor QLT0267 (1.5µM) prior to and following the infection. At the above concentration, virus replication was slightly, but not completely, blocked (Fig. 4-3A & 4-4A), providing a condition in which even a slight change in virus replication or cytopathic effects would be detectible. As shown in Fig. 4-8A, Ad-myr-Akt1, to some extent, increases viral protein expression. However, the effect on virus release and cardiomyocytes viability is particularly significant (Fig. 4-8B & 8C & 8E). It is evident that over-expression of myr-Akt1 in cardiomyocytes treated with QLT0267 overturns the protective effect of ILK inhibition, reduces cardiomyocyte viability, and augments CVB3 release, indicating an elevated death rate in infected cardiomyocytes. As shown in Fig. 4-8D, over-expression of active Akt1 has no measurable effect on virus-induced caspase-3 cleavage in CVB3/QLT0267 treated cardiomyocytes. Similar observations were made when Ad-myr-Akt1 transfected HeLa cells were treated with ILK inhibitor QLT0267 prior to virus infection (Fig. 4-9A &B).
vi. Beta-1 and beta-3 integrins are not involved in CVB3 infection in both HeLa and HL-1 cell lines

ILK has been shown to interact with the cytoplasmic domain of the $\beta_1$ and $\beta_3$ integrins linking extracellular matrix components to cytoplasmic signaling and to structural networks, thereby transmitting outside-in and inside-out signals that mediate essential biological process such as proliferation, migration, adhesion and survival [36,40]. Since our results indicated a regulatory role for ILK during CVB3 infection in cardiomyocytes, we sought to determine whether integrin subunits also play a role in CVB3 entry and replication. Therefore, mouse cardiomyocytes were treated with increasing doses of $\alpha_v\beta_1$ cyclic blocking peptide GRGDNP [H-Gly-Arg-Gly-Asp-Asn-Pro-OH] and $\alpha_v\beta_3$ cyclic binding peptide XJ735 [Cyclo(-Ala-Arg-Gly-Asp-3-aminomethylbenzoyl)] for 2 hours prior to infection as well as during virus incubation. Following the infection period, cells were replenished with fresh serum-free medium.

Cell lysates were collected at desired timepoints post-infection and viral protein expression (indication of virus entry) and virus-induced morphological changes (indication of virus replication) were evaluated. As shown in Figure 4-10A, blocking $\beta_1$ and $\beta_3$ integrins had no effect on virus replication and virus-induced cytopathic effects in infected cardiomyocytes.

To assure the effectiveness of RGD peptide treatment in a mouse cardiomyocytes model, in another experiment, we treated HL-1 cells with both blocking peptides for 24 hours and monitored the morphological changes using brightfield microscopy. The
morphological changes caused by disruption of extracellular matrix/integrins interaction are apparent in our cardiomyocyte culture system (Fig. 4-10B).

Similar results were obtained when HeLa cells were treated with increasing doses of monoclonal blocking antibody against integrin αvβ3 receptors and control mouse IgG1 (Fig. 4-10C). These findings suggest that integrin receptor β1 and β3 subunits are not involved in CVB3 replication in HL-1 mouse cardiomyocytes and HeLa cells.
4-4. DISCUSSION

CVB3 is the most prominent infectant of the myocardium causing acute myocarditis, which frequently leads to end-stage dilated cardiomyopathy. Treatment of viral myocarditis is a therapeutic dilemma to both clinicians and scientists, and, thus far, the only available effective cure is heart transplantation surgery. Successful exploration for an alternative therapeutic approach for viral myocarditis necessitates an understanding of virus-host cell interaction and cellular events during viral infection.

It is well known that CVB3 directly injures cardiac myocytes prior to infiltrative inflammatory responses and generally through the activation and subsequent cleavage of the components of apoptotic pathways including caspases [9-11,14,15]. Yet, there is growing evidence pointing towards the emerging hypothesis that other caspase-independent death pathways may also be responsible for the observed degenerative morphology in infected cell [13,15,41]. This initiative is supported by the report that treatment of CVB3-infected cells with the general caspase inhibitor zVAD.fmkk only partially eliminates virus-induced cytopathic effects while significantly blocking virus replication and progeny release [15].

During the course of viral infections, viruses release proteins that alter cellular function and structure. In response to the assault, host cells may activate pro-survival defenses aimed at preserving cell viability, opposing virus-induced cytopathic effects, and delaying or averting virus release. How viruses evolve strategies to hijack or delay such defense mechanisms and/or manipulate host cell signaling pathways to augment their own replication is currently the subject of intense research.
In recent years, extensive efforts have been targeted towards understanding the cross talk and interplay between pro- and anti-apoptotic signaling pathways activated by either virus or the host cell. Activation of the PI3K/Akt, Erk1/2 MAPK, and P38 MAPK pathways in CVB3 infection have been mapped and thoroughly studied [9,18,42-45]. Among them, Akt activation has been shown to be beneficial for virus replication, wherein inhibition of Akt significantly blocks virus replication, apparently through a caspase-independent pathway [18].

Akt acts as a key protein mediator for a myriad of distinct cellular responses associated with cell survival, proliferation, differentiation, migration, and apoptosis [16,17,46]. Complete Akt activation requires phosphorylation of both Thr-308 and Ser-473 residues [19]. It has been well established that PDK-1 is the kinase responsible for Akt phosphorylation on threonine site [20-22]. However, the actual identity of the upstream kinase for serine phosphorylation (generally named as PKD-2) remains elusive. To date, several kinases have been shown to function as the PDK-2 including mitogen-activated protein kinase-activated protein kinase-2 (MK2), p38 MAP kinase, PKC, the mammalian target of rapamycin (mTOR), and ILK. Recently, through several in vitro studies, ILK has been proposed as a potential upstream kinase responsible for Akt phosphorylation on Ser\(^{473}\) [24-28]. However, other genetic studies suggest that ILK kinase activity may not be essential for full Akt activation and that the observed dependency is cell and agonist specific [29-32,36].

ILK is a dual-functional protein that plays an essential role as an adaptor molecule linking components of extracellular matrix and integrins to the actin cytoskeleton. It also
plays a role as a serine/threonine protein kinase transmitting signals in response to various stimuli [34,35,47-49]. Increased levels of ILK expression have been reported in various cancers, making it a potential and attractive therapeutic target for cancer treatment [36,40,50]. However, not much information is available on the potential role of ILK during the episode of viral infections, in general, and enteroviral myocarditis, in particular. Recently, cDNA microarray and Northern blot analyses of extracellular matrix genes expression in the myocardium of mice infected with CVB3 have shown a 2.4 fold increase in ILK mRNA expression as compared with control group, pointing towards a potential role for ILK in disease progression [18]. Yet, the role and consequence of ILK up-regulation in viral myocarditis remains elusive.

Here, we propose a crucial role for ILK in regulation of CVB3-induced Akt activation in early phase of infection. In this report, we have provided evidence that in both mouse cardiomyocytes and HeLa cells, inhibition of ILK by highly potent and specific small-molecule ILK inhibitors, as well as kinase-inactive mutant forms of ILK significantly blocks virus-triggered Akt phosphorylation in serine site without having any effect on threonine phosphorylation. We have also shown that ILK plays a crucial role in the progression of disease by regulating both virus replication and virus-induced cytopathic changes in infected cells. ILK inhibition has a major effect on the CVB3 life cycle, leading to a significant decline in viral RNA transcription, viral protein synthesis, and virus progeny release. All these events eventually rescue cells from virus-induced cytopathic effects and considerably improve the viability of infected cells.
Previously, we reported that Akt activation is favorable to CVB3 replication and inhibition of Akt activation benefits the host cell by blocking virus replication. Here, we tested this hypothesis by transfecting QLT0267-treated cardiomyocytes with an adenoviral vector expressing a constitutive active form of Akt1 (Ad-myr-Akt1). Interestingly, over-expression of an active form of Akt1 dramatically reverses the protective effect of QLT0267 in infected cardiomyocytes, indicating that 1) The protective effects of ILK inhibition is through down-regulation of Akt activation and 2) The Akt activation during CVB3 infection is detrimental and disadvantageous to the host cell, while it is beneficial to virus replication. These findings give rise to the notion that the outcome of Akt activation in various systems is highly dependent on cell environment and the stimuli.

ILK has been also shown to anchor to cytoplasmic tails of integrin β1 and β3 at the inner side of plasma membrane, providing cell-cell and/or cell-extracellular matrix interaction in response to various stimuli. Cumulative evidence indicates that integrin subunits may play a role in several viral infections by facilitating virus entry [51-57]. There are reports of colocalization of human coxsackievirus-adenovirus receptor (CAR) with integrins αvβ3 and αvβ5 in the heart of patients diagnosed with end-stage dilated cardiomyopathy [58]. Agrez et al. have studied the role of integrin subunits in CVB1 infection and shown that over-expression of integrin αvβ5 enhances CVB1 lytic infection in human colon cancer cells [59].

In this study, to determine whether β1 and β3 integrins are involved in CVB3 entry, we treated mouse cardiomyocytes with RGD cyclic peptides to block αvβ1 and αvβ3...
integrins. Our data show that blocking $\beta_1$ and $\beta_3$ integrins has no effects on virus entry and replication, as well as virus-induced cytopathic effects. However, the findings do not rule out the potential involvement of other integrins in CVB3 entry.

To our knowledge, this is the first report of a potential regulatory role for ILK in a viral infection model. Here, we have provided evidence that ILK plays a critical role in CVB3 pathogenesis, by modulating virus replication and virus-induced cellular injury through an Akt-dependent mechanism. Further in vivo studies using ILK inhibitors will also provide valuable information on efficacy of ILK inhibitors and will provide us with new insights in our efforts to establish an effective therapeutic approach to treat enteroviral myocarditis.
REFERENCES:


Figure 4-1. CVB3 infection leads to Akt phosphorylation on Ser-473 and Thr-308 residues in mouse cardiomyocytes.

Following serum starvation for 24 hours, HL-1 cells were either sham- or CVB3-infected (MOI=100) for one hour. Cell lysates were collected at specified times following infection and subjected to Western blot to detect Akt phosphorylation on both the Ser-473 and Thr-308 sites. Beta-actin protein was measured to ensure equal protein loading. Note that Akt phosphorylation in HL-1 cells is concomitant with viral protein VP1 expression.
**Figure 4-2**

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<th>β-actin</th>
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<tbody>
<tr>
<td></td>
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</table>

<table>
<thead>
<tr>
<th>HeLa Cells</th>
</tr>
</thead>
</table>

161
Figure 4-2. CVB3 induces Akt phosphorylation via an ILK-dependent mechanism.

Mouse cardiomyocytes or HeLa cells were serum-starved for 24 hours. After being treated with either increasing doses of ILK inhibitors or transfection with kinase-inactive mutants of ILK, cells were infected with either CVB3 or control medium for one hour. Cell lysates were collected and subjected to Western blot analysis. A) ILK inhibition in HL-1 cells blocks virus-induced Akt phosphorylation on Ser-473 without having any effect on Thr-308 phosphorylation, indicating that only Ser-473 phosphorylation is mediated via ILK. B) Over-expression of both kinase-dead and kinase-deficient mutants of ILK in HeLa cells significantly decreases Akt phosphorylation on Ser-473.
Figure 4-3

A) CVB3 (16hr pi)

<table>
<thead>
<tr>
<th></th>
<th>KP392</th>
<th>QLT0267</th>
</tr>
</thead>
<tbody>
<tr>
<td>μM</td>
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</tr>
<tr>
<td>-</td>
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<tr>
<td></td>
<td>5</td>
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</tbody>
</table>

β-Actin
VP1
HL-1 Cells

B) CVB3 Release (PFU/ml x 10^6)

\[ *P < 0.005 \]

0 0.5 5 QLT (μM)

CVB3 (16hr pi)

C) CVB3 (16hr pi)

<table>
<thead>
<tr>
<th></th>
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<th>50</th>
<th>100</th>
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<tbody>
<tr>
<td>C</td>
<td>ILK</td>
<td>C</td>
<td>ILK</td>
</tr>
</tbody>
</table>

nM
siRNA (96hr)
ILK
β-Actin
VP1
Figure 4-3. ILK inhibition blocks viral VP1 synthesis and viral release in infected cardiomyocytes.

A) ILK inhibitors block VP1 synthesis in a dose-dependent manner. CVB3-infected HL-1 cells were treated with either DMSO or increasing doses of ILK inhibitors. Cell lysates were collected for Western blot to detect viral capsid protein VP1 expression. β-actin expression was measured to ensure of equal protein loading. B) Supernatants from infected cardiomyocyte cultures were collected and assayed for released infectious particles using agar overlay plaque assay. Comparison of infectious virus particles in the indicated conditions shows that ILK inhibitor markedly decreases viral progeny release from infected host cells. The data represent three independent experiments where * indicates P < 0.005 as compared to non-treated infected cells. C) Mouse cardiomyocytes were transfected with increasing concentrations of ILK siRNA and were then infected with CVB3 at 96 hours post-transfection. Cell lysates were subjected to Western blot analysis to measure virus replication (VP1). ILK siRNA at the concentration of 100 nM significantly blocked ILK expression as well as viral protein VP1 synthesis in HL-1 cells.
Figure 4-4

A) CVB3 (16h pi)

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</table>

- KP392 (μM)
- β-actin
- VP-1

HeLa Cells

B) CVB3 (16h pi)

|   | 0  | 5  |

- QLT (μM)

HeLa Cells

C) CVB3 (6hr pi)

<table>
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<tr>
<th></th>
<th>Con</th>
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<th>KD</th>
<th>KI</th>
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</thead>
</table>

- β-actin
- VP-1

HeLa Cells
Figure 4-4. ILK inhibition significantly decreases viral protein VP1 and viral RNA expression in infected HeLa cells.

A) Virus-infected HeLa cells were treated with either DMSO or increasing doses of ILK inhibitors. Both ILK inhibitors blocked viral protein synthesis in a dose-dependent manner. B) *In situ* hybridization of CVB3-infected HeLa cells at 16 hours post-infection using digoxigenin-labelled viral strand-specific riboprobe shows that ILK inhibitor QLT0267 noticeably blocked viral RNA transcription and replication in infected cells. C) Over-expression of kinase-dead or -deficient mutant of ILK also decreased viral protein VP1 expression in infected HeLa cells.
Figure 4-5

A)

CVB3 (18hr pi) + QLT (µM)

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<tbody>
<tr>
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HL-1 Cells

B)

% Cell Viability

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<td>CVB3 (18hr pi)</td>
<td></td>
<td></td>
<td>*</td>
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*P < 0.005
Figure 4-5, Cont’d...

C)

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<table>
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<td>CVB3</td>
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</table>
Figure 4-5. Specific ILK inhibitor QLT0267 markedly decreases CVB3-induced cytopathic effects in HL-1 mouse cardiomyocytes.

A) Photomicrographs of mouse cardiomyocytes treated with either the ILK inhibitor QLT0267 (5 μM) or DMSO prior to and after virus infection. ILK inhibition led to a significant decline in virus-induced cytopathic effects in HL-1 cells. B) ILK inhibition also increases the viability of virus-infected cells as measured by MTS assay. C) Virus-infected HL-1 cells treated with either ILK inhibitor or DMSO were stained with cyto-dye and propidium iodide (PI) for 30 minutes. The cell death was determined by fluorescence microscopy. Dead cells were stained with PI (red) while live cells were stained with Cyto-dye (green). As shown, the number of dead cells (red cells) is less in cell culture treated with ILK inhibitor.
Figure 4-6

A)

CVB3 (16hr pi)

0 0 5 QLT (μM)

HeLa Cells
Figure 4-6, Cont’d…

B)

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<th>1</th>
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HeLa Cells

Cleaved Caspase-3

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HeLa Cells

Pro-Caspase-3

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<tbody>
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<td>PCDNA</td>
<td>KD-ILK</td>
<td>KI-ILK</td>
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HeLa Cells

Pro-caspase-3
Figure 4-6. ILK inhibition significantly decreases virus-induced cytopathic effects in CVB3-infected HeLa cells.

A) Photomicrograph of HeLa cells treated with either the ILK inhibitor QLT0267 (5 μM) or DMSO. As shown, ILK inhibitor markedly improves the viability of infected HeLa cells as compared to the vehicle-treated group. B) Inhibition of ILK by specific inhibitor or ILK kinase-inactive mutants does not affect caspase-3 cleavage (similar band intensity for pro-caspase-3 and cleaved caspase-3 for all treatments) in infected HeLa cells, indicating that ILK regulates virus-induced cytopathic effects through a caspase-3-independent mechanism. The data represent three independent experiments.
Figure 4-7

A)

Ad-GFP
Ad-myr-Akt1

HA
P-Akt (S-473)
Akt
P-GSK3β
β-Actin

Serum

B)

Ad-GFP 48hr
Ad-GFP 48hr
Ad-myr-Akt1 48hr

HL-1 Cells
Figure 4-7. Adenoviral transfection of HL-1 mouse cardiomyocytes with a constitutive active form of Akt1 and GFP control.

A) Sub-confluent cells were infected with adenoviral vector expressing either a constitutive active form of Akt1 (Ad-myr-Akt1) or GFP (Ad-GFP) at an MOI of 100. To ensure transfection efficiency, transfected cells were serum-starved for 24 hours, cell lysates were collected, and Akt expression and phosphorylation, GSK3β phosphorylation (as indicator of Akt activity) and HA-tagged protein expression were assessed using Western blot analysis. B) Photomicrograph of HL-1 cells showing the morphology of Ad-myr-Akt1-transfected cells at 48 hours following transfection. As shown, Ad-myr-Akt1 or GFP over-expression has no cytotoxic effects on HL-1 cells. Note the high expression of GFP in Ad-GFP-transfected HL-1 cells.
Figure 4-8

**A)**

<table>
<thead>
<tr>
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<th>Ad-myr-Akt1</th>
</tr>
</thead>
<tbody>
<tr>
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<td>1.5</td>
<td>1.5</td>
</tr>
</tbody>
</table>

- VP1
- CVB3 (18hr pi)

**B)**

- Virus Release (PFU/ml x 10^6)
- QLT (μM)
- CVB3 (18hr pi)

*P < 0.005
Figure 4-8, Cont’d...

C)

% Cell Viability

0 5 5 5 5
QLT (μM)

CVB3 (18hr pi)

*P < 0.005

D)

Control Ad-GFP Ad-myr-Akt1

QLT (μM)

Cleaved-Caspase-3

+ + +

CVB3 (18hr pi)
Figure 4-8, Cont’d...

E) 

- Cyto-Dye
- PI

CVB3 (16hr pi)

QLT (μM)

Control	CVB3 (16hr pi)

<table>
<thead>
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<th>5</th>
<th>5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>Ad-GFP</td>
<td>Ad-myr-Akt1</td>
</tr>
</tbody>
</table>

Cyto-Dye

PI
Figure 4-8. Constitutively active form of Akt1 increases viral protein synthesis and virus progeny release and enhances cell death in infected cardiomyocytes.

A) HL-1 cells were infected with either Ad-myr-Akt1 or Ad-GFP for 48 hours. In QLT0267-treated HL-1 cells, over-expression of an active form of Akt1 increases viral protein (VP1) expression, B) significantly enhances viral progeny release, and C) augments virus-induced cell death as compared with Ad-GFP group. D) Ad-myr-Akt1 has no noticeable effect on virus-induced caspase-3 cleavage in QLT0267-treated HL-1 cells. E) Cells transfected with either Ad-myr-Akt1 or Ad-GFP were pre-treated with either the ILK inhibitor or DMSO and were then infected with CVB3. After 16 hours, cells were stained with Cyto-dye and propidium iodide (PI) for 30 minutes. Cell death was evaluated by fluorescent microscopy. Dead cells were stained with PI (red) while live cells were stained with Cyto-dye (green).
Figure 4-9

A)

HeLa Cells

B)

CVB3 18hr + QLT (μM)

0 5

5 5

Ad-GFP  Ad-Akt1
Figure 4-9. Constitutively active form of Akt1 reverses the protective effect of ILK inhibition in CVB3-infected HeLa cells.

A) Photomicrograph of HeLa cells at 48 hours post adenoviral infection. As shown, Ad-myr-Akt1 or GFP over-expression has no cytotoxic effects on HL-1 cells. B) Photomicrograph presenting the morphology of adenoviral-transfected HeLa cells treated with either ILK inhibitor QLT0267 (5 μM) or DMSO. As shown, over-expression of active form of Akt1 reverses the protective effects of QLT0267 on infected HeLa cells.
Figure 4-10

A)

CVB3 (16 hr pi)

<table>
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<tr>
<th>GRGDNP (μM)</th>
<th>XJ735 (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 50 150 300</td>
<td>5 15 30</td>
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</tbody>
</table>

HL-1 Cells

B)

<table>
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<tr>
<th>GRGDNP (μM)</th>
<th>XJ735 (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 300</td>
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HL-1 Cells
Figure 4-10, Cont’d...

C)

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<tr>
<th>CVB3 (9hr pi)</th>
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<tbody>
<tr>
<td>-</td>
<td>β-actin</td>
</tr>
<tr>
<td></td>
<td>VP1</td>
</tr>
</tbody>
</table>

HeLa Cells

- IgG
- αvβ3 Ab

Control
CVB3
HeLa Cells
Figure 4-10. β1 and β3 integrin subunits are not involved in CVB3 entry and replication in mouse cardiomyocytes and HeLa cells.

A) HL-1 Cardiomyocytes were treated with various doses of the GRGDNP (α,β1 blocker) or XJ735 (α,β3 blocker) cyclic peptides for 1 hour prior to and during virus incubation. As shown none of the peptides had effects on viral protein (VP1) synthesis (upper panel) and virus-induced cytopathic features (lower panel) in infected cardiomyocytes. B) To ensure the efficiency of integrin blocking peptides, HL-1 cells were treated with 300 μM of GRGDNP (α,β1 integrin blocker) or 30 μM of XJ735 (α,β3 integrin blocker) for 24 hours. Note the disruption of ECM and cell-cell interactions and detachment of cardiomyocytes with early signs of anoikis. C) HeLa cells were treated with increasing doses of mouse monoclonal blocking antibody against α,β3 for 1 hour prior to and during virus incubation. α,β3 blocking antibody did not affect VP1 synthesis (upper panel) and virus-induced cytopathic effects (lower panel) in HeLa cells.
CHAPTER 5: NUCLEAR FACTOR KAPPA-B (NFkB) IN COXSACKIEVIRUS B3 INFECTION

Objective:

To determine whether CVB3 infection results in the activation of nuclear factor kappa B and to characterize the potential regulatory role of this transcription factor during the course of virus infection.

The candidate designed and performed or supervised all experiments presented in this section. The assistance of research technicians Agripina Suarez and Seti Boroomand in performing, protein gel analyses, NFkB-DNA binding assay, and tissue culture is greatly appreciated. Special thanks to research technician Zhongshu Luo for her assistance in amplification of NFkB constructs.

Based on the Manuscript:
Transient stimulation of coxsackievirus B3-infected cells with tumor necrosis factor-α (TNF-α) confers resistance to virus-induced injury through activation of the PI3K/Akt/NFkB pathway. Mitra Esfandiarei, Seti Boroomand, Agrapina Suarez, Maziar Rahmani, Xiaoning Si, and Bruce McManus (manuscript in preparation)
5-1. INTRODUCTION

Apoptosis or programmed cell death is one of the hallmarks of viral infections and results in cellular changes including nuclear chromatin condensation, nuclear DNA fragmentation, membrane blebbing, and cell shrinkage. In some infection models, apoptotic events appear to provide a defense mechanism through which the infected cell commits suicide prior to the completion of virus life cycle, limiting virus replication [1,2]. In such cases, the apoptotic bodies are eventually recognized and cleared by neighboring cells. However, in some other systems, virus-induced apoptosis seems to facilitate viral progeny release from infected cells enhancing viral spread and disease progression [3,4].

Coxsackievirus B3 (CVB3), a non-enveloped single positive-stranded RNA Enterovirus in the Picornaviridae family, is the most frequent cause of human viral myocarditis in neonates and young adults [5,6]. CVB3 can also infect other organs such as the pancreas, spleen, and brain causing severe pathological complications including pancreatitis, non-septic meningitis and encephalitis [6,7].

During the sub-acute phase of viral myocarditis (7-14 days post-infection), the host’s inflammatory responses contribute to the pathogenesis of CVB3-induced myocarditis [8-12]. It is well accepted that early innate immune response as well as direct virus-induced injury prior to immune infiltration are very important determinants of the extent of injury and disease progression [13-15]. Microscopic evaluation of tissues from infected mice early following infection (3-5 days) clearly indicates that cardiac myocytes are injured
prior to immune infiltration of tissue, as determined by distinctive histopathologic
c Characteristics [13,14,16].

Currently, our understanding of the initiation and development of viral myocarditis is
inadequate. Nevertheless, there is agreement on the idea that severity of disease is highly
dependent on the equilibrium between manifold and concurrent pro- and anti-apoptotic
processes. Our laboratory has carried out extensive work on CVB3-triggered death and
survival signaling pathways in the context of interaction between these pathways and
virus life cycle. We have characterized apoptotic signaling, in particular the caspases,
following CVB3 infection [17-19]. In addition, we have shown that CVB3 can activate
Akt in infected HeLa cells and mouse cardiomyocytes. Although Akt has been shown to
have an anti-apoptotic role, inhibition of its activity in virus-infected cells has a strapping
deleterious effect on viral RNA and protein expression, implying a fundamental role for
the PI3k/Akt pathway in CVB3 pathogenesis [20].

Akt modulates several cellular functions including cellular metabolism, proliferation, and
survival, and exerts its regulatory effects through a wide-range downstream effectors [21-
23]. Among, NFkB promotes the transcription of interleukin-1 & -8 (IL-1 & IL-8), matrix
metalloproteinase (MMPs), adhesion molecules ICAM and VCAM, and the family of
cellular inhibitors of apoptosis (c-IAPs). In a resting condition, NFkB is sequestered
within the cytoplasm by specific inhibitors of kappa B or IkBs. Following stimulation,
IkB proteins undergo phosphorylation, poly-ubiquitination, and degradation. Subsequently, liberated NFkB complex translocates to the nucleus, where it enhances the
transcription of specific target genes [24-26].
In recent years, the role of NFkB in viral infections has gained much more attention. Viruses like herpes simplex virus-1 (HSV-1) [27], human immunodeficiency virus (HIV) [28], Epstein-bar virus (EBV) [29], and influenza virus [30] have been shown to activate NFkB. Furthermore, inhibition of NFkB in these infection models results in a significant decrease in viral replication, signifying that these viruses activate NFkB in order to provide favorable conditions for their own replications. On the other hand, there are some indications of a pro-apoptotic function for NFkB during viral infection. Clarke et al. [31] have shown that reovirus-induced activation of NFkB results in a significant increase of apoptosis, a mechanism used by virus to promote viral progeny release from infected cells.

In the present study, we sought to determine whether CVB3 infection can activate NFkB in infected HeLa cells and whether this activation is through a PI3K/Akt-dependent pathway. We also investigated the potential role of NFkB transcription factor in regulating virus-induced cellular injury.
5-2. MATERIALS & METHODS

i. Cell culture and transient transfection

HeLa cells (HeLa S3) were obtained from the American Type Culture Collection (ATCC). Sub-confluent cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated newborn calf serum (Invitrogen, Burlington, ON) at 37°C, in a humidified incubator in 5% CO₂. Penicillin G (100 μg/ml) and streptomycin (100 μg/ml) (Invitrogen) were added to all culture media. For transient transfection of cells, 1-2 μg of cDNA was introduced using FuGENE 6 reagent (Roche Applied Science, Laval, PQ) according to the manufacturer's protocol.

ii. Virus infection

CVB3 (Nancy strain) was a kind gift from Dr. Reinhard Kandolf and was propagated in HeLa cells and stored at -80°C. The titer of virus was determined routinely prior to each experiment. Sub-confluent wild type or transfected HeLa cells were serum-starved for 24 hours prior to virus infection to eliminate the effect of serum growth factors. Cells were infected with either CVB3 at a multiplicity of infection (MOI) of 10 or culture medium for control groups. The control medium was extracted from HeLa cells in culture under identical conditions as those used for virus propagation to account for the potential effect of proteins within the media. Alternatively, for some experiments, cells were infected with virus at MOI=1 to allow longer infection cycle. Following 1-hour incubation at 37°C, cells were replenished with fresh medium. For inhibitor experiments, pretreatment
with inhibitor was performed one hour prior to infection and fresh inhibitors were added following medium changes, if needed.

iii. Antibodies and inhibitors

Rabbit polyclonal antibodies against IkB-α, phospho-IkB-α, NFkB p105/50, and phospho-IKKα/β were from cell signalling (Beverly, MA). Mouse monoclonal caspase-3, β-actin, nucleolin (C23), and rabbit polyclonal NFkB p65 antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-rabbit-IgG and anti-mouse-IgG conjugated with horseradish-peroxidase, and NFkB inhibitory peptide (SN-50), NFkB control peptide, PI3K inhibitor 2-morpholino-8-phenyl-4H-1-benzopyran-4-one (LY294002), and TNF-α were also purchased from Santa Cruz Biotechnology. Mouse monoclonal antibody against viral protein VP1 was obtained from Dako (Mississauga, ON). The MEK/ERK inhibitor U0126 was from Promega (Madison, WI).

iv. Electrophoretic mobility shift assay (EMSA) using IRDye™-labeled DNA

Nuclear extracts of mock- or virus-infected cells were prepared using the nuclear extraction kit from Active Motif (Carlsbad, CA) according to the manufacturer’s protocol. The DNA oligonucleotide end-labeled with infrared dye (IRDye™) containing the consensus nucleotide sequence for NFkB-DNA binding site were obtained form LICOR Inc. (Lincoln, NE). Nuclear extracts (3 μg) were incubated with infrared dye labeled double stranded NFkB oligonucleotide 5'-TTGTTACAAAGGACTTTCGGCTG

GGGACTTTCCAGGGAGGCGTGG-3' (the binding sites are underlined and printed in bold). To ensure the specificity of NFkB-DNA binding reaction, an unlabeled double-
stranded NFkB oligonucleotide was used as competitor DNA in the reaction. Binding reactions were performed in binding buffer containing 100 mM Tris PH 7.5, 500 mM NaCl, 50% (V/V) glycerol, and 5 mM EDTA PH 7.6 for 30 min at room temperature and in the dark. DNA-protein complexes were separated from free oligonucleotide in a 4% native polyacrylamide gel for 45 minutes at 100V. NFkB-DNA complex bands were detected using Odyssey Infrared Imaging System (Licor Inc.).

v. NFkB reporter (luciferase) gene assay

NFkB luciferase assay was performed using the NFkB Reporter Luciferase Assay System from Clontech (Mountain View, CA). Sub-confluent cells (2 x 10^5) were cultured in 12-well plates and incubated at 37°C for 24 hours. Cells were then transfected with pNFkB-Luc vector containing the firefly luciferase (Luc) gene and four tandem copies of the NFkB binding sites fused to a TATA-like promoter (P_{TAL}) region from the Herpes Simplex Virus thymidine kinase (HSV-TK) promoter. The pTAL-Luc vector containing no kappa B binding sites was used as control. To monitor transfection efficiencies, cells were co-transfected with a pSV-β-galactosidase control vector and the activity of β-galactosidase reporter enzyme were assayed using spectrophotometry.

vi. Western blot analysis

Cells either untreated or treated with different experimental reagents were washed twice with ice-cold PBS containing 5% phosphatase inhibitors (Active Motif Co.), and cell lysates were prepared as described previously [20]. Protein concentration was determined by the Bradford assay. Extracted protein (40-80 µg) was fractionated by electrophoresis
in 10% SDS-polyacrylamide gels, transferred to nitrocellulose membranes (Amersham Pharmacia Biotech), and blocked with PBS containing 0.1% Tween-20 and 5% nonfat dry milk for 1 hour. The membrane was incubated with specific primary antibody overnight at 4°C, followed by secondary antibody for 2 hours at room temperature. The immunoblots were visualized with enhanced chemiluminescence detection system according to the manufacturer’s protocol (Amersham Pharmacia Biotech).

vii. Cell viability assay

Sub-confluent cells were plated in 24-well culture plates, treated with either specific inhibitors or constructs, and then infected with CVB3. The CellTiter 96® AQueous Non-Radioactive Cell Proliferation Assay (MTS) was used to measure cell viability according to the manufacturer’s protocol (Promega, Madison, WI). As a complementary experiment, cellular death was also assessed using propidium iodide (PI) a cell non-permeable red fluorescent dye to stain dead cells according to manufacturer’s protocol (Oncogene Research Products, Boston, MA).

viii. Statistical analysis

Two-way analysis of variance with multiple comparisons and paired Student’s t-tests were performed. Values shown are the mean ± SD. A p value of <0.005 was considered significant.
5-3. RESULTS

i. Coxsackievirus B3 induces the activation of NFkB in infected cells

NFkB heterodimer consists of p65 and p50 subunits. In unstimulated cells, NFkB p50 is present as a precursor protein (p105) within the cytoplasm. Western blot analysis was used to detect NFkB p105 degradation within the cytoplasmic fraction and subsequent translocation of the p50/p65 complex into the nucleus of infected cells. As shown (Fig. 5-1A), CVB3 induces degradation of NFkB p105 within the cytoplasm of infected cells around 4 hours post-infection. Translocation of the NFkB p65 subunit is also detectable around 4 hours with a peak at 6 hours post-infection (Fig. 5-1B). As expected, p105 expression was not observed within the nucleus of infected cells. To ensure equal protein loading and the absence of cytoplasmic contamination, the expression of beta-actin and nucleolin (C23) were measured.

To validate the above findings, cells transfected with the NFkB-Luc and pTAL-Luc vectors were infected with CVB3 and NFkB luciferase activity was measured at various timepoints post-infection. As shown in Fig. 5-2A, CVB3 significantly increases NFkB-dependent luciferase activity around 4 hours post-infection. As a complementary experiment and to measure the DNA-binding activity of NFkB within the nucleus, nuclear extracts were also prepared at various timepoints following infection, and then incubated with IRDye™ 700 labeled DNA. NFkB-DNA complexes were separated on a native 4% polyacrylamide gel. As shown in Fig. 5-2B, CVB3 increased NFkB-DNA binding around 4 hours post-infection.
ii. Inhibition of CVB3-induced NFkB activation significantly decreases the viability of virus-infected cells

To determine the effect of NFkB activation on host cells, CVB3-infected HeLa cells were incubated with increasing doses of the specific NFkB inhibitory peptide (SN-50) or 100 μg/ml of the control peptide (MTS). Fluorescent microscopy using propidium iodide (PI) staining, confirmed that NFkB inhibition significantly diminished the viability of virus-induced infected cells, suggesting that NFkB activation has a beneficial and anti-apoptotic effect on infected HeLa cells (Fig. 5-3).

iii. CVB3 phosphorylates IKK-α but not IKK-β within the cytoplasm of infected HeLa cells

To characterize the upstream pathway of NFkB activation, we investigated the phosphorylation of IKKα/β and its downstream target IkB-α following CVB3 infection. As shown in Fig. 5-4, virus infection leads to phosphorylation of IKK-α, but not IKK-β, starting at 5 hours post-infection. IKK-α phosphorylation is associated with a slight phosphorylation and degradation of IkB-α (inhibitor of NFkB).

iv. Virus-induced NFkB activation is through PI3K and Erk1/2-dependent pathways

We have previously reported on the activation of the PI3K/Akt and Erk1/2 pathways in CVB3-infected cells [20,32]. Since both pathways have been implicated in NFkB activation, we sought to determine whether CVB3-mediated NFkB activation was also dependent on these pathways. Cells were transfected with NFkB-Luc and pTAL-Luc
vectors for 48 hours and then pretreated with specific PI3K and MEK-1/Erk1/2 inhibitors (50 μM LY294002 and 20 μM U0126, respectively). All inhibitors were added one hour prior to infection. As shown in Fig. 5-5A & 5-5B, both inhibitors significantly reduced virus-induced NFkB activation, suggesting that, in virus-infected cells, the NFkB activation is dependent on both PI3K and Erk1/2 pathways.

v. **TNF-α treatment of CVB3-infected cells significantly increases the activation of NFkB**

TNF-α is a strong activator of NFkB and its expression has been shown to increase in both acute (day 5) and chronic (day 14) stages of CVB3 myocarditis [33,34]. To determine the potential role of TNF-α in NFkB activation in virus-infected cells, cells were treated with TNF-α at the time of virus infection and NFkB activation was measured by Western blot analysis and luciferase assay. As expected, TNF-α markedly enhances NFkB activation in CVB3-infected cells (Fig. 5-6A & 6B).

vi. **TNF-α does not directly affect CVB3 replication within infected cells**

In this part of the study, we examined any potential direct effect of TNF-α on virus replication. Cells were treated with increasing doses (5-100 ng/ml) at the time of infection. As shown, TNF-α treatment had no effect on viral protein expression (VP1) within the infected cells (Fig. 5-7A). To assess the influence of such treatment on different phase of virus life cycle, in a separate experiment, 100 ng/ml TNF-α was introduced at different timepoints pre- and post-infection. Likewise, no significant difference was observed in viral protein synthesis among different conditions, indicating
that regardless of the dose and timing of treatment, TNF-α has no direct impact on CVB3 replication (Fig. 5-7B).

ei. **Short-term and early treatment of infected cells with TNF-α reduces virus-induced cytopathic effects and augments host cell viability**

TNF-α can activate both pro- and anti-apoptotic pathways. To determine the effect of this cytokine on virus-induced cellular injury, HeLa cells were treated with TNF-α (100 ng/ml) starting one hour prior to infection. Following one-hour virus incubation, medium containing viral particles and TNF-α was replaced with fresh serum-free medium. As shown in Fig. 5-8A, early transient treatment of infected cells distinctly decreases virus-induced cell death. To determine whether the observed beneficial effect is dependent on the NFkB activation, virus-infected cells were treated with increasing doses of specific NFkB inhibitory peptide (SN-50). As shown, suppression of NFkB activity significantly decreases host cell viability in a dose-dependent fashion, suggesting that TNF-α exerts its protective effects through the activation of NFkB, and TNF-α-induced NFkB activation has a beneficial role by enhancing the viability of infected cells (Fig. 5-8B). Interestingly, sustained and long-term presence of TNF-α, regardless of the starting time for treatment, has a deleterious effect on infected cells by increasing the cytopathic effect and cellular death (Fig. 5-8C), implying that the outcome of TNF-α activation is highly dependent on the duration of stimulation.
5-4. DISCUSSION

During viral infection, viruses encode highly effective products in order to optimize viral replication, to produce huge numbers of progeny virions, and to release infectious particles to launch a new round of infection. However, host cells activate pro-survival defenses aimed to preserve host cell viability in response to viral infection. Viruses may evolve strategies to block or delay such host defense mechanisms. How viruses interact with and manipulate host cell signaling pathways to augment their own replication is currently the subject of intense research. As reported earlier, CVB3 infection results in the activation of Akt through a PI3K/ILK-dependent pathway. Our previous findings have also revealed that Akt phosphorylation and activation seem to be of assistance to virus replication by increasing viral RNA and protein synthesis [20]. It has been suggested that viruses may activate the PI3K/Akt pathway in order to restrain apoptosis and to provide a short-term cellular survival during viral replication [35-37]. However, the exact mechanism and consequence of the Akt activation during CVB3 infection is not well-understood.

The PI3K/Akt pathway modulates the function of several downstream substrates involved in cell survival, growth, and proliferation [22]. Among them, the transcription factor NFkB has been shown to play an important role in transcription of several genes involved in inflammatory responses, cellular survival, and apoptosis [38]. The role of NFkB in the regulation of early myocardial damage in acute myocarditis is also of importance, since NFkB controls the transcription of a vast number of inflammatory cytokines, adhesion molecules, and anti-apoptotic proteins [38]. The family of NFkB proteins comprises the
p50, p52, p65 (Rel-A), Rel-B, and c-Rel subunits that eventually form homo- or hetero­dimer complexes [39,40]. Cumulative evidence suggests that the Rel-A (p65)/p50 dimer is the most abundant NFkB complex. Different stimuli such as tumor necrosis factor-α (TNF-α), interleukin-1 (IL-1), UV-radiation, cellular stress, and viral infections have been shown to induce NFkB activation [40,41]. NFkB can be activated through either classical or alternative pathway. The former involves the activation of the upstream IKK (inhibitor of IKB) complex composed of two catalytic subunits (IKK-α & IKK-β) and a regulatory subunit (IKK-γ) [42,43]. The alternative pathway, on the other hand, activates IKK-α independently of IKK-β and IKK-γ through the upstream kinase NFkB-inducing kinase (NIK) [44,45].

There is some indirect evidence implicating the NFkB transcription factor in the progression of viral myocarditis in the experimental mouse model. Matsumori et al. [46] have reported that NFkB inhibition prevents the development of viral myocarditis caused by encephalomyocarditis virus (ECMV), and down-regulates the expression of pro-inflammatory cytokines as well as the inducible nitric oxide synthase (iNOS) gene in mouse cardiac tissues. However, the evidence for activation of NFkB following CVB3 infection at different stages of myocarditis remains elusive. In the present study, we sought to characterize NFkB activation, its potential regulatory role, and the dependency of its activation on the PI3K/Akt pathway following CVB3 infection.

Our observations suggest that CVB3 induces the degradation of NFkB p105 (the precursor of p50) within the cytoplasm, which is associated with the translocation of NFkB p65 subunit into the nucleus of infected cells. Both luciferase reporter and
electromobility shift assays confirm that the liberated NFkB complex shows a strong DNA-binding activity. We were not able to detect any significant phosphorylation and degradation or either IkB-α or IkB-β following infection, indicating that CVB3 likely activates NFkB independently from IkBs.

Several studies have shown that induction of survival pathways can result in activation of NFkB [37,47,48]. In CVB3-infected cells, we were able to show that inhibition of PI3K and Erk1/2 significantly blocks virus-induced NFkB activation, highlighting the role of these kinases as the potential upstream activator of NFkB. However, the actual mechanisms underlying the regulatory events and existence of potential cross talk between PI3K/Akt and Erk1/2 pathways are not known and necessitate further investigations.

TNF-α is a strong activator of NFkB transcription factor [49,50]. The role of TNF-α in different infection models has been studied and shown to participate in regulation of apoptosis, inflammatory response, and cell survival, generally through the activation of NFkB transcription factor [51,52]. During the early phase of viral myocarditis, TNF-α, released by cardiac myocytes and residing macrophages, affects cellular functions in an autocrine and/or paracrine fashion. There is substantial evidence that TNF-α could have an either beneficial or deleterious effect on disease progression, and the delicate balance between these two opposing functions would determine the outcome of such stimulation.

Our findings demonstrate that TNF-α significantly intensifies virus-induced NFkB activation while having no effects on virus replication. When treated with TNF-α for a short time (one hour prior and one hour during virus incubation), infected cells
demonstrate a significant decrease in virus-induced cytopathic effects. On the other hand, a long-term TNF-α treatment, significantly enhanced virus-induced cell death.

In summary, the results of this study illustrate that 1) CVB3 activates NFkB transcription factor; 2) NFkB activation is through the PI3K/Akt and Erk1/2 pathways; 3) NFkB activation enhances the viability of infected cells; and 4) short-term and transient treatment of infected cell with TNF-α enhances cellular viability through NFkB activation while sustained presence of this cytokine is deleterious to the viability of virus-infected cells.

Currently, studies are ongoing to further dissect the ambivalent nature of NFkB activation by investigating the expression profile for downstream anti- and pro-apoptotic products such as the xIAP proteins, the cellular FLICE inhibitory protein (cFLIP), Fas, TNF-α, and caspase-8 in virus-infected cells. The new information will assist us to gain a better understanding of the regulatory mechanisms that control the delicate balance between two opposite functions of NFkB in CVB3 myocarditis.
REFERENCES:


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Figure 5-1

A)

<table>
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<th>CVB3 (hr pi)</th>
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</thead>
<tbody>
<tr>
<td></td>
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</tr>
</tbody>
</table>

**Cytoplasmic Fraction**

- NFkB p105
- NFkB p50
- β-Actin

**Nuclear Fraction**

- NFkB p105
- NFkB p50

B)

<table>
<thead>
<tr>
<th>Cytoplasm</th>
<th>Nucleus</th>
<th>CVB3 (hr pi)</th>
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</thead>
<tbody>
<tr>
<td>0 1 2 4 6 8</td>
<td>0 1 2 4 6 8</td>
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</table>

- NFkB p105
- NFkB p65
- NFkB p50
- β-Actin
- Nucleolin (c23)
Figure 5-1. CVB3 induces translocation of NFkB p65 into the nucleus.

HeLa cells were infected with CVB3 and cytoplasmic and nuclear extracts were isolated and subjected to Western blot analysis. A) NFkB p105 (the precursor of p50) is degraded around 4 hours post-infection. As expected, NFkB p105 is not present within the nuclear fraction while NFkB p50 subunit is detectible in both cytoplasmic and nuclear fractions. B) CVB3 induces translocation of p65 subunit into the nucleus of infected cells starting around 4 hours and with a peak at 6 hours post-infection. Beta-actin protein was measured to ensure equal cytoplasmic protein loading and the absence of cytoplasmic contamination within the nuclear fraction. Nucleolin (C23) was detected to ensure equal nuclear protein loading. The data represent three independent experiments.
Figure 5-2

A)  

![Graph showing relative luciferase activity over time.](image)

*P < 0.005

B)  

![DNA gel showing NFkB and Free DNA Probe.](image)
Figure 5-2. CVB3 infection enhances DNA-binding activity of NFkB.

A) HeLa cells were transiently transfected with an NFkB luciferase reporter construct or the control vector plus beta-galactosidase vector, and then infected with CVB3 (MOI=10). Luciferase activity was normalized to the corresponding beta-galactosidase activity in each sample and shown as relative luciferase activity. B) NFkB-DNA binding activity was also measured by EMSA using infrared dye labeled double-stranded NFkB oligonucleotide and 3 μg of nuclear protein. Virus infection increases NFkB binding activity around 4 hours post-infection. For the positive control group, HeLa cells were treated with 100 ng/ml TNF-α for 2 hours and nuclear fractions were prepared. To assure the specificity of NFkB-DNA binding reaction, the same amount of competitor unlabeled NFkB oligonucleotide was added to the positive control sample (first lane). The data are representative of three independent experiments.
Figure 5-3. Inhibition of NFkB activation in CVB3-infected HeLa cells significantly reduces the viability of infected cells.

HeLa cells were treated with the NFkB inhibitory peptide (SN-50) or the control peptide (MTS) starting at 1 hour prior to infection. Infected cells were stained with fluorescent dye propidium iodide (PI) At 6 hour post-infection and incubated at 37°C for 30 minutes. As shown, inhibition of NFkB increases cell death in a dose-dependent fashion. The data are representative of three independent experiments.
Figure 5-4. CVB3 infection results in phosphorylation of IKK-α but not IKK-β.

HeLa cell were infected with CVB3 (MOI=10) and whole cell lysates were collected at different timepoints. Virus infection leads to the phosphorylation of IKK-α, but not IKK-β, starting at 5 hours post-infection. IKK-α phosphorylation is associated with slight phosphorylation and degradation of IKB-α (inhibitor of NFkB). Presented blot is representative of three independent experiments.
Figure 5-5

A)

B)
Figure 5-5. Inhibition of the PI3K and Erk1/2 pathways markedly reduces the DNA-binding activity of NFkB.

HeLa cells were transfected with NFkB luciferase vector and pre-treated with either 50 μM of LY294002 or 20 μM of U0126 for one hour prior to virus inoculation and for the duration of post-infection incubation. A & B) The PI3K and Erk1/2 inhibitors decreased virus-induced NFkB activation in infected HeLa cells, indicating that NFkB activation is PI3K- and Erk1/2-dependent. The data represent three independent experiments (n=3), where a $p$ value of $< 0.005$ was considered significant.
A)

- + + CVB3 (6hr pi)
- - + TNF-α (100 ng/ml)

NFκB p105
NFκB p65
NFκB p50

Nucleoli

Nuclear Fraction

B)

<table>
<thead>
<tr>
<th></th>
<th>pTAL</th>
<th>pNFκB-Luc</th>
</tr>
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<tr>
<td>CVB3 (2hr pi)</td>
<td><img src="2000x2000" alt="Graph" /></td>
<td><img src="2000x2000" alt="Graph" /></td>
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*P < 0.005
Figure 5-6. TNF-α treatment increases NFkB activity in CVB3-infected cells.

HeLa cells were treated with 100 ng/ml TNF-α at the time of virus inoculation. Following 1-hour incubation, cells were replenished with fresh medium containing TNF-α. Cell lysates were collected at 6 hours post-infection. For luciferase assay, cell lysates were collected at 4 hours post-infection using specific reporter assay lysis buffer. A) TNF-α treatment enhances virus-induced NFkB p65 translocation into the nucleus of infected cells. Nucleolin has been measured to ensure equal protein loading. B) TNF-α treatment increases virus-induced NFkB-derived luciferase activity in infected HeLa cells. Values are the relative luciferase activity in infected cells. The data are representative of three independent experiments (n=3), where p < 0.005 considered significant.
Figure 5-7

A) Virus-infected HeLa cells were treated with increasing concentrations of TNF-α for 5 hours. As confirmed by Western blot analysis, TNF-α has no effect on virus replication in HeLa cells. B) HeLa cells were incubated with 100 ng/ml TNF-α starting at various times pre- and post-infection, and viral protein (VP1) synthesis was measured by Western blot analysis.
Figure 5-8, Cont’d...

C)

![Graph showing cell viability and TNF addition](image)

- Control
- CVB3 (16 hr pi)

- *P < 0.005

- 1 = TNF added at 0 hr pi
- 2 = TNF added at -2 hr pi
- 3 = TNF added at 2 hr pi

% Cell Viability vs. ng/ml
Figure 5-8. Short-term and early treatment of infected cells with TNF-α significantly restrains virus-induced cytopathic effects and augments host cell viability.

A) HeLa cells were treated with 100 ng/ml of TNF-α for 2 hours (treatment begins one hour prior to infection and ends at one hour post-infection). Phase-contrast microscopy of HeLa cells treated with TNF-α shows a significant decrease in virus-induced cytopathic effects at 8 hours post-infection. B) HeLa cells were treated with TNF-α as described in section A, infected with CVB3 at MOI=1, and incubated at 37°C for 16 hours post-infection in the presence or absence of NFκB inhibitor or peptide control. Inhibition of TNF-α-induced NFκB activation markedly reduced the viability of infected cells in a dose-dependent manner. C) Long-term (16 hours) stimulation of virus-infected cells with TNF-α significantly enhances cell death, indicating a detrimental effect for sustained TNF-α presence. The data represent three independent experiments (n=3), where p<0.005 was considered significant.
CHAPTER 6: SUMMARY, CONCLUSION AND FUTURE DIRECTIONS

6-1. SUMMARY

CVB3 is the most prominent infectant of the myocardium causing acute myocarditis, frequently leading to end-stage dilated cardiomyopathy. Treatment of viral myocarditis is a therapeutic dilemma to both clinicians and scientists, and, thus far, the only available effective cure is heart transplantation surgery. Successful exploration for an alternative therapeutic approach for viral myocarditis necessitates an understanding of virus-host cell interaction and cellular events during viral infection.

The studies presented in this dissertation have a focus on potential role of the PI3K/Akt pathway as a critical determinant of virus replication and virus-induced cellular injury in an acute model of coxsackievirus B3 infection. Three major constituents of the PI3K/Akt pathway have been investigated: Akt as the central mediator of the pathway; integrin-linked kinase as the potential upstream activator of Akt; and nuclear factor kappa B as one downstream candidate through which Akt may exert its regulatory effects.

In the present studies, we report that CVB3 enhances Akt phosphorylation and activation through a PI3K-dependent pathway. Replication-deficient CVB3 (UV-irradiated) fails to induce Akt phosphorylation indicating that post-entry viral replication and progeny production are required for Akt phosphorylation and activation in infected cells. Remarkably, inhibition of Akt results in a significant decrease in viral replication and release. We have also concluded that the regulatory role of Akt in virus replication is independent of the anti-apoptotic function of Akt.
We have also provided evidence that inhibition of integrin-linked kinase, an upstream kinase of Akt, significantly blocks virus-triggered Akt phosphorylation in serine site without having any effect on threonine phosphorylation. ILK inhibition has a major effect on CVB3 life cycle leading to significant decline in viral RNA transcription, viral protein synthesis, and virus progeny release. All these events eventually rescue cells from virus-induced cytopathic effects and considerably improve the viability of infected cells. Our data support the notion that the protective effects of ILK inhibition are highly dependent on the subsequent containment of Akt activation in virus-infected cell.

CVB3 infection also results in the activation of the NFkB transcription factor through a PI3K-dependent pathway. Our observations suggest that NFkB activation enhances the viability of infected cells. Further studies are needed to uncover the potential mechanism underlying the observed protective effects of NFkB activation during the course of CVB3 infection. The synopses of the experimental findings, central hypothesis and specific aims of this study are presented in Fig. 6-1.

6-2. CONCLUSION

Recent advances in cell and molecular biology have shed light on different aspects of viral pathogenesis. Both direct virus- and immune-mediated injuries are believed to contribute to the development of destructive morphology within myocardium and disease progression in viral myocarditis. The fact that CVB3 can establish persistence/latency long after infection in both human and mouse indicates the existence of a host- and/or virus-driven mechanism to shut off viral protein synthesis and RNA replication and conveys more emphasize on the importance of the interplay between virus and host cell
components. Successful viral replication relies on strategies that amend host cell signalling, particularly those controlling apoptosis and survival. Activation of anti-apoptotic pathways may help to maintain a short-term cell survival required for replication of progeny viruses, whereas the late apoptotic events may provide a condition necessary for virus release.

The time course for ILK and Akt activation in infected cells corroborates the concept that these activations rely on post-entry viral replication. Inhibition of ILK significantly blocks virus-induced Akt activation and cellular injury, whereas over-expression of an active form of Akt, subverts the protective effect of ILK inhibition in infected cells, adding more certainty to the notion that Akt activation is a detrimental factor to host cell viability during the acute phase of CVB3 infection. These findings highlight the potential therapeutic value of both ILK and Akt in viral myocarditis.

The unexpected and interesting results of this study have prompted us to revisit and revise our early hypothesis. According to our new findings, we propose that coxsackievirus B3 activates the PI3K/Akt pathway in an attempt to enhance its replication, and that Akt activation is apparently beneficial to virus life cycle while enhancing the viability of virus-infected cells.

6-3. FUTURE DIRECTIONS

At least three distinct mechanisms have been suggested for PI3K activation; 1) through dimerization and activation of receptors that possess intrinsic tyrosine kinase activity (e.g. insulin and IGF receptors), 2) through receptors that are coupled to intracellular
tyrosine kinases (e.g. IL-6 family receptors), and 3) via G protein-coupled receptors (e.g. beta-adrenergic receptor). Here, we have provided indirect evidence that CVB3 can activate PI3K in vitro. However, the mechanism fundamental to this activation has yet to be uncovered.

PI3K can directly interact with small guanine nucleotide-binding protein Ras (p21\textsuperscript{ras} G-proteins). Biologically active Ras-GTP has an innate GTPase activity that turns Ras-GTP to its inactive form Ras-GDP [1]. This equilibrium is highly regulated by Ras GTPase-activating protein (Ras-GAP) that stimulates the intrinsic GTPase activity of Ras-GTP. A previous study in our laboratory has shown that Ras-GAP is cleaved around 5 hours post-infection in CVB3-infected HeLa cells [2]. It is presumed that in CVB3-infected cells, Ras-GAP cleavage may lead to the accumulation of active Ras-GTP in proximity of the plasma membrane where it can activate the downstream PI3K pathway. Further experiments will evaluate this hypothesis and clarify the upstream event leading to CVB3-induced PI3K activation.

As reported in chapter three, inhibition of Akt significantly decreases virus replication in vitro, while simultaneously increasing caspase-3 and PARP cleavage and apoptosis. Treatment of virus-infected cells with the general caspase inhibitor zVAD.fmk blocks CVB3-induced caspase-3 and PARP cleavage, and reduces apoptotic morphology, but fails to restore viral replication. These findings signify that Akt controls virus replication and apoptosis through a distinct caspase-independent mechanism. CVB3 has a rather short life cycle and Akt activation may be a strategy used by the virus to postpone apoptosis, securing the time required for complete virus replication. On the other hand,
Akt activation may be a host-driven defensive response to the insult. The latter is of importance when terminally- differentiated cells like neurons and cardiac myocytes are targeted.

In addition, the consequence of Akt activation during a long-term and chronic infection is not clear. In experimental models of CVB3 myocarditis, there are reports of viral persistence within the myocardium even months after initiation of the acute phase [3-7]. In such conditions, the virus has developed strategies to survive for long period without killing the infected cell. During the latency, few viral proteins are expressed in the absence of viral progeny production [8]. Akt may contribute to the maintenance of the latent state by suppressing apoptosis and elimination of virus-infected cells by immune cells. The consequence of long-term Akt activation on cardiomyocyte homeostasis and morphology during the state of virus latency is of importance. Although short-term Akt activation can be considered as a compensatory response to the insult, sustained and aberrant activation of Akt may lead to maladaptive changes in cardiac function and myocyte enlargement.

Chronic viral infection in the heart may ultimately lead to dilated cardiomyopathy that is characterized by enlargement of the heart chambers, impaired ventricular function, and congestive heart failure [9,10]. Transgenic mice with cardiac-specific expression of the mutant full-length copy of CVB3 genome (that expresses a low level of viral RNA and protein) present with left and right atrial enlargement, cardiomyocyte hypertrophy, and abnormal excitation-contraction coupling comparable to that in a pressure-overload model of cardiomyopathy [11,12].
Numerous studies in an experimental mouse model have demonstrated that cardiomyocyte-targeted over-expression of a constitutively active form of Akt can induce a noticeable increase of myocardial mass and cardiac hypertrophy [13-16]. Thus, further investigation of the potential relation between aberrant virus-induced Akt activation and hypertrophic response seems worthwhile. Future in vivo experiments will allow the dissection of the specific role of Akt during both acute and chronic phases of viral myocarditis.

Although this study has provided valuable information concerning NFkB activation and the effect of this event on virus-induced cytopathic effects, there is still a void in clarifying the mechanism underlying the observed phenomenon. As noted before, NFkB exerts its anti-apoptotic functions by enhancing the production of survival proteins such as x-IAP and c-FLIP. Currently, studies are ongoing in our laboratory to determine the expression level of these proteins following virus infection and to assess the consequence of NFkB inhibition during the course of CVB3 infection.
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CVB3 activates Akt through a PI3K-dependent pathway.

Inhibition of the PI3K/Akt pathway decreases CVB3 replication and virus-induced CPE.

CVB3-induced Akt phosphorylation is via ILK activation, and ILK inhibition results in significant decrease in viral replication and virus-induced CPE.

CVB3 activates NFkB through a PI3K-dependent pathway. NFkB activation is beneficial to the host cell and enhances the viability of infected cells.

Figure 6-1. Synopsis of experimental findings, central hypothesis, and specific aims

(abbreviation: CPE, cytopathic effects)