Abstract—Viral myocarditis is a major cause of unexpected and sudden cardiac death in children and young adults. Among viruses, coxsackievirus B3 (CVB3) is the most common agent for myocarditis. Recently, more consideration has been given to the role of signaling pathways in pathogenesis of enteroviral myocarditis, providing new platform for identifying a new potential therapeutic target for this, so far, incurable disease. Previously, we reported on the role of the protein kinase-B/Akt in CVB3 replication and virus-induced cell injury. Here, we report on regulation of virus-induced Akt activation by the integrin-linked kinase in infected mouse cardiomyocytes and HeLa cells. This study also presents the first observation that inhibition of ILK in CVB3-infected cells significantly improves the viability of infected cells, while blocking viral replication and virus release. Complementary experiments using a constitutively active form of Akt revealed that the observed protective effect of ILK inhibition is dependent on the associated downregulation of virus-induced Akt activation. To our knowledge, this is the first report of such beneficial effects of ILK inhibition in a viral infection model and conveys new insights in our efforts to characterize a novel therapeutic target for treatment of entero-viral myocarditis. (Circ Res. 2006;99:354-361.)

Key Words: viral myocarditis • CVB3 • integrin-linked kinase • cell death • PKB/Akt

Myocarditis, an inflammatory disease of heart muscle, is a major cause of unexpected and sudden cardiac death in people under 40 years of age. More than 20 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. Among them, coxsackievirus B3 (CVB3), a small nonenveloped single-stranded RNA enterovirus in Picornaviridae family, has been implicated in 25% to 40% cases of acute myocarditis and dilated cardiomyopathy in infants and young adolescents.

CVB3-induced myocarditis is known historically as an immune-mediated disease. However, direct CVB3-induced injury during acute phase of disease and before target-organ immune cell infiltration has been shown to be a very important determinant of disease progression and prognosis. The fate of infected cells and severity of disease are related to the balance between multiple and contemporaneous proapoptotic and antiapoptotic processes, both viral and host cell in origin.

In both cell cultures and experimental animal models, CVB3 infection leads to the release of mitochondrial cytochrome c and subsequent cleavage of executioner caspases in the cytoplasm of infected cells. These events result in morphological features of apoptotic cell death and virus-induced cytopathic effects (CPE). Host cell signaling may rebalance cellular homeostasis, block apoptotic cell death, and diminish viral progeny release. Of the host cell signals, the phosphatidylinositol 3-kinase/Akt (PI3K/Akt) pathway has been implicated in survival, metabolism, proliferation, and apoptosis.

Previously, we reported on activation of Akt during CVB3 infection through a PI3K-dependent pathway. However, much remains to be uncovered about the actual mechanisms underlying virus-induced Akt activation and its significance in virus-induced cardiac cell death.

Full Akt activation requires phosphorylation of Thr308 residue on the catalytic domain and Ser473 residue on its C-terminal hydrophobic domain. The 3-phosphoinositide-dependent kinase 1 (PDK-1), the kinase responsible for Akt phosphorylation on Thr308, has been identified and thoroughly investigated. Several studies in PDK-1 knockout cells have emphasized the existence of a distinct Ser473 kinase. Cumulative evidence suggests that integrin-linked kinase (ILK), a serine-threonine protein kinase containing 4 ankyrin-like repeats at the N terminus, a central pleckstrin

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354
homology (PH)-like domain, and a catalytic domain at C terminus,23–27 is the upstream kinase responsible for Akt phosphorylation on Ser473 in vitro.28–32 Conversely, genetic studies in mouse fibroblasts, Caenorhabditis elegans, and Drosophila revealed that ILK kinase activity may not be required for complete Akt activation.33–36

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 CPE, all of which are important events in the pathogenesis of viral myocarditis.

Materials and Methods

Cell Culture and Virus Infection

HeLa cells (HeLa S3) were obtained from the American Type Culture Collection (ATCC). Mouse atrial cardiomyocyte cell line (HL-1) was established and provided by Dr William Claycomb (Louisiana State University Health Sciences Center, New Orleans). CVB3 (Nancy strain) was a kind gift from Dr Reinhard Kandolf (University Hospital Benjamin Franklin, Berlin, Germany). (See the online data supplement, available at http://circres.ahajournals.org.)

Constructs

Adenoviral constructs encoding constitutively active form of murine Akt1 tagged with the HA epitope (Ad-Myr-Akt1) and control green fluorescence protein (GFP) (Ad-GFP) were kindly provided by Dr Kenneth Walsh (Whitaker Cardiovascular Institute, Boston University School of Medicine, Mass) and Dr Jason Dyck (University of Alberta, Edmonton, Canada) and described previously.37 ILK cDNAs including His-V5–tagged kinase-dead ILK (S343A) and His-V5–tagged kinase-deficient ILK (E359K) have been explained elsewhere31,32 (see the online data supplement).

ILK Inhibition

HeLa and HL-1 cells were pretreated with various doses of specific ILK inhibitors KP392 and QLT0267 (QLT Inc, Vancouver, British Columbia, Canada) for 2 hours before infection. Inhibitor-containing medium was removed and replaced by serum-free medium during virus incubation to avoid any possible interference with virus binding. For ILK RNA inhibition, a 21-base pair double-stranded small interfering RNA (siRNA) molecule targeting the PH domain of ILK or a control nonspecific siRNA were used as previously described.30

Western Blot and Kinase Assay

For Western blot, 40 to 80 μg of extracted protein was fractionated by 9% to 10% sodium dodecyl sulfate-polyacrylamide gels, and protein expression, phosphorylation, and cleavage were measured. Alternatively, 250 μg of protein was used for immunoprecipitation and kinase assay as described previously31,32 (see the online data supplement for more details).

Viral RNA Synthesis and Viral Release

In situ hybridization technique and agar overlay assay were used to measure virus RNA replication and viral progeny release, respectively. (See the online data supplement for a detailed description.)

Statistical Analysis

Two-way analysis of variance with multiple comparisons, and paired Student t tests were performed. Values shown are the mean±SD. A probability value of <0.005 was considered significant.

Results

CVB3 Infection Enhances ILK Kinase Activity and Akt Phosphorylation in HL-1 Mouse Cardiomyocytes

To characterize the activation dynamics of Akt, mouse cardiomyocytes were grown in Claycomb medium. Confluent HL-1 cells represent the contractile phenotype of adult cardiomyocytes (see Movie in the online data supplement). Cells were infected with CVB3 and cellular extracts were collected at various time points postinfection. CVB3 infection enhanced ILK kinase activity (Figure 1B) and increased Akt phosphorylation on Ser473 and Thr308, with a phosphorylation peak around 4 hours postinfection that coincided with the expression of viral protein (VP1) in infected cells (Figure 1A).
Virus-Induced Akt Phosphorylation on Ser473 Is ILK Dependent

To examine whether ILK is an upstream kinase regulator of Akt-Ser473 phosphorylation, cells were pretreated with increasing doses of specific small-molecule ILK inhibitors KP392 and QLT0267, and Akt phosphorylation on both sites was assessed. ILK inhibition significantly blocked Ser473 phosphorylation, while having no effect on Thr308 phosphorylation (Figure 1C and 1D). Similarly, transient overexpression of both kinase-dead (S343A) and kinase-deficient (E359K) mutants of ILK in HeLa cells resulted in considerable inhibition of virus-induced Ser473 phosphorylation (supplemental Figure I).

Inhibition of ILK Significantly Blocks CVB3 Replication

As previously reported, blocking Akt phosphorylation leads to a significant decrease in virus replication in vitro. Here, we have assessed the effect of ILK inhibition on various phases of CVB3 replication. ILK inhibitors KP392 and QLT0267 markedly diminished viral protein expression and progeny release in a dose-dependent manner (Figure 2A and 2B). ILK Inhibition by siRNA (100 nmol/L for 96 hours) also reduced viral replication (Figure 2C).

Similarly, in infected HeLa cells, ILK inhibition caused a substantial decline in VP1 expression and viral RNA synthesis (Figure 3A and 3B). Furthermore, HeLa cells were also transfected with 2 kinase-inactive mutants of ILK (S343A and E359K). Overexpression of ILK inactive forms substantially blocked virus replication (Figure 3C), which was associated with apparent downregulation of virus-induced Akt phosphorylation (supplemental Figure I).

ILK Inhibition Suppresses Virus-Induced CPE and Enhances Host Cell Viability

In virus-infected cells, excessive viral replication is associated with the activation of apoptotic pathways. We have previously shown that the late virus-induced apoptosis/CPE and the secondary necrosis facilitate viral progeny release, a process necessary for disease progression. On the other hand, in a variety of experimental models, ILK inhibition has been shown to promote cell cycle arrest and disrupts cell adhesion and migration leading to cell death.

Taking into account that ILK inhibition markedly reduced virus replication, it was important to determine whether inhibition of ILK in virus-infected cells would also induce cardiomyocyte cell death. To examine this hypothesis, we investigated the effect of ILK inhibition on virus-induced CPE and host cell viability. ILK inhibition significantly decreased virus-induced CPE but enhanced the viability of...
infected cardiomyocytes in a dose-dependent manner, as determined by morphological features and CellTiter 96 AQueous Assay (MTS) (Figure 4A and 4B). Furthermore, fluorescent staining of infected HL-1 cells using the Double Live/Dead staining method confirmed that ILK inhibition resulted in significant decrease in virus-induced cell death (Figure 4C).

Additionally, experiments in HeLa cells using ILK inhibitors also corroborated our findings in mouse cardiomyocytes, suggesting that ILK inhibition was beneficial to virus-infected host cells by overturning virus-induced CPE (supplemental Figure IIA). Notably, inhibition of ILK had no significant effect on virus-induced caspase-3 cleavage in infected HeLa cells, while significantly diminishing cellular cytopathic features (supplemental Figure IIB and IIC), giving more credibility to the previously proposed hypothesis that a caspase-independent pathway is also involved in the process of destructive morphological changes caused by CVB3.13,15

Constitutively Active Form of Akt1 Subverts the Protective Effects of ILK Inhibition in Infected Cardiomyocytes

Previously, we provided evidence that Akt activation is 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 suppression of virus-induced CPE, mouse cardiomyocytes were transfected with either a constitutively active form of Akt1 (Ad-Myr-Akt1) or GFP (Ad-GFP) at the multiplicity of infection of 100. To ensure transfection efficiency, transfected cells were serum starved for 24 hours, cell lysates were collected, and Akt expression and phosphorylation, GSK3-β phosphorylation (as indicator of Akt activity), and HA-tagged protein expression were assessed using Western blot analysis (Figure 5A). To exclude any potential cytotoxic effect caused by adenoviral infection, and to evaluate GFP expression (transfection efficiency), transfected cells were observed at 48 hours following transfection. Note the high expression of GFP in Ad-GFP–transfected HL-1 cells (Figure 5B).
inhibitor QLT0267 (1.5 μmol/L). As shown before, at the above concentration, virus replication is slightly, but not completely, blocked (Figures 2A and 3A), providing a condition in which even a slight change in virus replication or cytopathic effects would be detectible. Although overexpression of an active form of Akt1, to some extent increased viral protein expression (Figure 6A), the effect on virus release and cardiomyocytes viability was particularly significant. It was evident that overexpression of Myr-Akt1 overcame the protective effect of ILK inhibition; augmented CVB3 release (Figure 6B), and reduced cellular viability (Figure 6C), indicating an elevated rate of cardiomyocyte death (Figure 6D). Remarkably, Overexpression of active Akt1 had no measurable effect on virus-induced caspase-3 cleavage in CVB3/QLT0267-treated cardiomyocytes (supplemental Figure III). Similar observations were made when Ad-Myr-Akt1 transfected HeLa cells were treated with ILK inhibitor QLT0267 (supplemental Figure IVA and IVB).

**Inhibition of αvβ1 and αvβ3 Integrins With RGD Peptides Does Not Inhibit CVB3 Infection**

ILK has been shown to interact with the cytoplasmic domain of the β1 and β3 integrins linking extracellular matrix components to cytoplasmic signaling and structural networks. Because our results characterized a regulatory role for ILK during CVB3 infection, we sought to determine whether integrin subunits also played a role in CVB3 infection. Hence, mouse cardiomyocytes were treated with increasing doses of αvβ1 cyclic blocking peptide GRGDNP (H-Gly-Arg-Gly-Asp-Asn-Pro-OH) and αvβ3 cyclic binding peptide XJ735 (Cyclo-Ala-Arg-Gly-Asp-3-aminomethylbenzoyl) before infection as well as during virus incubation. Following the infection period, cells were replenished with fresh serum-free medium.

At desired time points postinfection, viral protein expression (indication of virus entry) and virus-induced morphological changes (indication of virus replication) were evaluated. Blocking β1 and β3 integrin function with RGD peptides had no effect on virus replication and virus-induced cytopathic effects in infected cardiomyocytes (Figure 7).

To ensure the effectiveness of RGD peptide treatment, in a separate experiment, cardiomyocytes were treated with both blocking peptides for 24 hours and morphological changes were monitored using bright-field microscopy. The disruption of extracellular matrix/integrin interaction and cellular de-

![Figure 6](https://circres.ahajournals.org/content/108/5/358/F6.large.jpg)

**Figure 6.** Constitutively active form of Akt1 increases viral protein synthesis and virus progeny release and enhances cell death in infected cardiomyocytes. A, In QLT0267-treated HL-1 cells, overexpression of an active form of Akt1 increased viral protein synthesis. HL-1 cells were transfected with either Ad-Myr-Akt1 or Ad-GFP for 48 hours and then treated with ILK inhibitors before CVB3 infection. B, The active form of Akt1 significantly enhanced CVB3 release from QLT0267-treated cells. C, Constitutively active form of Akt1, augmented virus-induced cell death. The viability of QLT-treated HL-1 in the presence or absence of an active form of Akt1 was measured using a standard MTS assay. D, Virus-infected HL-1 cells were stained with Cyto-dye and propidium iodide (PI) for 30 minutes at 16 hours post-infection (dead cells, red; live cells, green). Original magnification, ×200.
Cardiomyocytes were treated with increasing doses of GRGDNP peptides had no measurable effects on CVB3 replication. HL-1 activation requires phosphorylation of both Thr308 and p-Ser473. Among them, are the MAPK-activated protein kinase-2 (MK2), p38 MAP kinase, protein kinase C (PKC), and the mammalian target of rapamycin (mTOR). Recently, through several in vitro studies, ILK has also been proposed as a potential upstream kinase responsible for Akt phosphorylation on Ser473.28–32 However, other genetic studies suggest that ILK kinase activity may not be essential for full Akt activation (at least in vertebrates) and that the observed dependency is cell and agonist specific.33–36

Increased level of ILK expression has been reported in various cancers, making it a potential and attractive therapeutic target for cancer treatment.39,40,46 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, the cDNA microarray and Northern blot analyses of extracellular matrix gene expression in myocardium of mouse infected with CVB3 have shown a 2.4-fold increase in ILK mRNA expression as compared with control group, pointing toward a potential role for ILK in disease progression.47 However, the role and consequence of ILK upregulation in viral myocarditis remains elusive.

Here, we suggest a crucial role for ILK in regulation of CVB3-induced Akt activation in early phase of infection. Our findings in mouse cardiomyocytes (HL-1) and human epithelial cells (HeLa) showed that inhibition of ILK activity and expression by various means significantly blocked virus-triggered Akt phosphorylation on Ser473 without having any effect on Thr308 phosphorylation. ILK inhibition had a major effect on CVB3 life cycle leading to a significant decline in viral RNA transcription, viral protein synthesis, and virus progeny release. All these events eventually rescued cells from virus-induced CPE and considerably improve the viability of infected cells. Overexpression of an active form of Akt1 dramatically reversed the protective effect of QLT0267 in infected cells, indicating that (1) protective effects of ILK inhibition was through down regulation of Akt phosphorylation and (2) Akt activation during CVB3 infection was detrimental and disadvantageous to the host cell but was beneficial to virus replication. These findings suggest that the outcome of Akt activation may be highly dependent on cell environment and the type of agonist.

Following its activation, ILK interacts with several components of the focal adhesion complex through its catalytic domain.23,39 This interaction is necessary to establish a strong association between ILK and actin cytoskeleton, leading to cell proliferation and spreading. Inhibition of Rho GTPases such as RhoA, Rac, and Cdc42 as well as of F-actin assembly can block CVB3 replication in Caco-2 cell line because of the inhibition of virus movement toward the tight junction and subsequent movement through the cytosol of infected cells.48 ILK is a crucial regulator of actin rearrangements by activating Rho GTPases and by phosphorylating components of the focal adhesion complex including α- and β-Parvin and PINCH.30,49,50 It is therefore possible that ILK may regulate CVB3 movement within the infected cell by modulating focal adhesions and actin cytoskeleton rearrangements, which is crucial for virus movement and replication. Currently, re-
search is ongoing in our laboratory to investigate this hypothesis.

ILK has also been shown to anchor to cytoplasmic tails of integrin $\beta_1$ and $\beta_3$, regulating 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 $\alpha_2\beta_1$ and $\alpha_6\beta_1$ in the heart of patients diagnosed with end-stage dilated cardiomyopathy.58 Agrez et al59 have studied the role of integrin subunits in CVB3 infection and shown that overexpression of integrin $\alpha_5\beta_1$ enhances CVB3 lytic infection in human colon cancer cells.

Our findings demonstrated that inhibiting $\beta_1$ and $\beta_3$ integrin function with RGD peptides had no effects on CVB3 entry and replication, as well as virus-induced CPE. However, the data do not rule out the potential involvement of other motifs on $\beta_1$ and $\beta_3$ integrins as well as a potential role for other integrin subunits in CVB3 entry and replication.

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 and knockout mouse models will also provide valuable information on the efficacy of ILK inhibition and should provide a foundation to establish and develop an effective therapeutic approach to treat enteroviral myocarditis.

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Disclosure
None.

References


Novel Role for Integrin-Linked Kinase in Modulation of Coxsackievirus B3 Replication and Virus-Induced Cardiomyocyte Injury
Mitra Esfandiarei, Agripina Suarez, Ansel Amaral, Xiaoning Si, Maziar Rahmani, Shoukat Dedhar and Bruce M. McManus

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Supplementary Text

Material and Methods:

Cell culture and virus infection

All cell cultures were incubated at 37°C in a 5% CO₂ water-jacketed incubator. HL-1 cells were grown in culture dishes pre-coated with 0.00125 percent fibronectin (Calbiochem, San Diego, CA) in 0.02 percent gelatin (Sigma-Aldrich, Oakville, ON), and in Claycomb Media™ (JHR Bioscience, Lenexa, KS) supplemented with 10 percent heat-inactivated fetal bovine serum, 100 μg/mL penicillin G (Invitrogen, Burlington, ON), 100 μg/mL streptomycin (Invitrogen), 2 mmol/L L-glutamine (Sigma-Aldrich), and 0.1 mmol/L norepinephrine (Sigma-Aldrich) to maintain the contractile phenotype of adult cardiomyocytes. The titer of 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 1-hour incubation at 37°C, cells were replenished with fresh medium. For inhibitor experiments, pretreatment with inhibitor was performed 1-2 hours prior to infection and fresh inhibitors at the specified concentrations were added following medium changes, if needed.

Transient transfection

HeLa and HL-1 cells were infected with adenoviral constructs encoding constitutively active form of murine Akt1 tagged with the HA epitope (Ad-Myr-Akt1) and control GFP (Ad-GFP) 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. For cDNA 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 control pCDNA3.1 using Effectene reagent (Invitrogen) according to the manufacturer's instruction. Transfected cells were used for various experiments at 48 hours post-transfection.

For ILK siRNA experiment, 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.

**ILK kinase assay**

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. For ILK kinase assay, 250 μg of protein lysates were immunoprecipitated with ILK antibody-conjugated protein A Sepharose overnight at 4°C while shaking. Kinase assays were carried out using glycogen synthase kinase fusion protein as a substrate for ILK. Phosphorylated protein was separated on a 11% SDS-PAGE gel and specific anti-phospho-GSK3β antibody.

**Antibodies and reagents**
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 α₃β₃ monoclonal blocking antibody was from Chemicon International (Temecula, CA). RGD cyclic peptide GRGDNP (α₁β₃ blocker) was obtained from Calbiochem (Mississauga, ON). RGD cyclic peptide XJ735 (α₃β₃ blocker) was from Bachem (Torrance, CA).

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.

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 the agar overlay plaque assay as previously described^{1,2}. 
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). Cellular viability was also assessed using the Live/Dead Double Staining kit (Oncogene Research Products, Boston, MA) according to the manufacturer’s instruction. The kit utilizes Cyto-dye, a cell-permeable green fluorescent dye to stain live cells; and propidium iodide (PI), a cell non-permeable red fluorescent dye to stain dead cells.

References:


Supplementary Data

Figure Legends:

Movie file. Contraction of HL-1 cardiomyocytes in culture plate.

Mouse cardiomyocytes were grown in culture dishes pre-coated with 0.00125 percent fibronectin in 0.02 percent gelatin and in Claycomb Media™. As shown in movie, confluent HL-1 culture represents the contractile feature similar to the adult cardiomyocyte.

Figure S1. Inhibition of ILK significantly blocks CVB3-induced Akt-Ser^{473} phosphorylation in HeLa cells.

HeLa cells were transiently transfected with the kinase-dead ILK (S343A) and kinase-deficient ILK (E359K) mutants for 48 hours. Transfected HeLa cells were infected with virus and Akt phosphorylation was measured by Western blotting. ILK inhibition markedly blocked virus-induced Akt phosphorylation on ser^{473} site.

Figure S2. ILK inhibition significantly decreases virus-induced cytopathic effects in CVB3-infected HeLa cells.

A) Photomicrograph of HeLa cells treated with either ILK inhibitor QLT0267 (5 µmol/L) or DMSO. As shown, ILK inhibitor markedly improved viability of infected HeLa cells as compared to vehicle (DMSO)-treated group (original magnification: X 200). B & C) Inhibition of ILK by specific inhibitors or ILK kinase-inactive mutants did not affect caspase-3 cleavage (similar band intensity for pro-caspase-3 and cleaved caspase-3 for all treatment) in infected
HeLa cells, indicating that ILK regulates virus-induced cytopathic effects through a caspase-3-independent mechanism.

**Figure S3. ILK inhibition has no detectable effect on caspase-3 cleavage in virus-infected mouse cardiomyocytes.**

Sub-confluent mouse cardiomyocytes were infected with adenoviral vector expressing either a constitutively active form of Akt1 (Ad-Myr-Akt1) or GFP (Ad-GFP) at the MOI of 100. Transfected cells were pre-treated with specific ILK inhibitor QLT0267 (1.5 µmol/L) and then infected with CVB3. Cell lysates were collected, and then subjected to Western blot analysis to measure caspase-3 cleavage. As shown, over-expression of an active form of Akt1 in QLT0267-treated cells had no significant effect on caspase-3 cleavage and apoptosis in infected HL-1 cells.

**Figure S4. 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 had no cytotoxic effect on HL-1 cardiomyocytes (original magnification: X 200). B) Photomicrograph presenting the morphology of adenoviral-transfected HeLa cells treated with either ILK inhibitor QLT0267 (5 µmol/L) or DMSO. As shown, over-expression of active form of Akt1 reversed the protective effects of QLT0267 in infected HeLa cells (original magnification: X 200).

**Figure S5. Efficiency of integrin blocking peptide treatment in HL-1 mouse cardiomyocytes.**
To ensure the efficiency of integrin blocking peptides, HL-1 cells were treated with 300 µmol/L of GRGDNP (ανβ1 integrin blocker) and 30 µmol/L of XJ735 (ανβ3 integrin blocker) for 24 hours. Note the disruption of ECM and cell-cell interactions and detachment of cardiomyocytes with early signs of anoikis.

**Figure S6. Inhibition of ανβ3 with blocking antibody does not inhibit CVB3 infection in HeLa cells.**

HeLa cells were treated with increasing doses of mouse monoclonal blocking antibody against ανβ3 for 1 hour prior to and during virus incubation. The ανβ3 blocking antibody did not affect CVB3 replication and virus-induced cytopathic effects in HeLa cells (original magnification: X 200).
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- **P-Akt-Ser^473**
- **β-Actin**
Figure S2

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Cleared Caspase-3

Pro-Caspase-3

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Pro-caspase-3

Pro-caspase-3

HeLa Cells

Histogram
### Figure S3

<table>
<thead>
<tr>
<th>Control</th>
<th>Ad-GFP</th>
<th>Ad-Myr-Akt1</th>
<th>QLT0267 (µmol/L)</th>
<th>CVB3 8hr</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.5</td>
<td>1.5</td>
<td>1.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Pro-Caspase-3**

**Cleaved-Caspase-3**

**HL-1 Cells**
Figure S4

A)

B)

CVB3 18hr + QLT0267 (µmol/L)

<table>
<thead>
<tr>
<th></th>
<th>0</th>
<th>5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ad-GFP</td>
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<td></td>
</tr>
<tr>
<td>Ad-GFP</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ad-Akt1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ad-Akt1</td>
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<td></td>
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</tbody>
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Ad-GFP 48hr
Ad-GFP 48hr
Ad-Akt1 48hr
Ad-Akt1 48hr

Ad-GFP
Ad-Myr-Akt1
Figure S5

<table>
<thead>
<tr>
<th></th>
<th>GRGDNP</th>
<th>XJ735</th>
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<tr>
<td>0</td>
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<td>30</td>
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<tr>
<td>µmol/L</td>
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<td>µmol/L</td>
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HL-1 Cells
Figure S6

CVB3 9hr

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<tbody>
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<td>ανβ3 Ab (µg/mL)</td>
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<tr>
<td>β-actin</td>
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<td></td>
<td></td>
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<tr>
<td>VP1</td>
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<td></td>
</tr>
</tbody>
</table>

HeLa Cells

Control

CVB3

- IgG ανβ3 Ab