Adenoviral Gene Transfer of Activated Phosphatidylinositol 3'-Kinase and Akt Inhibits Apoptosis of Hypoxic Cardiomyocytes In Vitro

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Background—The intracellular signaling pathways that control cardiomyocyte apoptosis have not been fully defined. Because insulin-like growth factor-1 (IGF-1) prevents cardiomyocyte apoptosis, we examined the role of its downstream signaling molecules in an in vitro model of hypoxia-induced cardiomyocyte apoptosis.

Methods and Results—Treatment of rat neonatal cardiomyocytes with IGF-1 increased activity of both phosphatidylinositol 3'-kinase and its downstream target, Akt (also known as protein kinase B or PKB). Cardiomyocytes were subjected to hypoxia for 24 hours, and apoptosis was assessed by DNA laddering, TUNEL staining, and ELISA for histone-associated DNA fragments. IGF-1 treatment (100 nmol/L) reduced cardiomyocyte apoptosis, and this effect was inhibited by simultaneous treatment with a PI 3-kinase inhibitor. Cardiomyocytes were infected with either a control adenovirus (Ad.EGFP) or adenoviruses carrying constitutively active forms of PI 3-kinase (Ad.BD110) or Akt (Ad.myr-Akt-HA). Ad.BD110 significantly inhibited apoptosis of hypoxic cardiomyocytes compared with Ad.EGFP (61.0 ± 4.6% less DNA fragmentation than in Ad.EGFP-infected cells, \( P = 0.0001 \)). Ad.myr-Akt-HA even more dramatically inhibited apoptosis of hypoxic cardiomyocytes (90.9 ± 1.4% less DNA fragmentation than in controls, \( P < 0.0001 \)).

Conclusions—IGF-1 activates PI 3-kinase and Akt in cardiomyocytes. Activated PI 3-kinase and Akt are each sufficient to protect hypoxic cardiomyocytes against apoptosis in vitro. Adenoviral gene transfer provides a useful tool for investigating the role of these signaling pathways in cardiomyocyte apoptosis. (Circulation. 1999;100:2373-2379.)

Key Words: kinase • viruses • growth substances • signal transduction

Cardiomyocyte programmed cell death (apoptosis) has been identified in a wide variety of cardiovascular disorders, including myocardial infarction and heart failure (reviewed in Reference 1). Apoptosis is particularly prominent in models of ischemia-reperfusion injury, affecting a significant proportion of cardiomyocytes in the involved area. Although apoptosis might appear to be an attractive target for therapeutic intervention, the overall functional contribution of apoptosis to disease progression has not been defined in these conditions. Identifying the intracellular signaling pathways that control apoptosis in cardiomyocytes and developing approaches to modulating these pathways could help clarify their role in experimental models of cardiac disease and potentially lay the foundation for novel therapeutic strategies. The goals of this study were to identify signaling pathways that modulate cardiomyocyte apoptosis in vitro and to examine the ability of somatic gene transfer to manipulate these pathways.

Insulin-like growth factor-1 (IGF-1) blocks apoptosis in many settings, and this ability is generally dependent on activation of phosphatidylinositol 3' (PI 3')-kinase. Recent studies have demonstrated that Akt (also known as protein kinase B or PKB) is the target of PI 3-kinase that is both necessary and sufficient to mediate inhibition of apoptosis in cerebellar neurons. Akt can inhibit caspase-mediated cell death through at least 3 mechanisms: phosphorylation of the death agonist Bcl-XL/Bcl-2-associated death promoter (BAD), releasing Bcl-2 family members; direct phosphorylation of caspase-9; and phosphorylation of the FKHRL1 transcription factor, blocking Fas ligand expression. Therefore, PI 3-kinase appears to be a critical component in a signal transduction pathway mechanistically linking viability factors, such as IGF-1, to caspases through activation of the serine-threonine kinase, Akt.

The ability to manipulate the activity of these molecules in cardiomyocytes would facilitate an examination of their...
functional effects. Relatively specific pharmacological inhibitors of PI 3-kinase have been identified.\textsuperscript{8,9} In contrast, no inhibitors of Akt have been discovered to date, nor are there reagents that specifically and effectively activate either PI 3-kinase or Akt. In the present study, we used adenoviral gene transfer to directly examine the role of these molecules in an in vitro model of hypoxia-induced cardiomyocyte apoptosis.

Methods

In Vitro Model of Cardiomyocyte Hypoxia

Cardiomyocytes were prepared from 1- to 2-day-old rats by use of the Neonatal Cardiomyocyte Isolation System (Worthington Biochemical Corp.), plated in 12-well plates or on glass coverslips in 6-well plates at 1\times10^5 or 5\times10^5 cells/well, respectively, and cultured in RPMI 1640/5% FCS/10% horse serum (HS) for 72 hours before infection. Cells were then infected in RPMI 1640 alone for 2 hours with or without adenoviral vector, as indicated. An equal volume of RPMI 1640/5% FCS/10% HS was then added to each well, and cells were cultured for an additional 46 hours. For hypoxia, the medium was then changed to serum-free RPMI 1640 saturated with 95% N\textsubscript{2}/5% CO\textsubscript{2}, and cells were placed in a 37°C airtight box for 24 hours. O\textsubscript{2} concentrations were monitored (Ohmeda oxygen monitor, type 5120). For normoxic controls, culture medium was changed to RPMI 1640/5% FCS/10% HS, and cells were placed in a 37°C/5% CO\textsubscript{2} incubator for 24 hours before analysis.

TUNEL Staining

TUNEL staining was performed with the Apoptag kit (Intergen), according to the manufacturer’s recommendations. Cardiomyocytes were identified by simultaneous immunostaining with the anti-sarcomeric \( \alpha \)-actinin monoclonal antibody (mAb) EA-53 (Sigma). To quantify the number of apoptotic cardiomyocytes, nuclei were counterstained with Hoechst 33258 (Sigma), and the total numbers of nuclei and TUNEL-positive nuclei were counted in 8 to 10 low-power fields in 3 independent experiments. More than 1500 nuclei were counted under both normoxic and hypoxic conditions. These data are given in the text as the mean\( \pm \)SD.

DNA Fragmentation

Myocytes (1\times10^5) were trypsinized and resuspended in 0.75 mL of lysis buffer (100 mmol/L Tris-HCl [pH 8.5], 5 mmol/L EDTA, 0.2% SDS, 200 mmol/L NaCl, 100 \( \mu \)g/mL proteinase K). Cell lysates were incubated at 37°C for 4 hours with agitation and precipitated with an equal volume of isopropanol (1:1) at \(-20^\circ\text{C}\) for 4 to 18 hours, followed by phenol/chloroform extraction and ethanol precipitation at \(-80^\circ\text{C}\) for \( \geq 15\) minutes. Pellets were washed with 7% ethanol, air-dried, and resuspended in TE buffer (10 mmol/L Tris-HCl [pH 8.0], 1 mmol/L EDTA). RNase (Boehringer Mannheim) was added to a final concentration of 10 \( \mu \)g/mL. One microgram of each DNA sample was labeled with 0.5 \( \mu \)Ci [\( ^{32} \)P]dCTP (DuPont NEN) in the presence of 5 U of Klenow polymerase (NEB, 10 minutes in NEB Klenow buffer) without additional unlabeled dNTPs. The reaction was terminated (10 mmol/L EDTA, 75°C for 10 minutes) and subjected to 1.8% agarose gel electrophoresis and autoradiography.

Cell Death ELISA

Histone-associated DNA fragments were quantified by ELISA (Boehringer Mannheim). All cells from each well were collected by trypsinization and pipetting, pelleted (800 rpm, Sorvall T6000B, 5 minutes), lysed, and subjected to the capture ELISA according to the manufacturer’s protocol. Data were normalized to the amount of DNA fragmentation seen with hypoxic Ad.EGFP-transduced cardiomyocytes. Each experiment was carried out in triplicate and repeated in \( \geq 3\) independent experiments.

Kinase Assays

PI 3-Kinase

PI 3-kinase activity was measured as described previously.\textsuperscript{9} Cell lysates were immunoprecipitated with either anti-phosphotyrosine mAb (PY20) for endogenous PI 3-kinase activity or anti-myct (Santa Cruz Biotech) for BD110, which is not recognized by PY20. PI 3-kinase activity was measured in a reaction mixture containing phosphatidylinositol (Avanti) and \( [\gamma-^{32} \)P]ATP (DuPont NEN). After 5 minutes, the reaction was stopped by the addition of HCl and chloroform:methanol and analyzed by thin-layer chromatography. PI 3-kinase activity was detected by the appearance of a specific radioactive spot corresponding to \( [\gamma-^{32} \)P]PI-3-P. We also verified that phosphorylation was occurring at the 3 position by using the specific PI 3-kinase substrate, PI-4,5-P\textsubscript{2} (data not shown).

Akt Assay

Akt kinase assays were performed as described previously.\textsuperscript{3} Cell lysates were immunoprecipitated with anti-Akt1 mAb (Upstate Biotech), used in kinase reactions with histone H2B (Boehringer Mannheim) as substrate and \( [\gamma-^{32} \)P]ATP, and subjected to SDS-PAGE. The lower-molecular-weight area in the gel (with radiolabeled Histone H2B) was separated for direct autoradiography. The higher-molecular-weight area in the gel (with Akt) was transferred to a membrane for immunoblotting with anti-Akt1 mAb (Transduction Laboratories).

Immunohistochemistry

Cardiomyocytes were fixed with 10% neutral buffered formalin (22°C, 5 minutes), permeabilized (\(-20^\circ\text{C}\) methanol/acetic acid [1:1], 10 minutes), and incubated with primary mAb to c-\( \alpha \)-myc or c-\( \alpha \)-actin (30 minutes, 22°C). Cells were rinsed in PBS, incubated with anti-mouse IgG (Fab-specific) conjugated to tetramethylrhodamine (Sigma) (30 minutes, 22°C), and mounted.

Recombinant Adenoviral Vectors

Three recombinant first-generation type 5 adenoviruses were used for these studies. Ad.EGFP has been described previously.\textsuperscript{11} Ad.BD110 contains the myc-tagged BD110\textsuperscript{12} expression cassette in E1 and an EGFP (Clontech) expression cassette in E3 and was created through homologous recombination between pAE1A-CMV-BD110 and pBHGI1-EGFP in 293 cells, as previously described.\textsuperscript{13} The myr-Akt-HA-expressing adenovirus was constructed by first adding the src myristylation signal to a cDNA clone expressing HA epitope–tagged Akt in pCAV-6, transferring the resulting insert into pACCMCLPA, and obtaining homologous recombinants through cotransfection with pJM17 (Microbix Systems) in 293 cells. Recombinant plaques were isolated and propagated in 293 cells, and transgene expression and appropriate kinase activity were verified in cardiomyocytes. Viral titer was determined by plaque assay in 293 cells. Stock titers were \( \sim 10^9\) pfu/mL for each vector, with a particle/pfu ratio of \( \sim 10^2\). Wild-type adenovirus contamination was excluded by the absence of both PCR-detectable E1 sequences and cytotoxic effects on the nonpermissive A549 cell line.

Statistical Analysis

Data are represented as the mean\( \pm \)SEM of \( \geq 3\) independent experiments and were compared by ANOVA. The null hypothesis was rejected at \( P<0.05\).

Results

Hypoxia-Induced Apoptosis in Cardiomyocytes

Transient hypoxia (24 hours) induced apoptosis that was evident by TUNEL staining, DNA laddering, and ELISA for histone-associated DNA fragments, in agreement with previous reports\textsuperscript{14–16} (Figure 1). Colocalization of TUNEL-positive cells with immunohistochemical staining for sarcomeric \( \alpha \)-actinin confirmed that the apoptotic cells were
cardiomyocytes, rather than contaminating fibroblasts (Figure 1A). After hypoxia, 97% TUNEL-positive nuclei localized to α-actinin–positive cells and represented 21 ± 2% of the nuclei in these cells. In contrast, only 2 ± 1.7% of α-actinin–positive cells were TUNEL-positive in normoxic cultures. Only 3.0 ± 1.2% of the TUNEL-positive nuclei occurred in the absence of α-actinin staining. Further evidence that cardiomyocytes were the predominant contributor to the apoptosis observed in this model was provided by 2 additional experiments in which cultures treated with BrdU to increase the proportion of cardiomyocytes16 yielded similar results (data not shown). Importantly, infection of cardiomyocytes with recombinant control adenovirus (Ad.EGFP) did not affect cardiomyocyte apoptosis (Figure 1B and 1C).

Modulation of Hypoxia-Induced Apoptosis in Cardiomyocytes

IGF-1 effectively blocked cardiomyocyte apoptosis, consistent with previous reports15 (Figure 2a). Although IGF-1 is a known activator of PI 3-kinase, we documented that this signaling mechanism was preserved in hypoxic cardiomyocytes. In hypoxic myocytes, PI 3-kinase was activated by IGF-1 treatment and significantly though incompletely inhibited by wortmannin (Figure 2b) and LY294002 (data not shown). Hypoxic cardiomyocytes treated with both IGF-1 and wortmannin displayed significantly more residual PI 3-kinase activity than cells treated with wortmannin alone (data not shown). We tested the hypothesis that the IGF-1 survival benefit was mediated through PI 3-kinase by treating cultures with both IGF-1 and the specific PI 3-kinase inhibitors wortmannin and LY294002. LY294002 significantly inhibited the ability of IGF-1 to prevent apoptosis in hypoxic cardiomyocytes, and a more modest effect was seen with wortmannin (Figure 2a). The ability of LY294002 to inhibit the survival benefit from IGF-1 strongly suggests that this benefit is mediated, at least in part, through PI 3-kinase.

However, the combined treatments generally failed to induce the same amount of DNA laddering as seen in the hypoxic control cells. This incomplete restoration of apoptosis may reflect either incomplete inhibition of PI 3-kinase or the involvement of other IGF-1–activated signaling mechanisms. Of note, LY294002 and wortmannin did not significantly affect baseline cardiomyocyte apoptosis, although in some experiments DNA laddering under hypoxic conditions appeared to be modestly increased by PI 3-kinase inhibition.

33258 (middle). Hypoxia induced an increase in TUNEL-positive cardiomyocytes (right). Representative data from 1 of 3 independent experiments are shown. B, Agarose gel electrophoresis. Hypoxia induced an increase in DNA laddering in both uninfected and Ad.EGFP-infected cultures (10 pfu/cell). Representative data from 1 of 3 independent experiments are shown. C, Cell death ELISA. To quantify apoptosis, a commercially available ELISA was used to detect histone-associated DNA fragments (Boehringer-Mannheim). Results are expressed as percentage of that seen with hypoxic, Ad.EGFP-infected cardiomyocytes. Hypoxia induced a significant increase in DNA fragmentation. Ad.EGFP infection did not affect amount of DNA fragmentation seen in either normoxic or hypoxic cultures. Pooled data from 4 independent experiments are shown.

Figure 1. Apoptosis in neonatal rat cardiomyocytes in vitro. A, TUNEL assay of cardiomyocytes in normoxic or hypoxic cultures. TUNEL-positive cells (bottom) were identified as cardiomyocytes by simultaneous immunohistochemical staining with mAb to α-actinin (top). Nuclei were counterstained with Hoechst 33258 (middle). Hypoxia induced an increase in TUNEL-positive cardiomyocytes (right). Representative data from 1 of 3 independent experiments are shown. B, Agarose gel electrophoresis. Hypoxia induced an increase in DNA laddering in both uninfected and Ad.EGFP-infected cultures (10 pfu/cell). Representative data from 1 of 3 independent experiments are shown. C, Cell death ELISA. To quantify apoptosis, a commercially available ELISA was used to detect histone-associated DNA fragments (Boehringer-Mannheim). Results are expressed as percentage of that seen with hypoxic, Ad.EGFP-infected cardiomyocytes. Hypoxia induced a significant increase in DNA fragmentation. Ad.EGFP infection did not affect amount of DNA fragmentation seen in either normoxic or hypoxic cultures. Pooled data from 4 independent experiments are shown.
Although these data suggest the involvement of PI 3-kinase activation in the antiapoptotic effect of IGF-1, we cannot exclude the possibility that IGF-1 and PI 3-kinase modulate apoptosis through independent mechanisms by this pharmacological approach.

To overcome this limitation, we used adenoviral gene transfer to directly examine the effects of specific signaling molecules. We constructed a recombinant adenoviral vector carrying a constitutively active form of PI 3-kinase, BD110. BD110 contains the p110 catalytic domain of PI 3-kinase fused in-frame with the binding domain (AA 474 to 552) of the regulatory subunit, p85, leading to constitutive binding and enzymatic activation. This virus also encodes an independent expression cassette carrying EGFP that allows direct identification of transduced cells by fluorescence microscopy (Figure 3). Infection of cardiomyocytes with Ad.BD110 conferred activation of both PI 3-kinase and Akt (Figure 4a and 4b). Treatment of cardiomyocytes with IGF-1 also activated Akt (Figure 4b). These results both established the biological activity of Ad.BD110 and confirmed that Akt is a downstream target of PI 3-kinase in cardiomyocytes activated by IGF-1 treatment (Figure 4b). Because Akt is the PI 3-kinase substrate essential for its survival benefit in other cells, we created a recombinant adenovirus carrying a constitutively active Akt (myr-Akt). This virus was also able to mediate robust transgene expression in cardiomyocytes and increased Akt activity even in the absence of IGF-1 (Figure 4b).

Infection of cardiomyocytes with Ad.BD110 at a multiplicity of infection (MOI) of 10 plaque-forming units (pfu)/cell did not affect survival of unstimulated cells (Figure 5a). Infection at an MOI ≥50 induced apoptosis of even unstimulated cardiomyocytes, suggesting that massive overexpression of this pleiotropic kinase is not well tolerated (data not shown). However, cardiomyocytes infected at an MOI of 10 pfu/cell were substantially protected against hypoxia-induced apoptosis (61.0 ± 4.6% less DNA fragmentation versus Ad.EGFP-infected cells, P<0.0001) (Figure 5a). Of note, at this MOI, virtually all of the cardiomyocytes were transduced, as evident by EGFP expression. The protection afforded by Ad.BD110 was abolished by simultaneous treatment with the PI 3-kinase inhibitor LY294002 (10 μmol/L).

![Figure 2. IGF-1 effects in cardiomyocytes. a, IGF-1 inhibits cardiomyocyte apoptosis. Cardiomyocytes were treated with IGF-1 (100 nmol/L), LY294002 (10 μmol/L, bottom), and wortmannin (100 nmol/L, top), alone or in combination as indicated. Cells in each group were cultured under normoxic or hypoxic conditions for 24 hours, as indicated. IGF-1 treatment reduced DNA laddering seen in hypoxic cultures. Specific PI 3-kinase inhibitors wortmannin and LY294002 did not affect basal DNA fragmentation. Simultaneous treatment with IGF-1 and LY294002 significantly inhibited protective effect of IGF-1. More modest inhibition was seen with wortmannin treatment. Representative data from 1 of 4 independent experiments are shown. b, IGF-1 activates PI 3-kinase in hypoxic cardiomyocytes. Hypoxic cardiomyocytes were treated with IGF-1 (100 nmol/L) or wortmannin (100 nmol/L) for 8 hours before harvest. Cell lysates were immunoprecipitated with a monoclonal antibody to phosphotyrosine (PY20), and PI 3-kinase activity was assessed as described in Methods. Endogenous PI 3-kinase activity was increased by IGF-1 treatment and inhibited by wortmannin. Representative data from 1 of 3 independent experiments are shown.

![Figure 3. Ad.BD110 mediates coexpression of BD110 and EGFP in cardiomyocytes. After infection with Ad.BD110, cardiomyocytes express both EGFP (right) and BD110 (left) detected with mAb to myc epitope incorporated into construct. Representative data from 1 of 3 independent experiments are shown.](http://circ.ahajournals.org/doi/abs/10.1161/01.CIR.102.8.2576?journalCode=cir)
In contrast, Ad.myr-Akt-HA did not increase cardiomyocyte apoptosis even at an MOI of 80 pfu/cell (Figure 5b and data not shown). In hypoxic cultures, Ad.myr-Akt-HA significantly inhibited hypoxia-induced DNA fragmentation in a dose-dependent manner at an MOI from 10 to 80 pfu/cell (Figure 5c). The maximum reduction in cardiomyocyte apoptosis seen with z-VAD.fmk treatment (93.5 ± 2.0%, \( P < 0.0001 \) versus uninfected controls) was comparable to that seen with Ad.myr-Akt-HA infection and greater than that achieved with BD110 at an MOI of 10 pfu/cell. Thus, caspase inhibition also blocks apoptosis of hypoxic cardiomyocytes and is quantitatively sufficient to account for the myocyte survival benefit seen with IGF-1 treatment as well as Akt or BD110 expression.

Discussion

We examined the effects of intracellular signaling molecules downstream of the IGF-1 receptor in an in vitro model of transient cardiomyocyte hypoxia. We found that IGF-1 activates PI 3-kinase and Akt in cardiomyocytes. Pharmacological studies suggested that PI 3-kinase was involved in the ability of IGF-1 to inhibit apoptosis of hypoxic cardiomyocytes, and we used adenoviral gene transfer to directly examine the ability of activated PI 3-kinase or its downstream target, Akt, to modulate apoptosis. Expression of activated forms of either PI 3-kinase or Akt in cardiomyocytes was sufficient to inhibit hypoxia-induced apoptosis in vitro. To the best of our knowledge, this is the first demonstration of a critical role for these signaling molecules in cardiomyocyte survival.

Interestingly, Ad.BD110 only incompletely prevented hypoxia-induced DNA fragmentation at an MOI of 10 pfu/cell and killed cardiomyocytes at high MOI. Incomplete protection could reflect either low transduction efficiency with population heterogeneity, the inability of this signaling pathway to completely protect against apoptosis, or inadequate activation of this signaling pathway. The last explanation appears to be the most likely. The EGFP coexpression cassette revealed that virtually all the cardiomyocytes were transduced by Ad.BD110 at an MOI of 10 pfu/cell, consistent with previous reports. We examined the effects of intracellular signaling molecules downstream of the IGF-1 receptor in an in vitro model of transient cardiomyocyte hypoxia. We found that IGF-1 activates PI 3-kinase and Akt in cardiomyocytes. Pharmacological studies suggested that PI 3-kinase was involved in the ability of IGF-1 to inhibit apoptosis of hypoxic cardiomyocytes, and we used adenoviral gene transfer to directly examine the ability of activated PI 3-kinase or its downstream target, Akt, to modulate apoptosis. Expression of activated forms of either PI 3-kinase or Akt in cardiomyocytes was sufficient to inhibit hypoxia-induced apoptosis in vitro. To the best of our knowledge, this is the first demonstration of a critical role for these signaling molecules in cardiomyocyte survival.

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activation of Akt or PI 3-kinase was probably mediated through caspase inhibition and tested the effects of a direct caspase inhibitor, z-VAD.fmk, in this model. In hypoxic cardiomyocytes, z-VAD.fmk blocked apoptosis to an extent comparable to the maximal benefit achieved through adenoviral activation of the PI 3-kinase/Akt signaling pathway. These results suggest that caspase inhibition could account for the benefits seen with adenoviral gene transfer of activated PI 3-kinase or Akt but do not establish a direct connection between these pathways in cardiomyocytes. It also remains to be seen whether the importance of these pathways will be conserved with other proapoptotic stimuli as well as in other in vitro and in vivo models.

Critical unanswered questions include the fate and functional capacity of cardiomyocytes in which apoptosis has been inhibited through activation of these signaling molecules. It is possible that these cells will simply die through another mechanism, such as necrosis or oncosis. Even if such cardiomyocytes survive, they may not function normally. Many pathways may contribute to hypoxia-induced myocyte dysfunction, and thus, inhibition of apoptosis could increase survival without improving the function of the surviving cells. Remarkably, the majority of cardiomyocytes transduced with BD110 at an MOI of 10 pfu/cell or myr-Akt at higher MOI continued to contract spontaneously and vigorously even after 24 hours of hypoxia, whereas virtually all cells transduced with the control virus stopped beating after hypoxia (data not shown). Rigorously defining the functional implications of specific apoptosis-related signaling pathways will be an important focus of future studies that should be facilitated by the gene transfer approach illustrated in the present study.

The present data are consistent with previous studies documenting the ability of IGF-1 to block apoptosis in many cell types, including cardiomyocytes, and to mediate beneficial effects in animal models of myocardial ischemia, whether delivered through peptide injection or transgenically overexpressed in the heart. Although it is tempting to speculate that activation of PI 3-kinase and Akt may play a role in the IGF-1–mediated benefits seen in these animal models, IGF-1 has systemic effects that could confound such studies. The recombinant vectors described in this report should provide useful tools for examining the relative contribution of local activation of PI 3-kinase and Akt to the observed benefits of hypoxia-induced DNA fragmentation in a dose-dependent manner at MOI from 10 to 80 pfu/cell (P<0.0001 and n=12 for each, vs Ad.EGFP-infected cultures). At an MOI of 80 pfu/cell, Ad.myr-Akt-HA reduced hypoxia-induced DNA fragmentation by 90.9±1.4%. Data are pooled from 4 experiments, with each condition tested indicated number of times. c, z-VAD.fmk prevents cardiomyocyte apoptosis. Uninfected cardiomyocytes were treated with z-VAD.fmk or vehicle alone, subjected to 24 hours of hypoxia, and analyzed for apoptosis by use of histone-associated DNA fragment ELISA. Results are expressed as percentage of that seen with uninfected, hypoxic cultures. At concentrations of 20 (n=9), 40 (n=6), or 80 (n=3) μmol/L, z-VAD.fmk reduced myocyte DNA fragmentation by up to 93.5±2.0% (P<0.0001 for all, vs uninfected cultures). Data are pooled from 3 experiments with each condition tested indicated number of times.

Figure 5. Pathways controlling apoptosis of hypoxic cardiomyocytes. a, PI 3-kinase is sufficient to prevent apoptosis of hypoxic cardiomyocytes. Cardiomyocyte cultures were infected with Ad.BD110 or Ad.EGFP at an MOI of 10 pfu/cell, or left uninfected. Forty-six hours later, cultures were subjected to hypoxia for 24 hours, and then apoptosis was assessed by ELISA for histone-associated DNA fragments, as described above. Results are expressed as percentage of that seen with Ad.EGFP-infected, hypoxic cardiomyocytes. Constitutively active form of PI 3-kinase, BD110, significantly reduced cardiomyocyte apoptosis (61±4.6% less, P<0.0001 vs Ad.EGFP-infected, hypoxic cultures, n=19). Simultaneous treatment with specific PI 3-kinase inhibitor LY294002 (10 μmol/L) abolished antiapoptotic effect of BD110 (P<0.0001 vs BD110 alone, n=7). Data are pooled from 7 experiments, with each condition tested indicated number of times. b, myr-Akt-HA prevents cardiomyocyte apoptosis. As above, cells infected with Ad.myr-Akt-HA or Ad.EGFP were subjected to 24 hours of hypoxia and analyzed as above. In hypoxic cultures, Ad.myr-Akt-HA significantly inhibited hypoxia-induced DNA fragmentation in a dose-dependent manner at MOI from 10 to 80 pfu/cell (P<0.0001 and n=12 for each, vs Ad.EGFP-infected cultures). At an MOI of 80 pfu/cell, Ad.myr-Akt-HA reduced hypoxia-induced DNA fragmentation by 90.9±1.4%. Data are pooled from 4 experiments, with each condition tested indicated number of times. c, z-VAD.fmk prevents cardiomyocyte apoptosis. Uninfected cardiomyocytes were treated with z-VAD.fmk or vehicle alone, subjected to 24 hours of hypoxia, and analyzed for apoptosis by use of histone-associated DNA fragment ELISA. Results are expressed as percentage of that seen with uninfected, hypoxic cultures. At concentrations of 20 (n=9), 40 (n=6), or 80 (n=3) μmol/L, z-VAD.fmk reduced myocyte DNA fragmentation by up to 93.5±2.0% (P<0.0001 for all, vs uninfected cultures). Data are pooled from 3 experiments with each condition tested indicated number of times.
IGF-1 in vivo. Understanding the role of specific pathways in cardiomyocyte apoptosis may help identify targets and novel therapeutic strategies for intervention in conditions such as myocardial infarction and heart failure.

Acknowledgments
This work was supported in part by grants from the NIH (HL-50361 and HL-57623 to Dr Hajjar; HL-59521 and HL-61557 to Dr Rosenzweig). Dr Rosenzweig is an Established Investigator of the American Heart Association. Dr Matsui is supported by an American Heart Association Fellowship. The authors thank Drs Thomas Force, Kazuyoshi Yonezawa, and Kenta Hara for their insightful suggestions. The technical assistance of Drs Zhaobin Kang and Quifen Qui is greatly appreciated, as is Paula Kaltofen’s help with manuscript preparation.

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_Circulation_. 1999;100:2373-2379
doi: 10.1161/01.CIR.100.23.2373

_Circulation_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the
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