Akt Promotes Survival of Cardiomyocytes In Vitro and Protects Against Ischemia-Reperfusion Injury in Mouse Heart

Yasushi Fujio, MD, PhD; Thao Nguyen, BS; Detlef Wencker, MD; Richard N. Kitsis, MD; Kenneth Walsh, PhD

Background—IGF-1 has been shown to protect myocardium against death in animal models of infarct and ischemia-reperfusion injury. In the present study, we investigated the role of the IGF-1–regulated protein kinase Akt in cardiac myocyte survival in vitro and in vivo.

Methods and Results—IGF-1 promoted survival of cultured cardiomyocytes under conditions of serum deprivation in a dose-dependent manner but had no effect on cardiac fibroblast survival. The cytoprotective effect of IGF-1 on cardiomyocytes was abrogated by the phosphatidylinositol 3-kinase (PI 3-kinase) inhibitor wortmannin. Wortmannin had no effect on cardiomyocyte viability in the absence of IGF-1. IGF-1–mediated cytoprotection correlated with the wortmannin-sensitive induction of Akt protein kinase activity. To examine the functional consequences of Akt activation in cardiomyocyte survival, replication-defective adenoviral constructs expressing wild-type, dominant-negative, and constitutively active Akt genes were constructed. Transduction of dominant-negative Akt blocked IGF-1–induced survival but had no effect on cardiomyocyte survival in the absence of IGF-1. In contrast, transduction of wild-type Akt enhanced cardiomyocyte survival at subsaturating levels of IGF-1, whereas constitutively active Akt protected cardiomyocytes from apoptosis in the absence of IGF-1. After transduction into the mouse heart in vivo, constitutively active Akt protected against myocyte apoptosis in response to ischemia-reperfusion injury.

Conclusions—These data are the first documentation that Akt functions to promote cellular survival in vivo, and they indicate that the activation of this pathway may be useful in promoting myocyte survival in the diseased heart. (Circulation. 2000;101:660-667.)

Key Words: myocytes ■ apoptosis ■ Akt ■ ischemia ■ reperfusion

Myocyte loss, both necrotic and apoptotic, is a feature of many heart pathological conditions. Because adult cardiomyocytes possess minimal capacity to reenter the cell cycle, the control of myocyte loss through suppression of cell death pathways represents a logical strategy to prevent heart failure. It has been shown that transgenic mice overexpressing insulin-like growth factor-1 (IGF-1) display less myocyte apoptosis after myocardial infarction and have reduced wall stress and ventricular dilatation. IGF-1 administered to rats also reduces myocardial apoptosis and injury in response to ischemia followed by reperfusion. More recently, it has been shown that IGF-1 functions as a cell survival factor for cultured cardiac myocytes exposed to doxorubicin in the absence of serum. Therefore, analysis of the IGF-1 signaling pathways, especially with respect to cell survival, may lead to the development of clinical strategies to treat heart disease.

IGF-1 stimulates several signaling pathways, including phosphatidylinositol (PI) 3-kinase. Likewise, PI 3-kinase has a number of downstream targets, including the Akt protooncogene, a serine/threonine protein kinase that is activated by the inositol lipid products of the PI 3-kinase reaction. Activation of Akt has been shown to promote survival of some cell types, including neurons and lymphocytes. In other cell types, however, activation of Akt or inhibition of PI 3-kinase has no effect on survival. Here, we investigated the role of PI 3-kinase–dependent activation of Akt in IGF-1–mediated cardiomyocyte survival in culture and tested whether constitutive activation of Akt could protect myocytes in vivo from reperfusion injury.

Methods

Reagents, Cell Culture, and Akt Assays

IGF-1 was purchased from Gibco BRL. Wortmannin was obtained from Sigma. Anti-Akt antibody and its corresponding immunogenic peptide came from Santa Cruz Biotechnology. Anti-hemagglutinin (HA) antibodies were from Boehringer Mannheim or Santa Cruz. Anti–β-galactosidase antibody was from Chemicon. Protein...
G-agarose was purchased from Boehringer Mannheim. Peroxidase-conjugated anti-mouse IgG was obtained from Amersham Life Science. Primary cultures of neonatal cardiac myocytes were prepared as described previously.2 Cultures were enriched with cardiac myocytes by preplating for 60 minutes to eliminate fibroblasts. More than 90% of the cells were identified as cardiac myocytes by immunocytochemistry with anti-sarcomeric α-actin (Sigma). Attached cells were passaged once and used as the fibroblast fraction. Cell viability was determined with the trypan blue exclusion assay.11 Surviving cell number is expressed relative to the initial cell number. Alternatively, cells were scored for condensed or pyknotic nuclei after fixation in 3.7% formaldehyde and staining with Hoechst 33342. In some assays, cells were pretreated with wortmannin for 30 minutes and incubated in the medium containing wortmannin in the presence or absence of IGF-1. Dimethyl sulfoxide was used as the vehicle for the wortmannin at a concentration of 0.1% (vol/vol). Control cultures received the vehicle alone. Akt kinase assays were performed on anti-Akt or anti-HA immunoprecipitates from cell lysates as described previously with histone H2B.13 Immunoblots were performed as described previously.14

DNA Ladder and TUNEL Analyses With Cultured Myocytes

DNA ladder experiments were performed as described previously.15 In brief, DNA was purified by the NaI method after treatment with proteinase K and RNase A. DNA (10 μg) was separated on agarose gel electrophoresis. For immunofluorescent microscopy, cells were fixed with 3.7% formaldehyde in PBS for 20 minutes. After 2 washes with PBS, cells were permeabilized with 0.2% Triton X-100 and washed 3 more times with PBS. After incubation with the first antibody (anti-α-sarcomeric actin, Sigma) at 37°C for 60 minutes, cells were washed 3 times in PBS, then incubated with secondary antibody (rhodamine-labeled anti-mouse IgG, Pierce) at 37°C for 60 minutes. This was followed by staining with Hoechst 33342. Finally, terminal deoxynucleotidyl transferase (TdT)–mediated dUTP nick end-labeling (TUNEL) staining was performed for detection of apoptotic cells according to the manufacturer’s protocol (Boehringer).

Adenoviral Constructs

Wild-type, dominant-negative, and constitutively active forms of Akt were tagged with the HA epitope to assess whether adenovirus-mediated myrAkt gene delivery inhibited ischemia-reperfusion–induced myocyte apoptosis in vivo. To document the cytoprotective effects of IGF-1 under the conditions of our assays, neonatal rat cardiac myocytes were cultured in serum-free medium in the presence or absence of IGF-1, and cell viability was determined by trypan blue exclusion at various time points (Figure 1A). In the absence of IGF-1, cardiac myocyte cultures displayed a time-dependent decrease in viability with >50% loss of live cells by 72 hours. However, cardiac myocyte viability was maintained in the presence of IGF-1. In contrast to the myocyte fraction of cells, passaged nonmyocyte cells (fibroblasts) prepared from neonatal heart were resistant to death induced by serum deprivation (Figure 1B). The insensitivity of cardiac fibroblasts to serum deprivation–induced cell death has been reported previously.19 Cardiomyocyte protection was dependent on the dose of IGF-1 (Figure 1C). IGF-1–induced cytoprotection was abrogated by treatment with 200 nmol/L wortmannin (Figure 1D), suggesting that the survival effects of IGF-1 are regulated by effector proteins down-stream of PI 3-kinase. In the absence of IGF-1, wortmannin had no effect on cardiomyocyte viability.

Results

IGF-1 Protects Cardiac Myocytes Under Conditions of Serum Deprivation

Serum deprivation induces apoptosis in cultured cardiomyocytes.8,19 To document the cytoprotective effects of IGF-1 under the conditions of our assays, neonatal rat cardiac myocytes were cultured in serum-free medium in the presence or absence of IGF-1, and cell viability was determined by trypan blue exclusion at various time points (Figure 1A). In the absence of IGF-1, cardiac myocyte cultures displayed a time-dependent decrease in viability with >50% loss of live cells by 72 hours. However, cardiac myocyte viability was maintained in the presence of IGF-1. In contrast to the myocyte fraction of cells, passaged nonmyocyte cells (fibroblasts) prepared from neonatal heart were resistant to death induced by serum deprivation (Figure 1B). The insensitivity of cardiac fibroblasts to serum deprivation–induced cell death has been reported previously.19 Cardiomyocyte protection was dependent on the dose of IGF-1 (Figure 1C). IGF-1–induced cytoprotection was abrogated by treatment with 200 nmol/L wortmannin (Figure 1D), suggesting that the survival effects of IGF-1 are regulated by effector proteins down-stream of PI 3-kinase. In the absence of IGF-1, wortmannin had no effect on cardiomyocyte viability. To provide additional evidence that these treatments influence cell death, cultures were fixed and stained with Hoechst 33342 and anti-HA or anti–β-galactosidase antibody. The same sections were also stained with TUNEL to detect apoptotic cells according to the manufacturer’s protocol (Boehringer).

Secondary antibodies labeled with rhodamine (Pierce) were used for the detection of HA-Akt–positive or β-galactosidase–positive cells. Myocyte identity was indicated by staining with anti-α-sarcomeric actin antibody, by use of Cy5-labeled secondary antibody (Accurate Chemical and Scientific Corp). Alternatively, sections were stained with X-gal. For animals subjected to ischemia-reperfusion injury, sections corresponding to the left ventricular free wall were analyzed by first viewing the fluorescein channel to locate the region of injury as indicated by TUNEL-positive nuclei. TUNEL-positive microscopic fields were then assessed for cells staining positive for HA-Akt or β-galactosidase transgenes. Once transgene-positive high-power fields (×400) were identified, all myocytes within the field (∼200) were scored for transgene expression and TUNEL staining. Two or 3 randomly chosen transgene-positive, high-power fields were examined for each mouse. The percentages of transgene-positive and -negative myocytes that were TUNEL-positive were compared in each group to assess whether adenovirus-mediated myrAkt gene delivery inhibited ischemia-reperfusion–induced myocyte apoptosis in vivo. For sham-operated animals, sections of the left ventricular wall were first scored for HA-tagged Akt, then for TUNEL staining in sections corresponding to the left ventricular free wall. A separate group of mice was subjected to sham operation, coronary occlusion, or coronary occlusion followed by reperfusion. Regions of myocardial perfusion under each condition were assessed by staining with Evans blue dye (Sigma) as described previously.18

Statistical Analysis

All data were evaluated with a 2-tailed, unpaired Student’s t test and expressed as the mean±SEM. A value of P<0.05 was considered significant.

Adenovirus Transduction In Vivo, Transgene Detection, TUNEL, and Evans Blue Staining

Eleven C57BL/6J mice 6 to 8 weeks old were anesthetized with methoxyflurane and ventilated with a rodent respirator attached to a nose cone. A mixture of Adeno-myrAkt (6×1010 pfu/mouse) plus Adeno-β-gal (1×1010 pfu/mouse) or Adeno-β-gal (7×1010 pfu/mouse) alone in 15 μL of PBS with 10% glycerol was quickly injected into the apex and anterolateral wall of the heart with a 26-gauge needle.17 Twenty hours later, animals were subjected to sham operation or left coronary artery occlusion for 45 minutes, followed by 4 hours of reperfusion. Hearts were frozen immediately after euthanasia, and 10-μm sections were prepared with a Microm HM505E (Zeiss). Sections were fixed with 4% paraformaldehyde in PBS for 15 minutes and stained with Hoechst 33342 and anti-HA or anti–β-galactosidase antibody. The same sections were also stained with DNA ladder and TUNEL analyses with cultured myocytes.
Wortmannin-Sensitive Activation of Akt by IGF-1
Akt activity was determined in cultures of serum-deprived cardiac myocytes in the presence or absence of IGF-1. Akt protein kinase activity was assessed in immunoprecipitates of lysates of cultured cardiomyocytes with an antibody that reacts specifically with Akt but not Akt2, which is also expressed in the heart.20 Immunoprecipitated Akt activity was assayed with histone H2B as substrate. In addition, cell lysates were immunoprecipitated with anti-Akt antibody in the presence or absence of the immunogenic competitor peptides. Akt-associated phosphorylation of histone H2B was induced by IGF-1 (Figure 2A). The IGF-1–induced kinase activity was largely attributable to Akt, because kinase activity was reduced when lysates were immunoprecipitated in the presence of the immunogenic competitor peptide. The effect of wortmannin on IGF-1–induced Akt activity was also analyzed. Inclusion of 200 nmol/L wortmannin in the culture medium completely blocked IGF-1–activated Akt kinase activity (Figure 2B). These data suggest that Akt acts downstream of PI 3-kinase in response to IGF-1 in cardiomyocytes.

Adenoviral Transfection of Wild-Type, Dominant-Negative, or Constitutively Active Akt in Cultured Cardiomyocytes
To determine the functional significance of Akt activation in IGF-1–stimulated cardiac myocyte cultures, replication-defective adenoviral vectors expressing wild-type and mutant forms of Akt fused to the HA epitope were constructed (Figure 3A). The mutant Akt (T308A, S473A) cannot be activated by phosphorylation,21 and it functions in a dominant-negative manner.22 Cardiac myocytes were infected with adenovirus vectors at an MOI of 25, and expression of exogenous Akt proteins was demonstrated by Western immunoblot analysis with anti-HA antibody (Figure 3B). To analyze the kinase activities of adenovirally encoded Akt proteins, parallel serum-deprived cultures were incubated in the presence or absence of IGF-1. Specific Akt protein kinase activity was detected in extracts from cells infected with the wild-type Akt vector in the presence, but not the absence, of IGF-1 stimulation (Figure 3C). The vector expressing dominant-negative Akt was inactive with regard to kinase activity. In contrast, cells infected with the vector expressing constitutively active Akt displayed high levels of kinase activity in the presence or absence of IGF-1. Cardiac myocyte cultures were infected with an adenoviral vector expressing dominant-negative Akt mutant to test whether Akt is essential for IGF-1–mediated survival. Infec- tion with the dominant-negative Akt vector largely eliminated the increase in cell survival resulting from the inclusion of 25 or 50 ng/mL IGF-1 in the culture medium (Figure 4A). In the absence of IGF-1, Adeno-dnAkt did not affect cardiomyocyte survival, indicating the specificity of dnAkt action. The control adenoviral construct expressing β-galactosidase...
Constitutively active Akt protects cardiomyocytes from apoptosis. Cardiomyocytes were transfected with β-gal or constitutively active Akt (myrAkt). Cells were cultured in serum-deprived condition for 48 hours, and DNA fragmentation was analyzed. M indicates marker of 123-bp DNA ladder.

myocyte cultures infected with Adeno-βgal displayed the nucleosome spacing ladder after gel electrophoresis that is indicative of apoptosis (Figure 4C). This DNA ladder was diminished, but not eliminated, in serum-deprived cultures infected with Adeno-myrAkt, suggesting that this agent inhibits apoptotic cell death in the absence of exogenous IGF-1 stimulation.

TUNEL analyses were performed to examine the effects of the different Akt-expressing adenovirus vectors on cardiomyocyte survival in vitro. Cells were infected with the indicated adenovirus vector or mock-infected overnight, and then cultured in the serum-depleted medium in the presence of the indicated concentrations of IGF-1 for 48 hours (Figure 5). Apoptotic cells were identified by TUNEL and Hoechst staining, and staining with anti-α-sarcomeric actin was performed to confirm myocyte identity (Figure 5A). As shown in Figure 5B, increasing concentrations of IGF-1 reduced the frequency of myocyte apoptosis in mock- and Adeno-βgal–transfected cells. Infection with adenovirus expressing dominant-negative Akt blocked the protective effects of IGF-1. Infection with adenovirus expressing wild-type Akt facilitated IGF-1–mediated cardiomyocyte survival at low IGF-1 concentrations, whereas infection with adenovirus expressing constitutively active Akt reduced the frequency of myocyte apoptosis in the absence of IGF-1 or at low concentrations of IGF-1. Of note, Adeno-myrAkt did not have an additive effect on survival at saturating IGF-1 (50 ng/mL), suggesting further that Akt activation is an integral feature of the IGF-1 survival pathway in cardiomyocytes.

Constitutively Active Akt Protects Myocardium From Apoptosis After Reperfusion Injury

Reperfusion of ischemic myocardium is reported to accelerate myocyte apoptosis, and IGF-1 can protect myocardium...
Akt is necessary and sufficient for IGF-1–mediated myocyte survival. A, Representative image of TUNEL (TNL)-positive cardiac myocytes in culture. Cardiac myocytes were cultured in absence of serum for 48 hours. Cells were stained with Hoechst, TUNEL, and anti–α-sarcomeric actin antibody. B, Effects of Akt-expressing adenovirus vectors on cardiac myocyte survival. Cultured cells were infected with mock (no virus) or adenovirus vectors expressing β-galactosidase, wild-type, dominant-negative, or constitutively active Akt (β-gal, wtAkt, dnAkt, and myrAkt, respectively). After infection, cells were cultured in presence of indicated concentrations of IGF-1 for 48 hours. Myocyte viability was assessed by staining with Hoechst and TUNEL. Cultured cells were identified as cardiomyocytes by staining with anti–α-sarcomeric actin antibody. Data are shown as mean ± SEM (n = 4).
under these conditions. Thus, we investigated whether expression of constitutively active Akt could protect murine myocardium from reperfusion injury (Figure 6A). In this model, adenoviral constructs were injected directly into the apical and anterolateral free wall of the heart, a region that shows a large perfusion defect after occlusion of the left coronary artery as determined by staining with Evans blue dye (Figure 6B). Evans blue staining was uniform after removal of the ligature, consistent with restored perfusion (not shown). Direct adenovirus injection resulted in transgene expression localized to cells adjacent to the track of the needle and confined to myocardium subject to hypoperfusion (Figure 6C). To examine the effects of constitutive Akt expression on myocyte viability in vivo, mouse hearts were injected with Adeno-myrAkt or the Adeno-βgal control vector 20 hours before ischemia-reperfusion injury or sham operation. Sections of heart were analyzed for transgene expression by immunohistochemical detection of the HA epitope (myrAkt) or β-galactosidase, and cell death was assessed by TUNEL staining (Figure 6D and 6E). Cells with apoptotic nuclei were identified as cardiac myocytes by staining with anti-α-sarcomeric actin antibody (not shown). High-power microscopic fields (200 Hoechst-positive myocytes per field) were identified that contained transgene-positive cells. Systematic analyses of these fields revealed that myocardial cells expressing HA-tagged myrAkt displayed significantly fewer TUNEL-positive nuclei than immediately adjacent cells that were negative for transgene expression. Consequently, we investigated whether ex-
expression (Figure 6F). In contrast to cultured myocytes, no obvious morphological alterations were apparent in the cells expressing myrAkt (not shown). Mouse hearts injected with Adeno-βgal and subjected to ischemia-reperfusion injury displayed no differences in TUNEL-positive staining between cells that were positive or negative for β-galactosidase transgene expression (Figure 6F). TUNEL-positive cells were not identified in either HA-myrAkt–positive or –negative cells of sham-operated animals.

Discussion

Here, we report that IGF-1–mediated survival correlates with the wortmannin-sensitive activation of the protein kinase Akt in cultured cardiomyocytes. The functional significance of Akt activation was demonstrated by use of adenovirus-mediated transfer of wild-type and mutant Akt genes to cardiomyocytes. Dominant-negative Akt abolished IGF-1–mediated cardiac myocyte survival, whereas overexpression of wild-type or constitutively active Akt genes promoted cardiac myocyte survival. These data suggest that the PI 3-kinase–dependent activation of Akt is a key step in the IGF-1 signaling pathway that protects cardiomyocytes from death.

Previous studies have shown that permanent or temporary occlusion of the left coronary artery of the mouse results in myocyte apoptosis exclusively in the left ventricular free wall.12,15,24 Thus, we reasoned that the direct injection of the adenovirus expressing constitutively active Akt could minimize myocyte apoptosis in the affected region of the heart. Furthermore, because adenovirus-mediated gene transfer permits acute delivery of recombinant protein, we reasoned that this model would provide a more direct test of protein function than transgenic animals, in which secondary effects of chronic expression can confound analyses. After ischemia-reperfusion injury, the numbers of TUNEL-positive myocyte nuclei in myocardial cells expressing constitutively active Akt were decreased compared with adjacent, nontransduced myocardial cells. Furthermore, the observed decrease in cell death could be attributed to the Akt transgene, because hearts injected with Adeno-βgal revealed no difference in frequency of TUNEL staining in the β-galactosidase–positive and –negative cells. Although serum deprivation in vitro simulates a component of ischemia, ischemia-reperfusion is a more complex stimulus, involving deprivation of growth/survival factors, accumulation of metabolic waste products, changes in mechanical factors, and generation of toxic substances, such as reactive oxygen species.1,4,6,7 Our finding that Akt inhibits myocyte death induced not only by serum deprivation but also by ischemia-reperfusion in vivo suggests that this protein lies at the crossroads of multiple apoptotic stimuli activated during myocardial injury.

Signaling pathways that may also converge on Akt include the cardiomyocyte survival factors that function through the glycoprotein 130 (gp130) signal-transducing protein. For example, CT-119 and LIF25 can activate Akt in a wortmannin-sensitive manner,10 but the role of Akt in gp130-mediated survival has yet to be demonstrated. It might also be relevant that insulin administered with glucose and potassium (GIK therapy) can slow the rate of ischemic cell death after acute myocardial infarction, thereby reducing patient mortality.27 Like IGF-1, insulin can activate Akt through its interaction with the IGF-1 receptor.21 Therefore, Akt may function at a nodal point to coordinate growth factor signaling with myocyte survival and thus may represent a logical target for pharmacological or genetic therapies to promote myocyte survival during heart failure.

Acknowledgments

This work was supported by NIH grants AG-15052, HL-50692, and AR-40197 to Dr Walsh and HL-60665 and HL-61550 to Dr Kitsis. Dr Kitsis is the Charles and Tamara Krasne Faculty Scholar in Cardiovascular Research of the Albert Einstein College of Medicine. Dr Fujio is supported by the Study Group of Molecular Cardiology.

References


Akt Promotes Survival of Cardiomyocytes In Vitro and Protects Against Ischemia-Reperfusion Injury in Mouse Heart
Yasushi Fujio, Thao Nguyen, Detlef Wencker, Richard N. Kitsis and Kenneth Walsh

Circulation. 2000;101:660-667
doi: 10.1161/01.CIR.101.6.660

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circ.ahajournals.org/content/101/6/660

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation is online at:
http://circ.ahajournals.org/subscriptions/