Nuclear Targeting of Akt Enhances Kinase Activity and Survival of Cardiomyocytes

Isao Shiraishi, Jaime Melendez, Youngkeun Ahn, Maryanne Skavdahl, Elizabeth Murphy, Sara Welch, Erik Schaefer, Kenneth Walsh, Anthony Rosenzweig, Daniele Torella, Daria Nurzynska, Jan Kajstura, Annarosa Leri, Piero Anversa, Mark A. Sussman

Abstract—Heart failure is associated with death of cardiomyocytes leading to loss of contractility. Previous studies using membrane-targeted Akt (myristolated-Akt), an enzyme involved in antiapoptotic signaling, showed inhibition of cell death and prevention of pathogenesis induced by cardiomyopathic stimuli. However, recent studies by our group have found accumulation of activated Akt in the nucleus, suggesting that biologically relevant target(s) of Akt activity may be located there. To test this hypothesis, a targeted Akt construct was created to determine the antiapoptotic action of nuclear Akt accumulation. Nuclear localization of the adenovirally encoded Akt construct was confirmed by confocal microscopy. Cardiomyocytes expressing nuclear-targeted Akt showed no evidence of morphological remodeling such as altered myofibril density or hypertrophy. Nuclear-targeted Akt significantly elevated levels of phospho-Akt and kinase activity and inhibited apoptosis as effectively as myristolated-Akt in hypoxia-induced cell death. Transgenic overexpression of nuclear-targeted Akt did not result in hypertrophic remodeling, altered cardiomyocyte DNA content or nucleation, or enhanced phosphorylation of typical cytoplasmic Akt substrates, yet transgenic hearts were protected from ischemia-reperfusion injury. Gene array analyses demonstrated changes in the transcriptional profile of Akt/nuc hearts compared with nontransgenic controls distinct from prior characterization of Akt expression in transgenic hearts. Collectively, these experiments show that targeting of Akt to the nucleus mediates inhibition of apoptosis without hypertrophic remodeling, opening new possibilities for therapeutic applications of nuclear-targeted Akt to inhibit cell death associated with heart disease. (Circ Res. 2004;94:884-891.)

Key Words: Akt ■ apoptosis ■ nuclear ■ cardiomyocytes ■ transgenic

Programmed cell death, also known as apoptosis, occurs in a wide variety of cardiovascular disorders and is now recognized as a fundamental process that contributes to deterioration of cardiac function. Controlling myocardial cell loss by enhancing survival signal cascades leading to inhibition of apoptosis could be a useful strategy for slowing development of heart failure. Among numerous signaling pathways involved in regulation of cell survival cascades, the serine-threonine kinase Akt/PKB plays a crucial role. Cytosolic Akt is activated by phosphorylation mediated by PI3-kinase and 3-phosphoinositide– dependent kinase (PDK) that are stimulated by growth factors such as IGF-1 at cell membrane. After activation, Akt accumulates in the nucleus, phosphorylates multiple protein substrates, and is thought to regulate gene transcription. Akt activation also promotes glucose transport, glycogen and protein synthesis, and withdrawal from the cell cycle. However, cardiovascular-related Akt research has been predominantly fueled by the ability of activated Akt to enhance cell survival by promoting signaling cascades that lead to inhibition of cardiomyocyte apoptosis in vitro and in vivo. Akt-associated pathways and substrate proteins leading to antiapoptotic effects and cellular survival are complex, but recent studies suggest that biologically relevant targets of phospho-Akt action are localized in the nucleus. We previously reported a 2-fold higher level of phospho-Akt in cardiomyocyte nuclei of premenopausal women relative to men or postmenopausal women, and that estrogenic stimulation promotes accumulation of activated Akt in the nucleus. Higher levels of nuclear Akt activity in women and in estrogen-treated cardiomyocytes could partially account for the gender-associated differential in development of heart failure in women compared with middle-aged men. Protection from apoptotic stimuli in cardiomyocytes has been demonstrated using adenovirally mediated gene transfer.
of constitutively activated (myristolated) Akt. However, the constitutively activated form of Akt preferentially localizes at the cell membrane, resulting in supraphysiological levels of kinase activity. Constitutively activated forms of PI3-kinase or Akt induce oncogenic transformation in fibroblasts, as well as cardiac hypertrophy and/or failure, raising issues of feasibility and safety concerning the utility of Akt for therapeutic treatment of heart disease.

Antiapoptotic therapy for cardiomyocytes depends on successful inhibition of cell death without promoting activation of compensatory pathways leading to deleterious hypertrophic remodeling or cellular growth. Because biologically relevant target proteins of Akt kinase associated with inhibition of apoptosis may be localized in the nucleus, we explored the hypothesis that protection could be mediated by a nuclear-targeted Akt protein construct (Akt/nuc). Our results demonstrate the cytoprotective effects of Akt/nuc and support the potential interventional use of Akt/nuc as interventional treatment to combat apoptosis leading to heart failure.

Materials and Methods
Experimental methods are detailed in the expanded Materials and Methods in the online data supplement (available at http://circres.ahajournals.org). Briefly, an adenovirus expressing mouse cellular Akt upstream of nuclear localization signals (Akt/nuc) was created by PCR-based cloning technique with AdMax (MicroBix) Cre-mediated site-specific recombination system. Cell culture, immunohistochemistry and confocal microscopy, nuclear extract and cytosol preparation, immunoblot and kinase assays were previously described. In vitro apoptosis was induced by staurosorpin, 2-deoxyglucose, or hypoxia and was quantified by TUNEL staining. The transgene for creation of nuclear-targeted Akt mice was created by subcloning nuclear-targeted Akt cDNA construct downstream of the mouse α-myosin heavy chain gene promoter. The mouse model of myocardial infarction and Verification of Expression

Results
Construction of Nuclear-Targeted Akt Adenovirus and Verification of Expression
Akt was targeted to the nucleus by cloning full-length wild-type mouse Akt cDNA upstream of three in-frame nuclear localization signal (NLS) sequences. Akt/nuc protein was distinguished from endogenous Akt by a 3′ myc-tag (Figure 1A). The combined Akt-NLS-myc was subsequently subcloned into an adenoviral expression vector for efficient introduction of Akt/nuc into cardiomyocytes. Infection of neonatal rat cardiomyocyte cultures leads to nuclear accumulation of the adenovirally encoded Akt/nuc as demonstrated by both confocal microscopy (Figure 1B) and immunoblot with anti-myc antibody (Figure 1C).

Phosphorylated Akt/nuc Accumulates in the Nucleus of Infected Cardiomyocytes
Localization of phospho-Akt was performed on infected cardiomyocytes to determine the phosphorylation status of Akt at residue T308 corresponding to activation. Accumulation of Akt/nuc signal was confirmed by confocal microscopy of infected cardiomyocytes labeled with anti-Akt antibody (Figures 1D3 and 1D4) versus cells expressing nuclear localized β-gal as a negative control (Figures 1D1 and 1D2). Comparably prepared cells labeled with anti–phospho-Akt antibody also show nuclear immunoreactivity (Figures 1D6 and 1D7; green), consistent with activation of the adenovirally encoded Akt/nuc. In comparison, infection with either Akt/wt or Akt/myr resulted in predominantly cytosolic and/or membrane-associated Akt immunoreactivity with minimal nuclear labeling (data not shown).

Nuclear Extracts Show Elevation of Akt and Phospho-Akt After Infection With Akt/nuc Adenovirus
Nuclear accumulation of Akt/nuc prompted quantitative analysis to compare levels of Akt and phospho-Akt between
cardiomyocyte cultures expressing Akt/nuc versus adenovirus encoding nuclear-localized β-gal as a control for nonspecific effects. Nuclear-enriched fractions were prepared and subjected to immunoblot analysis using antibodies to total Akt as well as phosphorylation-specific antibodies directed against sites T308 and S473 that correlate with Akt activation (Figures 1E). Total Akt level in nucleus was substantially higher in Akt/nuc-expressing cultures compared with β-gal (64.2±21.9-fold). The same extracts also showed increases for both phospho-Akt308 and phospho-Akt473 level (2.0±0.4- and 4.5±1.4-fold, respectively) compared with β-gal expressing cardiomyocytes. All of the observed increases in Akt and phospho-Akt signal resulting from expression of Akt/nuc were highly significant (P<0.01).

Akt/nuc Increases Kinase Activity in the Nucleus
Phosphorylation of Akt correlates with kinase activation, so substrate phosphorylation analysis was performed on nuclear extracts of Akt/nuc-expressing cardiomyocyte cultures to confirm elevation of kinase activity. Akt kinase activity in immunoprecipitates from nuclear extracts (Figure 1F) shows significantly increased phospho-GSK signal (2.2±0.18-fold) after incubation with extracts from Akt/nuc expressing cardiomyocytes relative to control cultures expressing nuclear-localized β-gal.

Akt/nuc Does Not Induce Morphological Remodeling Associated With Hypertrophy
Cardiomyocyte cell area measurements were taken to determine whether accumulation of Akt/nuc causes cardiomyocyte enlargement as reported for myristolated Akt (Figure 2). Expression of myristolated Akt significantly enlarged cardiomyocytes compared with cells that were uninfected (182%, P≤0.001) or infected with adenovirus expressing β-gal (199%, P≤0.001), wild-type Akt (218%, P≤0.001), or Akt/nuc (194%, P≤0.001). Akt/nuc showed no increase in cell size compared with uninfected or β-gal–expressing cardiomyocytes. Thus, expression of Akt/nuc does not induce hypertrophic remodeling in cultured cardiomyocytes.

Akt/nuc Inhibits Apoptosis Induced by Staurosporine or Deoxyglucose
To determine whether Akt/nuc promotes cell survival in cardiomyocytes, cultured myocardial cells expressing Akt/nuc were treated either with 1.0 mmol/L deoxyglucose or 0.25 μmol/L staurosporine. Apoptotic DNA fragmentation was detected by TUNEL assay. Akt/nuc significantly inhibited apoptosis after treatment with deoxyglucose compared with β-gal expressing cardiomyocytes. Results of TUNEL assay (A), PARP cleavage (B), or hypoxia (C). A, For TUNEL assay, cardiomyocyte cultures were infected with adenoviruses expressing nuclear-localized β-gal (black columns) or Akt/nuc (gray columns) overnight followed by treatment with 1.0 mmol/L deoxyglucose or 0.25 μmol/L staurosporine to induce apoptosis. Results shown are derived from a minimum of 4 separate experiments. B, For PARP cleavage experiments, cardiomyocyte cultures were infected with adenoviruses expressing nuclear-localized β-gal or Akt/nuc overnight followed by treatment with 1.0 mmol/L deoxyglucose or 0.25 mmol/L staurosporine to induce apoptosis. Results are expressed as the percentage reduction of PARP cleavage in Akt/nuc-expressing cells relative to nuclear-localized β-gal expressing cultures. C, ELISA-based immunoassay quantitation of DNA fragmentation in cardiomyocytes infected with adenovirus encoding EGFP, Akt/myr, or Akt/nuc at different pfu/cell ratios. Results are expressed in optical density (O.D.) units based on colorimetric reaction. * and ** indicate statistical difference (P<0.05 and P<0.01) vs EGFP-infected cells, respectively.
with cells expressing β-gal (Figure 3A; 82.2±11.2 versus 46.8±16.6%; P<0.01). Akt/nuc also inhibited apoptosis of staurosporine-treated cells (Figure 3A; 78.0±17.5% versus 40.3±21.6%; P<0.01).

To test whether Akt/nuc prevents apoptosis via downregulation of caspases, we analyzed cleavage of poly ADP-ribose polymerase (PARP), a 116-kDa nuclear DNA repair enzyme substrate of caspase-3 during apoptosis. Cleavage of PARP by caspase-3 yields an 85-kDa fragment, indicating activation of caspases. Cardiomyocyte cultures were infected with Akt/nuc and then treated to induce apoptosis with staurosporine or deoxyglucose the next day. Level of PARP cleavage in Akt/nuc-expressing cells was standardized relative to identical-treated cells infected with adenovirus expressing β-gal. In the absence of apoptotic stimulation, cleaved PARP signal was decreased 22% in cultures expressing Akt/nuc relative to nuclear-localized β-gal (Figure 3B). In comparison, Akt/nuc was capable of decreasing PARP cleavage by 55% and 44% in cultures treated with either staurosporine or deoxyglucose, respectively. These reductions of PARP cleavage were highly significant (P<0.01), demonstrating that Akt/nuc can inhibit apoptotic activation of caspases.

**Akt/nuc Inhibits Apoptosis of Hypoxic Cardiomyocytes**

To examine whether Akt/nuc effectively inhibited cardiomyocyte apoptosis as well as constitutively activated (myristo- lated) Akt, we infected cultured cardiomyocytes with adenovirus encoding either Akt/nuc or myristolated-Akt and the cells were subjected to hypoxia for 24 hours (Figure 3C). Infection of cardiomyocytes with myristolated-Akt at a multiplicity of infection (MOI) of 80 plaque-forming units (pfu) per cell significantly inhibited apoptosis of hypoxic cardiomyocytes when being compared with myocytes infected with adenovirus encoding EGFP as a control (less DNA fragmentation; P<0.05). Infection of Akt/nuc adenovirus substantially blocked apoptosis of hypoxic cardiomyocytes even at a concentration of 20 pfu/cell (less DNA fragmentation versus enhanced green-fluorescent protein (EGFP)-infected cells; P<0.05). Apoptosis was most effectively inhibited with Akt/nuc at a concentration of 40 pfu/cell (P<0.01).

**Generation of Transgenic Mice Cardiac-Specific Expression of Nuclear-Targeted Akt**

Transgenic mice expressing nuclear-targeted Akt driven by α-myosin heavy chain gene promoter (Figure 4A) were created to determine whether Akt/nuc enhances survival of cardiomyocytes in vivo. Expression of Akt/nuc in transgenic mice was confirmed by immunostaining and immunoblot by using Akt and phospho-Akt antibodies (Figures 4B and 4C). Transgenic mice (lines 71 and 41) show higher expression of Akt compared with non-transgenic mice. Transgenic Akt/nuc bands show higher apparent mobility because the Akt cDNA is fused with the myc tag and nuclear localization sequence. Transgenic mice were healthy without increases in heart weight/body weight ratio and myocardial cell size compared with nontransgenic mice (data not shown).

**Akt/nuc Protects Against Ischemia-Reperfusion Injury**

To test whether cardiac-specific transgenic expression of nuclear-targeted Akt was sufficient to protect the heart against pathological damage in vivo, mice were subjected to a 30-minute coronary occlusion followed by 24-hour reperfusion. Myocardial infarct size was significantly reduced in Akt/nuc transgenic mice (5.8% of region at risk; Figures 4D and 4E), demonstrating that Akt/nuc protects against ischemia-reperfusion injury.

**Akt/nuc Does Not Increase Phosphorylation of Cytoplasmic Akt Substrates**

The possibility that Akt/nuc promotes enhanced phosphorylation of cytoplasmic substrates was examined by immunoblot assessment of two canonical targets of activated Akt: GSK-3β and BAD. In both cases, phosphorylation levels at residues known to be Akt targets in cardiomyocytes were
comparable between nontransgenic WT and Akt/nuc transgenic mice (Figure 5). In comparison, phosphorylation levels for GSK-3β and BAD were increased in cardiomyocytes isolated from transgenic mice created with cardiac-specific expression of IGF-1, a known stimulus for Akt activation. Similarly, overexpression of myristolated Akt promotes enhanced phosphorylation of GSK-3β and BAD, confirming these proteins as substrates for Akt-mediated phosphorylation. These results indicate phosphorylation of typical cytoplasmic target substrates in Akt/nuc transgenic mouse hearts are not increased, supporting the postulate that Akt/nuc is efficiently targeted to the nucleus.

Akt/nuc Does Not Influence Cardiomyocyte Ploidy or DNA Content
The potential for Akt/nuc to promote nuclear division and DNA synthesis was assessed by determination of nuclei per cardiomyocyte and cardiomyocyte DNA content (Figures 6A and 6B, respectively). In both cases, populations of cardiomyocytes isolated from Akt/nuc hearts were comparable to those of nontransgenic WT mice, indicating that expression of Akt/nuc does not promote nuclear replication or DNA synthesis.

Akt/nuc Alters Transcriptional Profile in Transgenic Mouse Hearts
Gene expression in Akt/nuc mouse hearts was compared with normal nontransgenic mice by gene array profiling. Age- and gender-matched hearts were grouped for analysis as samples of pooled Akt/nuc hearts (Tg A = 3 hearts, Tg B = 4 hearts) and nontransgenic hearts (WT C = 3 hearts, WT D = 4 hearts). Tg A or Tg B samples were hybridized in quadruplicate (with Cy3 and Cy5 dye reversal) to WT C and in quadruplicate to WT D. Intensity ratio values that differed from the median with a confidence interval >95% were compiled. The Table presents nonexpression sequence-tagged (est) genes that were significantly altered in at least three of four hybridizations for each group. For example, Tg A versus WT C showed increased expression in at least three of four separate hybridizations for prostaglandin D2 synthase (PDGS; lipocalin) and protein tyrosine phosphatase, nonreceptor type 2 (PTP) with increased expression for gastric inhibitory peptide (GIP). PDGS was significantly downregulated in all four groups, and PTP was also downregulated in three of four groups as confirmed by RT-PCR. Although GIP was significantly upregulated in all four groups, GIP was not detectable in Akt/nuc heart RNA using RT-PCR suggesting cross-hybridization occurred with another mRNA possessing a similar coding sequence. Four additional genes were differentially regulated in one of the four groups (not shown). Subsequent RT-PCR validation tests confirmed one of these genes, connective tissue growth factor (CTGF), was downregulated in three of the four comparison groups.

**Fold Change in Gene Expression Determined by Microarray and Confirmed by RT-PCR**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Tg A vs WT C</th>
<th>Tg A vs WT D</th>
<th>Tg B vs WT C</th>
<th>Tg B vs WT D</th>
</tr>
</thead>
<tbody>
<tr>
<td>GIP</td>
<td>1.571 (ND)</td>
<td>1.446 (ND)</td>
<td>1.526 (ND)</td>
<td>1.418 (ND)</td>
</tr>
<tr>
<td>PDGS</td>
<td>0.486 (0.139)</td>
<td>0.728 (0.403)</td>
<td>0.553 (0.192)</td>
<td>0.743 (0.587)</td>
</tr>
<tr>
<td>PTP</td>
<td>0.642 (0.625)</td>
<td>0.756 (0.863)</td>
<td>NS (0.747)</td>
<td>0.743 (0.884)</td>
</tr>
<tr>
<td>CTGF</td>
<td>NS (0.793)</td>
<td>0.749 (0.637)</td>
<td>NS (1.014)</td>
<td>NS (0.814)</td>
</tr>
</tbody>
</table>

Fold changes in gene expression that were significant at the 95% confidence level are shown. NS indicates not significant for microarray at 95% confidence level. Values in parentheses were determined by RT-PCR; ND, not detectable.

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**Figure 5.** Akt/nuc does not enhance phosphorylation of cytoplasmic target substrates. Immunoblot analysis of heart lysates shows phosphorylation levels for GSK-3β (top) or BAD (middle) are not markedly enhanced by expression of Akt/nuc. In contrast, phosphorylation of these proteins is increased in transgenic mice expressing IGF-1 or in cultured cardiomyocytes after adenoviral-mediated overexpression of Akt/myr. Equivalent loading of lanes in immunoblot is demonstrated by comparable levels of actin in all samples (bottom).

**Figure 6.** Akt/nuc does not affect cell nucleation or DNA content. A, Distribution of nuclear number per cardiomyocyte in nontransgenic control (black bars) or Akt/nuc (gray bars) is comparable except for a slightly higher fraction of mononucleated cells that are more prevalent in the Akt/nuc (P = 0.05). B, Histogram distribution demonstrates no difference in DNA content per cell between nontransgenic control (WT), Akt/nuc, or lymphocytes as a normal diploid cell reference.
Discussion

Akt myocardial biology involving overexpression has been studied with constitutively activated26,27,29,30,38,39 or dominant-negative kinase.38 Characterizing Akt activation in the myocardium produces two recurring themes: promotion of growth and antiapoptotic activity. Promotion of growth is observed in vivo, particularly in assessment of postnatal cardiac enlargement resulting from transgenic expression of Akt kinase in the myocardium,38–40 although hypertrophic growth of cardiomyocytes was also reported.36 Antiapoptotic effects are a consistent finding both in vivo26,29 and in vitro.36,37 Alternatively, myocardial Akt signaling has been examined through manipulation of insulin41 or IGF-1 stimulation.31,32 Shared phenotypic characteristics of apoptotic resistance and hypercellularity were observed after cardiac-specific overexpression of IGF-143 or constitutive Akt activation.38–40 However, other aspects of Akt overexpression showing hypertrophy,38–40 depressed contractile function38–40 and heart failure39 are not readily reconciled with the phenotype of IGF-1 overexpressing transgensics. One potential explanation for the phenotypic differences between constitutively activated Akt- versus IGF-1 overexpression in the heart may be the degree and distribution of Akt activity, prompting our investigation of the consequences for targeted activation of Akt in the nucleus of cardiomyocytes.

Hearts from IGF-1–overexpressing mice show accumulation of activated Akt in the nucleus compared with nontransgenic mice, which show differential nuclear Akt activity correlating with gender.31 Akt does not possess an inherent nuclear localization signal sequence and activated Akt may depend on cofactors such as TCL1 that mediate nuclear localization by facilitating Akt oligomerization.44,45 TCL1 is an oncogene of human T-cell leukemia, but related molecules responsible for nuclear translocation of Akt may exist in cardiomyocytes. Numerous cell survival and cell cycle transcription factor substrates for Akt phosphorylation are located in the nucleus, including forkhead family proteins,9,10 SRK,13 E2F,14 CREB,15 TSC2,46 p21Cip/WAF1,47 and telomerase reverse transcriptase.48 In fact, Akt/nuc enhances telomerase activity and antagonizes the phenotypic changes characteristic of cardiomyocyte aging and senescence much better than wild-type Akt (unpublished results, 2004). Studies to determine the ability of cardiac-specific expression of Akt/nuc to antagonize aging in the myocardium are in progress and thus far Akt/nuc expression in transgenic mice to over 1 year of age does not provoke overt cardiomyopathic effects.

Activated Akt in cardiomyocytes localizes to the nucleus and estrogentic stimulation increased nuclear accumulation of activated Akt as well as cytoplasmic localization of forkhead transcription factors.31 Although Akt/nuc successfully promotes cell survival, the expression of cellular Akt (nonactivated form) in a previous study did not inhibit apoptosis without stimulation of IGF-1.36 In comparison, the subcellular distribution of constitutively active Akt is dispersed among the cellular components with 40% on the cell membranes, 30% in the nucleus, and 30% in the cytosol, whereas overexpression of cellular wild-type Akt accumulates primarily (90%) in the cytosol.31 Furthermore, Akt/nuc inhibited hypoxia-induced apoptosis as efficiently as (or better than) myristinated Akt. However, phosphorylation of cytoplasmic targets including the apoptotic mediator BAD were unchanged in Akt/nuc transgenic hearts (Figure 5). These findings support our postulate that relevant targets for Akt phosphorylation that mediate cellular survival are present in nucleus.

Potent antiapoptotic effects of Akt/nuc meet and surpass the level of protection afforded by Akt/myr both in vitro and in vivo (Figures 3 and 4). Cardiac-specific transgenesis of nuclear-targeted Akt produced a profound infarct-sparing effect (~70% reduction versus wild type). Akt/nuc affords protection comparable in magnitude to early ischemic preconditioning (~75% reduction),49 the most powerful cardioprotective modality that has been described to date.

Two genes found to be downregulated in Akt/nuc transgenic hearts are PGDS and PTP, which are novel and distinct from genes previously reported to be influenced by expression of activated Akt in the myocardium.40 Estrogen has been reported to increase PGDS mRNA in rat brain,51 and we previously found estrogen increases nuclear localization of Akt.31 Complex regulation of estrogen of PGDS51 and significant differences in experimental design could account for decreased PDGS expression in our study versus increased estrogen-mediated expression in the brain. Thus, PGDS may be an interesting estrogen-regulated gene in the heart. PTNP2 (also known as TC-PTP) is homologous to PTP-1B.52 PTP-1B influences PI3-kinase,53 insulin-mediated signaling,54,55 and downregulation enhances IGF-1–mediated cell survival.56,57 Thus, decreased expression of PTP could help promote enhanced cardiomyocyte survival in Akt/nuc transgenic mice.

Ideally, therapeutic intervention in apoptotic cardiomyocyte death should exert minimal deleterious effects on cardiomyocyte structure or function. Experimental prevention of cell death in acute cardiac injury associated with apoptosis has been attempted using membrane-targeted myristoylated Akt,26–30 but supraphysiological levels of constitutively activated kinase34,35 cause cardiomyocyte hypertrophy16,34 and cardiomyopathic effects.38,39 Potent survival signaling without promoting hypertrophic remodeling or DNA synthesis makes Akt/nuc an interesting candidate for therapeutic intervention against cardiomyopathic injuries resulting from ischemic heart disease, chronic pressure or volume overload, hypoxia, or anticancer drugs.

Acknowledgments

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References


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Experimental Procedures

Adenovirus expressing Akt with nuclear localization signals. Adenovirus expressing mouse cellular Akt with nuclear localization signals (Akt/nuc) was created by PCR-based cloning technique using mouse cellular Akt as a template. The PCR product was subcloned into pShooter Vector plasmid (pCMV/myc/nuc, Invitrogen), which has myc and triplet nuclear localization signals at the 3'-end of the multiple cloning sites (Fig. 1A). The resultant product was subcloned into adenovirus shuttle vector pDC315 (Microbix) for use with the AdEasy Cre-mediated site-specific recombination system. 1 Adenoviral backbone (pBHGloxE1,2Cre, Microbix) and Akt/nuc shuttle vectors were co-transfected to 293 cells with Polyfect reagent (Qiagen). Fidelity of PCR-derived DNA fragments was confirmed by sequencing. Virus titer was determined by plaque assay in 293 cells.

Cell culture. Neonatal ventricular myocytes were isolated from the hearts of 2- or 3-day-old Sprague-Dawley rats as described. 2 Adenovirus infection was started 24-48 hours after the first medium change. Myocytes were incubated with adenovirus containing cell lysate for 2 hours. Following infection, cultures were washed twice with 1xAds and incubated with medium M199 with 2% horse serum and 0.25x Nutridoma (Boehringer Mannheim) overnight.

Immunohistochemistry and confocal microscopy. Immunostaining and confocal microscopy were performed as previously described. 3 Antibodies were obtained to detect total Akt (Cell Signaling Technologies), phospho-Akt (Biosource), and α-actinin (Sigma).
Nuclear extract and cytosol preparation. Cardiomyocyte nuclear extracts and cytosol preparation were prepared as previously described. 3

Immunoblots and kinase assay. Immunoblots and in vitro kinase assay for Akt were performed as previously described. 3

Cellular hypertrophy. Isolated cardiomyocytes were infected with adenovirus encoding either wild-type Akt (Akt/wt), myristoylated Akt (Akt/myr), or nuclear-targeted Akt (Akt/nuc) for 2 hours, refed with serum-free media for 24 hours, and immunostained with pan Akt antibody and c-myc antibody to identify cells infected with Akt/nuc. Simultaneous staining with Texas Red-conjugated phalloidin (Molecular Probes) revealed actin filaments. Images were acquired by confocal microscopy and cell size determinations were done using ImageSpace software (Molecular Dynamics). Control infections were performed with adenovirus encoding β-gal or with uninfected cells.

Induction of apoptosis and quantitation. After infection with adenovirus expressing Akt/nuc or β-gal as a control, isolated neonatal rat cardiomyocytes cultured on chamber slides were refed with M199 supplemented with 2% horse serum and left overnight. The next day, myocytes were treated with staurosporine (0.25 M) or 2-deoxyglucose (1.0 mM) in M199 with 2% horse serum and cultured overnight. Nuclear fragmentation of cardiomyocytes was detected in 4% paraformaldehyde-fixed cells by TUNEL staining with Apoptosis kit (R&D systems) according to the manufacturer's recommendation. Approximately 200 cells were randomly selected from different fields and percent of apoptotic nuclei was determined. Results shown are derived from a minimum of four separate experiments. For hypoxia, cardiomyocytes were cultured in RPMI 1640/5% FCS/10% horse serum for 46 hours after the infection with adenovirus for 2 hours. Then, the medium was changed to serum-free RPMI 1640 saturated with
95% N₂/5% CO₂, and cells were placed in a 37°C airtight box saturated with 95% N₂/5% CO₂ for 24 hours. O₂ concentrations were <0.1% (Ohmeda oxygen monitor, type 5120). For normoxic controls, culture medium was changed to RPMI 1640/5% FCS/10% HS, and cells were placed in 37°C/5% CO₂ incubator for 24 hours before analysis.

**PARP cleavage detection.** Myocytes were infected with adenovirus encoding Akt/nuc or β-gal for 24 hours, and then treated overnight with staurosporine (0.5 M) or 2-deoxy-D-Glucose (1.0 mM). Cells were washed twice with PBS and harvested with lysis buffer (50 mM Tris-HCl, pH 7.4, 1.0 % NP-40, deoxycholic acid 0.25 %, 150mM NaCl, 1.0 mM EGTA, 1.0 mM NaF, 1.0 mM Na₃VO₄) containing a cocktail of protease inhibitors including 1.0 mM phenylmethylsulfonil fluoride, cypermethrin 10 nM, okadaic acid 10 nM, phenylarsine oxide 100 M, pepstatin, aprotinin, TLCK, TPCK, and leupeptin. Cell lysates were centrifuged at 4°C, dissolved in SDS-sample buffer, and separated by 6% SDS-polyacrylamide gel electrophoresis. Separated proteins were transferred to Hybond WCL nitrocellulose membranes (Amersham Pharmacia). Membranes were incubated in blocking buffer (PBS, 3.0 % Fractionation V-bovine serum, 0.05 % Tween 20) for 2 hours at room temperature followed by overnight incubation with 6.0 µg/mL of rabbit polyclonal antibody against anti-PARP cleavage site (214/215; Biosource). After three washes in PBS with 0.05% Tween 20 for 10 min, blots were incubated with 1:2500 goat anti-rabbit IgG conjugated with horseradish peroxidase (Amersham Pharmacia) for 2 hours at 37°C. After three washes in PBS, 0.5% Tween 20 for 10 min, bound antibody was detected by enhanced chemiluminiscence. Correction for minor variation in protein loading was performed by standardization relative to glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

**DNA fragmentation.** *In vitro* model of cardiomyocytes hypoxia and DNA fragmentation assay was performed as previously described.⁴
**Generation of transgenic mice.** The nuclear-targeted Akt cDNA construct was subcloned downstream of the 5.5-kb mouse alpha-myosin heavy chain gene promoter. Genomic DNA was isolated from mouse-tail biopsies and was analyzed by Southern blotting. All experiments were approved by the Children’s Hospital Research Foundation Animal Care Review Board and San Diego State University Animal Care and Use Committee.

**Mouse model of myocardial infarction.** The mouse model of myocardial infarction has been previously described in detail. Briefly, myocardial infarction was produced by a 30-minute coronary occlusion followed by a 24-hour reperfusion, eliminating the possibility of insufficient reperfusion time. Following postmortem perfusion with 1% solution of 2,3,5-triphenyltetrazolium chloride (phosphate buffer, pH 7.4, 37°C), the coronary artery was ligated at the site of previous occlusion and the coronary vascular bed perfused with 5% phthalocyanine dye from the aortic root. The non-ischemic portion of the left ventricle was stained dark blue and the viable tissue within the region at risk bright red, whereas the infarcted tissue appears light yellow. Infarct size analysis was performed in a blinded fashion and both sides of each heart slice were examined for infarction and the respective areas averaged to arrive at the reported values. Infarct size was measured with Image Tool software and expressed as a percentage of the region at risk.

**Determination of protein phosphorylation.** Lysates were prepared from Akt/Nuc transgenic mice (n=6), IGF-1 transgenic mice (Igf¹/², n=6) and wild-type littermates (WT, n=6) at 4-6 weeks of age. Hearts were excised and left ventricular (LV) myocytes were enzymatically dissociated. Briefly, the heart was connected to a plastic cannula for retrograde perfusion through the aorta. The solutions were supplements of modified commercial minimal essential medium (MEM) Eagle-Joklik. HEPES-MEM containing (in mM) NaCl 117, KCl 5.7, NaHCO₃ 4.4, KH₂PO₄ 1.5, MgCl₂ 17, HEPES 21.1, and glucose 11.7 with amino acids and
vitamins, 2 mM L-glutamine, and 21 mU/mL insulin; pH was adjusted to 7.3 with NaOH. The re-suspension medium was HEPES-MEM supplemented with 0.5% BSA and 0.3 mM CaCl₂. The cell isolation procedure consisted of three main steps: (1) The collagenase (selected type I, Worthington Biochemical Corp) perfusion of the myocardium was performed at 37°C with HEPES-MEM gassed with 85% O₂, 15% N₂. (2) The heart was removed from the apparatus, the LV was cut into small pieces and these fragments were shaken in re-suspension medium at 37°C. (3) Intact cells were enriched by centrifugation. This procedure was repeated 2-3 times. WT myocytes were infected in vitro with an adenoviral vector carrying Akt/myr at a multiplicity of infection of 100 for 24 h in MEM. 9 Freshly isolated and cultured myocytes were lysed with 350 µL of lysis buffer (50 mM Tris-HCl, pH 7.5; 5 mM EDTA; 250 mM NaCl; and 0.1% Triton X-100) containing the protease inhibitors PMSF, 2 mM, aprotinin, 1 µg/mL, DTT, 5 mM, Na₃VO₄, 1 mM. Myocyte lysates were centrifuged at 14,000 rpm for 10 minutes. Equivalents of 70 µg of proteins were separated on 10%-12% SDS–PAGE. Proteins were transferred onto nitrocellulose filters, blocked with 5% dry milk, and exposed to mouse monoclonal anti-phospho GSK3beta (Upstate, Charlottesville, VA) and sheep polyclonal anti phospho-Bad (Cell Signaling, Beverly, MA). Both the antibodies were used at concentrations of 4 µg/ml. Mouse and sheep HRP-conjugated IgG were used as secondary antibodies (Santa Cruz, Santa Cruz, CA).

Microarray analysis. Hearts were snap frozen in liquid nitrogen at the time of harvest. RNA was extracted using Qiagen RNeasy kit (Qiagen, Chatsworth CA). Hearts were pooled for each group to provide enough RNA for multiple microarray hybridizations. RNA concentration was determined by optical density at 260 nm. Formaldehyde gel electrophoresis was performed to confirm distinct 28S and 18S ribosomal RNA. All samples had a 28S band twice the intensity of the 18S band (data not shown). Oligonucleotide microarray chips containing 70mer probes for 16,463 mouse genes were prepared using standard protocols. Oligomer probes (purchased from Qiagen) were derived from unique 3’ regions of genes in the UniGene Database Build Mm 102. Probes are routinely resequenced to confirm their identity. Updated clone information can be
Oligomer probes were spotted onto poly-L-lysine-coated glass slides using a robotic DNA arrayer (Beecher Instruments; Bethesda, MD). Total RNA (35 µg) was labeled with Cy3- and Cy5-conjugated dUTP (Amersham; Piscataway, NJ) using a reverse transcription reaction and hybridized to the oligonucleotide microarray chip. Chips were scanned using an Axon scanner (Axon Instruments; Foster City, CA), and images were analyzed using the Array Suite Software (Scanalytics; Fairfax, VA). Relative fluorescence intensity was measured for each labeled RNA with subtraction of local background values. A ratio of the relative intensities of each fluor bound to each probe was calculated. The distribution of the ratio of all genes was analyzed and normalized based on the median value of a panel of 100 control genes. The distribution of the ratio of all of the genes was then calculated, and intensity ratio values that differed from the median with a confidence interval >95% were scored as significant changes. To increase stringency, each hybridization experiment was performed in quadruplicate. A database tool, MAPS, was used to compile the overall list of genes consistently and significantly changed across the multiple hybridizations. Quantitative real-time PCR (RT-PCR) was used to verify altered gene expression levels. The primer sequences used in RT-PCR are shown in Table 1. First strand synthesis was conducted for 60 min at 48°C in a 10-µl reaction containing 100 ng total RNA, 5.5 mM MgCl2, 2 µM dNTPs, 4 units RNase inhibitor, and 12.5 units reverse transcriptase. The real-time PCR reaction was carried out in a total volume of 40 µl which included the first strand synthesis reaction to which was added 4 mM MgCl2, 8 mM dNTPs, 1X SYBR green PCR buffer (PE Biosystems), 0.4 µM gene specific primers, and 2.5 units AmpliTaq Gold DNA polymerase (PE Biosystems). The reaction was analyzed using an
Applied Biosystems PRISM 7700 detection system. mRNA expression was normalized to GAPDH and 18S RNA (Amplicon Technologies). Each reaction was performed in triplicate.

**Nuclear number and DNA content.** For nuclear number, myocytes were stained with propidium iodide and the fraction of mono-, bi-, tri-, and tetranucleated cells was determined by counting 500-1,000 myocytes in each animal. For determination of DNA content, values were obtained by measuring fluorescence of nuclei in isolated cells as previously described. DNA was stained with 10 µg/ml of propidium iodide in the presence of 1 mg/ml of RNase were assessed to quantitate the area of the signal and mean fluorescence. Area x mean fluorescence = total fluorescence from the nucleus = DNA content. Image analysis was performed with ImagePro software (Media Cybernetics, Silver Spring, MD).

**Statistics.** All determinations for significance unless otherwise noted were performed by Student’s t-test analysis of sample populations using Microsoft Excel (Microsoft). Values of \( p<0.05 \) were considered significant.

**References**


Table 1. Primers used for RT-PCR analysis of microarray data.

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>GenBank #</th>
<th>Primer Name</th>
<th>Primer Sequence</th>
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<tbody>
<tr>
<td>Protein Tyrosine Phosphatase N2</td>
<td>NM008977</td>
<td>PTPN2-F</td>
<td>5' CCTGACCATGGACCTGCAGT 3'</td>
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<td></td>
<td>PTPN2-R</td>
<td>5' AGAGAAGGTGCCAGAGCAGC 3'</td>
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<td>Gastric Inhibitory Peptide</td>
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<td>GIP-R</td>
<td>5' GTGCAGGCGCAGCAAAATTT 3'</td>
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<td>Prostaglandin D2 synthase</td>
<td>AB 006361</td>
<td>PGSYNTH-F</td>
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<td>PGSYNTH-R</td>
<td>5' TGAATAGCGAGCGTACTCGCAT 3'</td>
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