

Akt Activation Preserves Cardiac Function and Prevents Injury After Transient Cardiac Ischemia In Vivo

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Background—The serine-threonine kinase Akt is activated by several ligand-receptor systems previously shown to be cardioprotective. Akt activation reduces cardiomyocyte apoptosis in models of transient ischemia. Its role in cardiac dysfunction or infarction, however, remains unclear.

Methods and Results—We examined the effects of a constitutively active Akt mutant (myr-Akt) in a rat model of cardiac ischemia-reperfusion injury. In vivo gene transfer of myr-Akt reduced infarct size by 64% and the number of apoptotic cells by 84% ($P < 0.005$ for each). Ischemia-reperfusion injury decreased regional cardiac wall thickening as well as the maximal rate of left ventricular pressure rise and fall (+dP/dt and -dP/dt). Akt activation restored regional wall thickening and +dP/dt and -dP/dt to levels seen in sham-operated rats. To better understand this benefit, we examined the effects of myr-Akt on hypoxic cardiomyocyte dysfunction in vitro. myr-Akt prevented hypoxia-induced abnormalities in cardiomyocyte calcium transients and shortening. Akt activation also enhanced sarcolemmal expression of Glut-4 in vivo and increased glucose uptake in vitro to the level seen with insulin treatment.

Conclusions—Akt activation exerts a powerful cardioprotective effect after transient ischemia that probably reflects its ability to both inhibit cardiomyocyte death and improve function of surviving cardiomyocytes. Akt may represent an important nodal target for therapy in ischemic and other heart disease. (*Circulation*. 2001;104:330-335.)

Key Words: apoptosis ■ signal transduction ■ gene therapy ■ ischemia

Ischemia-induced cardiac dysfunction reflects a combination of myocyte death and dysfunction. Cardiomyocyte death itself is an aggregate of both apoptotic and necrotic cell death, although overlap can exist between the morphological features of these categories.¹ Defining the signaling pathways that control survival and function of ischemic cardiomyocytes may provide insights into mechanisms of cardiac injury while identifying potential candidate targets for intervention.

The serine-threonine kinase Akt is a powerful survival signal in many systems² and is activated by several cardioprotective ligand-receptor systems, including insulin,^{3,4} insulin-like growth factor (IGF)-1,⁵⁻⁸ and gp130 signaling.^{9,10} Our group has shown that a constitutively active Akt mutant (myr-Akt) is sufficient to block apoptosis in hypoxic rat cardiomyocytes in vitro.¹¹ More recently, Fujio et al¹² demonstrated that Akt activation also reduced the number of TUNEL-positive nuclei in cardiomyocytes expressing the transgene after ischemia in vivo. The effects of Akt activation on overall apoptosis, infarction, or cardiac function, however, were not assessed. Although it is encouraging that Akt

activation can reduce DNA fragmentation in vitro^{11,12} and in vivo,¹² the relevance of these observations to clinically meaningful end points remains undefined.

To address these issues, we used adenoviral vectors (Ads) to express activated Akt in rat hearts subjected to transient ischemia in vivo. Not only did Akt activation reduce the total number of apoptotic cardiomyocytes, it also substantially reduced infarct size and even more dramatically improved regional cardiac function. Studies undertaken in an in vitro model of hypoxic cardiomyocyte dysfunction suggest that Akt is an important determinant not only of cell survival but also of the function of surviving cells.

Methods

Adenoviral Vectors

Ad.EGFP. β -gal contains cytomegalovirus-driven expression cassettes for β -galactosidase and enhanced green fluorescent protein (EGFP) substituted for E1 through homologous recombination.¹³ Ad.Akt(AAA) is structurally identical except that it encodes a dominant-negative Akt mutant rather than β -galactosidase and was constructed by use of the Akt(AAA) cDNA kindly provided by Dr

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Woodgett (Ontario Cancer Institute, Toronto, Canada).¹⁴ Ad.myr-Akt and Ad.EGFP (described previously¹¹) use a similar Ad backbone and mediate expression of HA-tagged constitutively active Akt or EGFP, respectively. Ads were amplified in 293 cells, particle count was estimated from OD₂₆₀, and titer was determined by plaque assay. Stock titers were $\approx 10^{11}$ pfu/mL, with a particle/pfu ratio of 20 to 80. Wild-type adenovirus contamination was excluded by the absence of polymerase chain reaction–detectable E1 sequences.

Animal Model

Male 250- to 300-g Sprague-Dawley rats were anesthetized (pentobarbital), intubated, and ventilated (SAR-830, CWE Inc), and 200 μ L of buffer containing 1.2×10^{12} particles/mL Ad or buffer alone was injected via left thoracotomy into the anteroapical myocardium. Forty-eight hours later, left thoracotomy was again performed, and the left anterior descending coronary artery (LAD) was ligated with 6-0 silk suture ≈ 4 mm from its origin with a slipknot. Ischemia was confirmed by myocardial blanching and ECG evidence of injury. Five minutes into ischemia, 300 μ L of fluorescent microspheres (10- μ m FluoSpheres, Molecular Probes) was injected into the left ventricular (LV) cavity. After 30 minutes, the LAD ligature was released and reperfusion visually confirmed. For sham ischemia-reperfusion injury (IR), thoracotomy was performed without LAD ligation. Overall survival was $\approx 90\%$ at 24 hours.

Infarct Size

Rats were euthanized 24 hours after ischemia. Hearts were frozen in liquid N₂ and sectioned from apex to base (Jung Frigocut 2800E, Leica) into four 2-mm sections, each separated by six 10- μ m sections. Two-millimeter sections were used to quantify the area at risk (AAR) and the infarct area. To delineate the infarct, sections were incubated in 5% (wt/vol) triphenyltetrazolium chloride (TTC, Sigma) in PBS (pH 7.4) at 37°C for 20 minutes. For each section, the AAR and infarct area were measured from enlarged digital micrographs with NIH Image. Percent myocardial infarction (%MI) was calculated as the total infarction area divided by the total AAR for that heart.

DNA Laddering

Fresh tissues (without TTC staining) were microdissected under UV light into ischemic and nonischemic regions and processed simultaneously. All tissue from each region was lysed (100 mmol/L Tris [pH 8.5], 5 mmol/L EDTA, 0.2% SDS, 200 mmol/L NaCl, 100 μ g/mL proteinase K) at 37°C for 18 to 20 hours. DNA was prepared, labeled with [α -³²P]dCTP, and subjected to electrophoresis, and autoradiography was performed as described.¹¹

TUNEL Staining

Terminal dUTP nick end-labeling (TUNEL) staining was performed with Apoptag (Intergen) according to the manufacturer's instructions, with Hoechst 33258 (Sigma) nuclear counterstaining. Nuclei were counted in 8 to 10 microscopic fields from a 10- μ m midventricular section for each heart used to assess infarct size. The mean number of nuclei per mm² was multiplied by the section area to calculate the total nuclei for that section. Virtually all TUNEL-positive nuclei were confined to a well-circumscribed area within the ischemic zone. TUNEL-positive nuclei from this region were counted in 8 to 10 microscopic fields, and the mean number of nuclei per mm² was multiplied by the area of the apoptotic region to calculate the number of TUNEL-positive nuclei for that section. More than 1500 nuclei were counted for each section.

Immunohistochemistry

After TUNEL staining, sections were incubated with primary antibody to α -actinin (30 minutes, 22°C), rinsed in PBS, and incubated with anti-mouse IgG conjugated to tetramethylrhodamine (Sigma) (30 minutes, 22°C).

Immunoblotting

SDS-PAGE was performed under reducing conditions on 12% separation gels with a 4% stacking gel. Proteins were transferred to polyvinylidene difluoride membrane (Bio-Rad). Blots were incubated with primary antibodies to hemagglutinin (HA) (12CA5, Boehringer-Mannheim), Akt (Transduction Laboratory), phospho-Akt (Ser473, NEB), or glucose transporter (Glut)-4 (1F8,¹⁵ kindly provided by Dr Kandror from the Boston University School of Medicine) for 18 to 20 hours at 4°C. Blots were incubated with horseradish peroxidase–conjugated secondary antibody, and signal was detected with enhanced chemiluminescence (NEN Life Science). For immunoblotting of sarcolemmal Glut-4, the P2 fraction was prepared as previously described.¹⁵

Hemodynamic Measurements

Twenty-four hours after ischemia or sham operation, a subset of rats underwent thoracotomy and placement of a 1.8F LV pressure transducer (Millar Instruments). Piezoelectric crystals (0.5-mm, Sonometrics) were placed on the anterior epicardial and endocardial LV surfaces. Regional wall thickening (anterior epicardial to endocardial) was calculated from digitally acquired piezoelectric crystal position data. Pressure measurements were digitized at 1.0 kHz and analyzed with commercially available software (Sonolab, Sonometrics) to derive the maximal rates of pressure rise (+dP/dt) and fall (–dP/dt).

In Vitro Cardiomyocyte Hypoxia Model

Cardiomyocytes were prepared from 1- to 2-day-old rats and subjected to transient hypoxia for up to 24 hours as previously described.¹¹ Cardiomyocyte shortening and intracellular calcium transients were analyzed in contractile cells stimulated at 1 Hz with biphasic pulses (Grass Instruments) after loading with fura 2 (Molecular Probes) as previously described.¹⁶

Akt Kinase Activity

Myocardial tissue was lysed, immunoprecipitated with anti-Akt antibody, and used to measure Akt kinase activity with the Akt Kinase Assay Kit (NEB) with GSK-3 α/β as a substrate according to the manufacturer's instructions.

Glucose Uptake

Cardiomyocytes were cultured for 18 hours in serum-free RPMI and incubated with 10 mmol/L 2-deoxy-D-glucose (Sigma; 4 hours, 37°C). Cells were washed with buffer (mmol/L: NaCl 140, KCl 2.7, CaCl₂ 1, KH₂PO₄ 1.5, Na₂HPO₄ 8, and MgCl₂ 0.5, pH 7.4) and incubated with buffer/0.1% BSA (20 minutes, 37°C) and then 0.5 μ Ci/well of deoxy-D-glucose-2-[1,2-³H(N)] (NEN) in buffer/0.1% BSA (10 minutes, 37°C). After a washing with 100 μ mol/L phloretin (Sigma) in buffer/0.1% BSA, cells were harvested with 0.1% SDS and counted in a scintillation counter.

Statistical Analysis

Data are presented as mean \pm SD. Data were compared by 2-tailed *t* test or ANOVA as appropriate with Statview (Abacus Concepts) or MS Excel '98. The null hypothesis was rejected for *P* < 0.05.

Results

Adenoviral Gene Transfer In Vivo

Direct injection of Ads resulted in regional transgene expression in an area composing $\approx 50\%$ of the area subjected to ischemia (data not shown). Immunoblotting confirmed the regional expression of the HA-tagged myr-Akt construct¹¹ (Figure 1a, top) and an increase in phosphorylated Akt (Figure 1a, middle). Akt kinase activity also substantially increased in the Akt-injected regions (Figure 1b). The overall level of Akt expression was similar in all regions (Figure 1a, bottom).

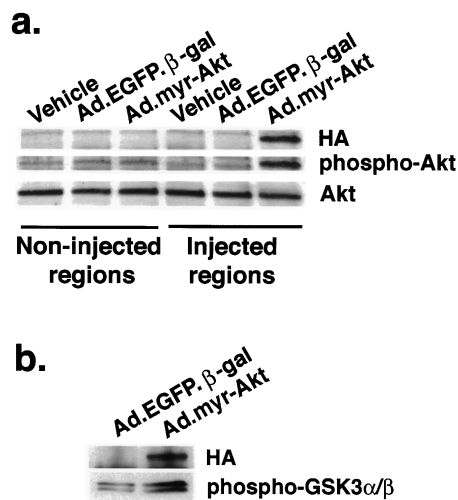


Figure 1. Adenoviral gene transfer in vivo. **a**, Akt expression and activation were assessed 48 hours after injection to reflect status at initiation of ischemia. Expression of HA-tagged myr-Akt was detected only in injected region (top). Amount of phosphorylated Akt was substantially increased by Ad.myr-Akt (middle). Overall level of immunoreactive Akt was not changed (bottom). **b**, Akt activity was measured in myocardium injected with Ad.myr-Akt or control virus with GSK-3α/β as substrate as described in Methods. myr-Akt significantly increased Akt activity in injected area.

Infarct Size and AAR

The ischemic area induced by LAD ligation (%AAR) did not differ among the 3 groups (data not shown). Akt activation, however, reduced infarct size by 64% compared with the vehicle-injected group (%MI $22 \pm 4\%$ versus $60 \pm 4\%$; Figure 2). This reduction was highly significant compared with either vehicle- or Ad.EGFP.β-gal-injected animals.

Apoptosis

Simultaneous α-actinin staining confirmed that apoptotic nuclei were predominantly in cardiomyocytes (Figure 3a, top, data not shown). The total number of nuclei did not differ among the 3 groups (data not shown). The number of apoptotic nuclei, however, was reduced 84% compared with buffer alone and 76% compared with Ad.EGFP.β-gal (Figure 3a). DNA laddering in the ischemic regions of Ad.myr-Akt-injected animals was also attenuated compared with that seen in the ischemic regions of control virus-injected rats (Figure 3b, top). Simultaneously processed samples from nonischemic regions revealed no DNA laddering (Figure 3b, bottom).

Cardiac Function In Vivo

Cardiac function was analyzed at 24 hours in 24 separate animals (Figure 4a) because this invasive assessment precluded morphological analyses. Twelve rats (7 buffer, 5 Ad.myr-Akt) underwent sham operation, and 12 (6 Ad.EGFP.β-gal, 6 Ad.myr-Akt) were subjected to IR. LV systolic and end-diastolic pressures were not different among the 4 groups. In the absence of IR, Akt did not affect any of the measured parameters. The maximal rates of pressure rise (+dP/dt) and fall (−dP/dt) were significantly reduced by IR in control Ad-infected rats (Figure 4a, $P < 0.05$ for both). Of note, extensive previous physiological measurements have

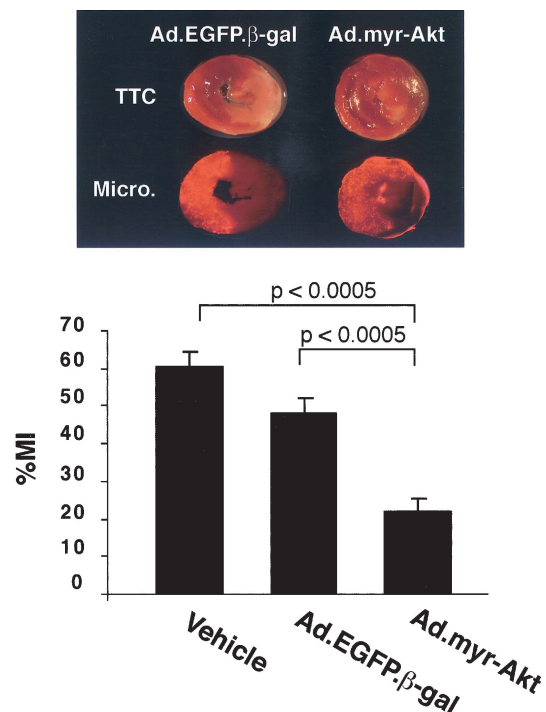


Figure 2. Infarct size. Micrograph, Representative fluorescent microsphere distribution (bottom) and TTC staining (top) for 1 section from animals injected with Ad.EGFP.β-gal (left) or Ad.myr-Akt (right). Graph, Injection of Ad.EGFP.β-gal or Ad.myr-Akt did not significantly affect AAR (data not shown). Animals transduced with myr-Akt had 64% reduction in infarct size vs vehicle-injected group ($22 \pm 4\%$ vs $60 \pm 4\%$). Cumulative data from 24 animals (8 in each group).

documented that control virus does not affect in vivo cardiac function.^{17,18} Akt activation in IR, however, significantly increased both +dP/dt and −dP/dt compared with the control Ad ($P < 0.002$ for both), restoring both to levels seen in sham-operated controls. We also evaluated cardiac wall motion with piezoelectric crystals placed on the epicardial and endocardial surfaces of the LV anterior wall. IR reduced systolic thickening in the ischemic region in controls, whereas myr-Akt expression preserved thickening at levels comparable to sham-operated animals (Figure 4b).

Cardiomyocyte Function In Vitro

Given the disproportionate improvement in cardiac function in vivo, we examined the functional effects of Akt activation in surviving cardiomyocytes subjected to transient hypoxia in vitro. Expression of myr-Akt blocked hypoxia-induced myocyte dysfunction, preserving contractile function (dL/dt) and calcium handling (τ) comparable to normoxic cultures at 24 hours (Figure 5a). Baseline function of normoxic cardiomyocytes was not affected by myr-Akt expression (data not shown). To demonstrate the kinase-dependence of this protective effect, we used a dominant negative Akt construct [Ad.Akt(AAA)]. Hypoxic cardiomyocytes expressing Akt(AAA) were all dead at 24 hours (data not shown) but demonstrated accelerated, early functional deterioration (Figure 5b).

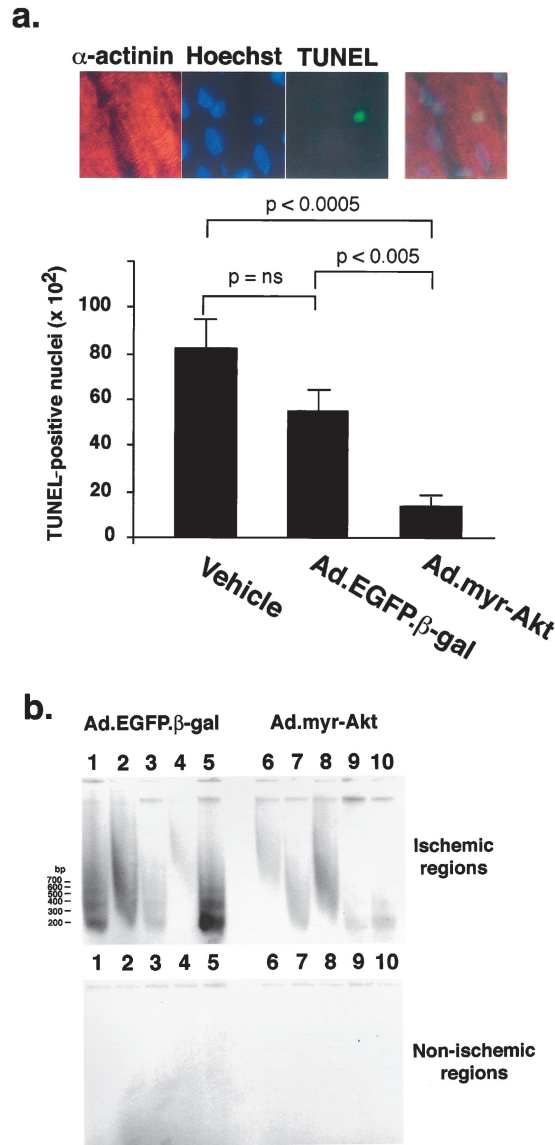


Figure 3. myr-Akt expression reduces DNA fragmentation in vivo. **a.** Micrograph: 24 hours after ischemia, sections were subjected to immunostaining for α -actinin, TUNEL staining, or nuclear counterstaining with Hoechst 33258. Right, Merged images. Representative data from 1 of 24 animals. Graph, Total nuclei did not differ among animals injected with vehicle alone, Ad.EGFP- β -gal, or Ad.myr-Akt (data not shown). In contrast, number of TUNEL-positive nuclei decreased 84% and 76% in Ad.myr-Akt-injected animals vs animals receiving vehicle or Ad.EGFP- β -gal, respectively. Cumulative data from 24 animals (8 each). **b.** DNA laddering in ischemic (top) and nonischemic (bottom) regions from Ad.EGFP- β -gal- (1 to 5) or Ad.myr-Akt-injected (6 to 10) animals. Laddering was attenuated in ischemic regions by myr-Akt (top). Simultaneously processed DNA from nonischemic regions revealed no laddering in either group (bottom).

Downstream Targets

No change in overall levels of immunoreactive BAD, phospho-BAD, GSK-3, phospho-GSK-3, or Bcl-2 was detected in Akt-injected hearts (data not shown). In contrast, Akt activation induced increased sarcolemmal expression of Glut-4, which was even more marked after ischemia (Figure 6, top). Sarcolemmal translocation of Glut-4, which can serve

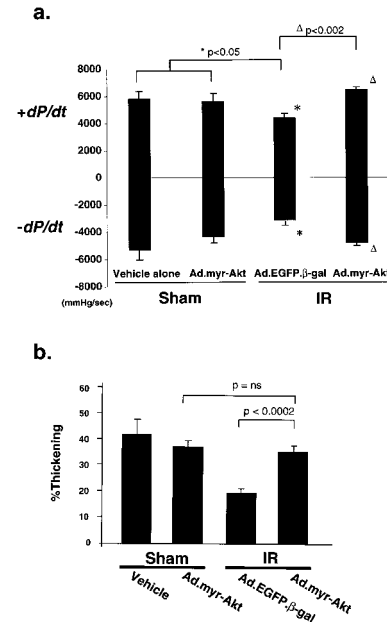


Figure 4. Cardiac function in vivo. **a.** +dP/dt and -dP/dt were derived from measures of LV pressure 24 hours after IR or sham operation. myr-Akt prevented decrease in +/-dP/dt seen in control animals after IR. **b.** Anterior systolic thickening was measured by microsonometry with epicardial and endocardial piezoelectric crystals. myr-Akt expression preserved systolic thickening at levels comparable to sham-operated animals ($P=NS$) and significantly better than Ad.EGFP- β -gal-transduced animals after IR.

as a direct target of Akt in some settings,¹⁹ is a well-documented effect of Akt activation in muscle.^{14,20} Correspondingly, Akt activation enhanced cardiomyocyte glucose uptake in vitro to levels seen with insulin stimulation (Figure 6).

Discussion

We examined the effects of expression of a constitutively active Akt mutant in an in vivo model of cardiac ischemia-reperfusion. We found that gene transfer achieved local expression of HA-myr-Akt in the ischemic region, increasing the proportion of phosphorylated Akt and Akt activity. The overall level of immunoreactive Akt was not appreciably changed. This could reflect either downregulation of endogenous Akt or preferential binding of the antibody to the unphosphorylated molecule. Akt activation substantially protected the heart from injury. Infarct size was reduced by 64%. Regional systolic thickening and the maximal rate of pressure rise and fall were restored to levels seen in sham-operated animals. These data establish an important role for Akt in the adult heart in vivo and suggest that much of the short-term anatomic and functional impairment in this setting is responsive to Akt activation. These data also suggest but do not prove that Akt activation could contribute to the cardioprotective effects of agents that activate Akt, such as insulin-, IGF-1-, and gp130-dependent cytokines.

Akt activation dramatically improved cardiac function. To determine whether this reflected simply infarct reduction or a direct functional benefit, we explored the effects of myr-Akt on hypoxic cardiomyocyte dysfunction in vitro. In this system, most cardiomyocytes survive hypoxia but exhibit

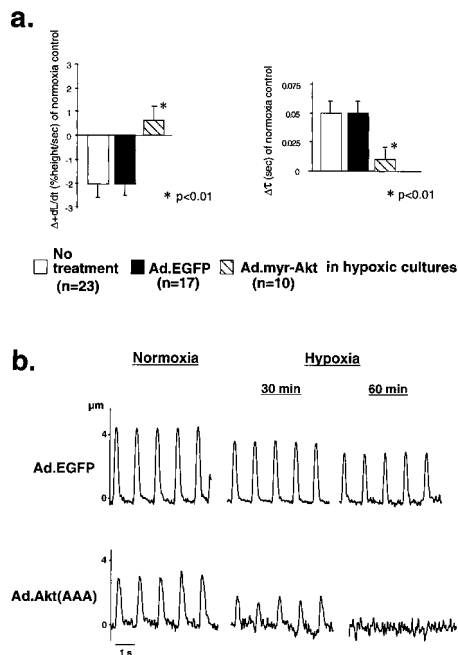


Figure 5. Cardiomyocyte function in vitro. After 24 hours of hypoxia, Ad.myr-Akt improved myocyte contraction and calcium handling vs uninfected or Ad.EGFP-infected hypoxic controls. Cumulative data show change in cardiomyocyte shortening-relengthening and calcium flux at 24 hours vs normoxic cardiomyocytes (a). Ad.myr-Akt preserved +dL/dt at levels comparable to normoxic controls and significantly better than hypoxic uninfected or Ad.EGFP-infected cells at 24 hours. myr-Akt also prevented prolongation of calcium transient reflected by τ despite 24 hours' hypoxia, preserving calcium flux at a level comparable to normoxic controls. Data are cumulative, with each condition tested indicated number of times. Cardiomyocytes expressing dominant negative Akt demonstrated markedly accelerated hypoxia-induced dysfunction at 30 and 60 minutes vs control virus-infected cells (b). Representative tracing from 1 of 3 experiments.

abnormalities of excitation-contraction coupling that may model the clinically important cardiac dysfunction seen in viable but underperfused myocardium.¹¹ Remarkably, Akt activation preserved the function of hypoxic cardiomyocytes at levels comparable to those of normoxic controls. In contrast, dominant negative Akt accelerated hypoxia-induced cardiomyocyte dysfunction and death. Thus, Akt is an important modulator of function in cardiomyocytes, and its ability to improve function in surviving cells is probably a contributing mechanism to the in vivo benefits observed. The broader implications of this observation are that Akt and possibly other apoptosis-related signaling pathways may modulate overall cardiac function in vivo by affecting both cardiomyocyte death and the function of surviving cells.

Many targets of Akt affect cell survival and/or inflammation and thus could modulate IR. We found no direct evidence for involvement of many of these, however. Neither expression of Bcl-2²¹ nor phosphorylation of BAD²² or GSK-3 β ²³ was altered by myr-Akt expression in the heart (data not shown). Nevertheless, we cannot exclude the possibility that subtle changes in localization or phosphorylation of these targets contribute to our observations. Akt activation of eNOS^{24,25} could reduce IR injury through NO inhibition of

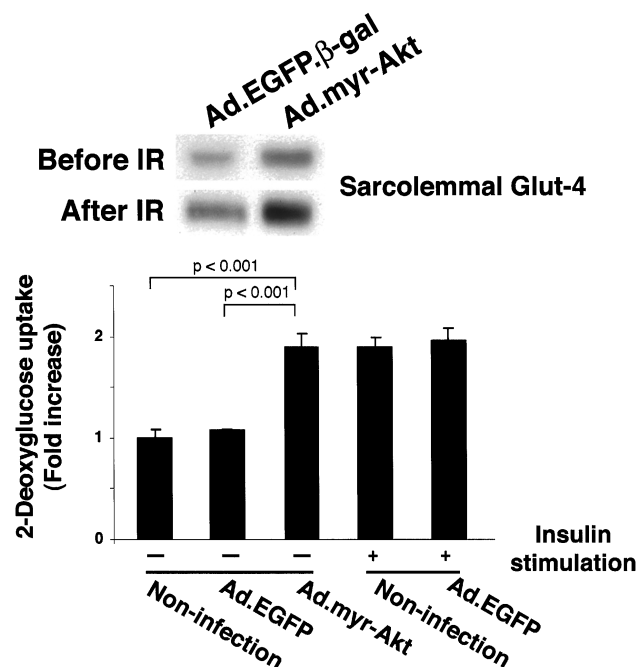


Figure 6. Akt targets. Cardiac sarcolemmal Glut-4 expression was increased by Akt activation in vivo both before and after IR (top). Representative data from 1 of 3 independent experiments. Bottom, myr-Akt enhanced cardiomyocyte glucose uptake in vitro to levels seen with insulin treatment (200 nmol/L). Representative data from 1 of 3 independent experiments performed in triplicate.

neutrophil infiltration. Myeloperoxidase activity, a specific measure of neutrophil infiltration, however, was not decreased in Akt-infected hearts (data not shown). Akt can also directly inhibit caspase-9.²⁶ Previous studies using pharmacological caspase inhibitors in IR have reported reductions in TUNEL-positive nuclei comparable to ours but significantly smaller reductions in infarct size.^{27,28} This comparison suggests that other targets of Akt activation play a role in the benefits we observed. Moreover, Akt phosphorylates human but not mouse or rat caspase-9.²⁹ In contrast, Akt-induced translocation of the dominant cardiac glucose transporter, Glut-4, occurs in both rodent and human skeletal muscle.^{14,20} Akt significantly increased sarcolemmal Glut-4 expression in vivo and cardiomyocyte glucose uptake in vitro to levels seen with pharmacological insulin treatment. These observations provide confirmation of activation of Akt-mediated signaling and may also provide a clue to one potential mechanism contributing to our observations. Increased glucose uptake can mitigate both ischemia-induced cardiac dysfunction³⁰ and cardiomyocyte apoptosis,³¹ perhaps through the more favorable bioenergetics of glycolytic metabolism. Intriguingly, the survival benefits of Akt activation are lost when glucose is removed from the media during hypoxia (data not shown), suggesting but not proving the functional relevance of these observations. Given the complexity of Akt signaling, however, it appears unlikely that any one substrate accounts entirely for our observations. Precise definition of the contribution of specific pathways in vivo will require additional investigation, perhaps in genetically manipulated murine models.

Some limitations of this study should be highlighted. We examined only short-term effects of Akt. Whether the observed benefits will be sustained or will extend to other clinically important cardiac conditions is unknown. Nevertheless, our data demonstrate that activation of Akt can mediate a powerful protective effect in IR injury on clinically relevant end points. Akt may represent an important control point determining not only cardiomyocyte survival but function as well.

Acknowledgments

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