Nuclear Targeting of Akt Enhances Ventricular Function and Myocyte Contractility

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Abstract—Cytoplasmic overexpression of Akt in the heart results in a myopathy characterized by organ and myocyte hypertrophy. Conversely, nuclear-targeted Akt does not lead to cardiac hypertrophy, but the cellular basis of this distinct heart phenotype remains to be determined. Similarly, whether nuclear-targeted Akt affects ventricular performance and mechanics, calcium metabolism, and electrical properties of myocytes is unknown. Moreover, whether the expression and state of phosphorylation of regulatory proteins implicated in calcium cycling and myocyte contractility are altered in nuclear-targeted Akt has not been established. We report that nuclear overexpression of Akt does not modify cardiac size and shape but results in an increased number of cardiomyocytes, which are smaller in volume. Additionally, the heart possesses enhanced systolic and diastolic function, which is paralleled by increased myocyte performance. Myocyte shortening and velocity of shortening and relengthening are increased in transgenic mice and are coupled with a more efficient reuptake of calcium by the sarcoplasmic reticulum (SR). This process increases calcium loading of the SR during relengthening. The enhanced SR function appears to be mediated by an increase in SR Ca\textsuperscript{2+}-ATPase\textsubscript{2a} activity sustained by a higher degree of phosphorylation of phospholamban. This posttranslational modification was associated with an increase in phospho–protein kinase A and a decrease in protein phosphatase-1. Together, these observations provide a plausible biochemical mechanism for the potentiation of myocyte and ventricular function in Akt transgenic mice. Therefore, nuclear-targeted Akt in myocytes may have important implications for the diseased heart.

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Key Words: Akt ■ myocyte mechanics ■ myocyte size and number

Protein kinase B, also referred to as Akt, phosphorylates multiple cytoplasmic and nuclear substrates implicated in cell survival and growth of several organs including the heart.\textsuperscript{1} Although myocyte survival and cellular hypertrophy may be viewed as important adaptations of the overloaded heart against the onset of ventricular decompensation,\textsuperscript{2} the targeted expression of constitutively activated Akt to the myocardium has resulted in cardiac hypertrophy\textsuperscript{3-7} and ventricular dysfunction.\textsuperscript{6} In these cases, however, transgene activity was widespread throughout cardiomyocytes at non-physiological levels, raising the possibility that the nuclear accumulation of Akt may retain the antiapoptotic effects of this serine–threonine kinase, without promoting organ hypertrophy and alterations in cardiac performance. In this regard, hearts of mice expressing nuclear-targeted Akt show no evidence of myopathy\textsuperscript{8} in contrast to other cardiac-specific Akt transgenics created with constitutively activated kinase. Targeting of Akt to myocyte nuclei preserves cell viability through the phosphorylation of survival factors within the nucleus that interfere with apoptotic death signaling.\textsuperscript{1,8-11} Thus, whether Akt is expressed in the cytoplasm or in the nucleus, cardiomyocyte apoptosis is inhibited, but whether the absence of cardiac hypertrophy in the latter condition affects differently ventricular hemodynamics is a relevant unanswered question.

In the models of Akt-induced cardiac hypertrophy, the analysis of myocardial and/or myocyte contractility cannot discriminate the effects of increased cell size from those related to the overexpression of Akt on the mechanical properties of the heart and its parenchymal cells.\textsuperscript{7,12} Similarly difficult is the interpretation of changes in sarcoplasmic reticulum (SR) Ca\textsuperscript{2+}-ATPase\textsubscript{2a} (SERCA2a) and other biochemical parameters in hypertrophied myocytes from Akt transgenic mice.\textsuperscript{12} Additionally, the risk of provoking cardiac hypertrophy, a major factor of heart failure in humans,\textsuperscript{13} precludes the feasibility of genetic manipulation of the heart with cytoplasmically overactive Akt constructs. Therefore, we have defined the cardiac phenotype of a transgenic mouse
cardiomyocytes, which are smaller in volume. (Figure 1E and 1F). Therefore, nuclear-targeted Akt does not phospho-Akt at Ser473 increased only in nuclei. This was myocytes had a 10-fold increase in total Akt protein but TG and WT (Figure 1D). Additionally, TG similar in TG and WT; together, there were 27% more LV number of mononucleated and binucleated myocytes in the/LV of TG mice was 60% and 26% higher than in WT, (Figure 2). The pressure values in combination with the anatomical measurements of wall thickness and chamber radius allowed us to compute midwall systolic and diastolic stress. Diastolic and systolic stress did not differ between TG and WT. Therefore, nuclear-targeted Akt overexpression is characterized by enhanced cardiac function.

Myocyte Mechanics and Ca2+ Transients
Isolated myocytes were field stimulated at 1-Hz pacing rate and their mechanical properties were determined by using a video-edge track detection system.15 LV myocytes from TG mice showed enhanced contractile function (Figure 3A and 3B). In comparison with WT myocytes, TG myocytes had an 18% increase in fractional shortening, a 20% increase in maximal rate of contraction (−dP/dt), and a 28% increase in maximal rate of relaxation (+dP/dt). Timing parameters of contraction were similar in both groups of cells, but velocity of shortening and relengthening was faster in TG myocytes.

To define the mechanisms underlying the potentiated myocyte contractility associated with nuclear-targeted Akt, intracellular Ca2+ handling was analyzed.16 Myocytes from TG and WT were loaded with Fluo-3 and were field stimulated at 1 Hz (Figure 3C). Ca2+ transient amplitude was comparable in TG and WT myocytes, but Ca2+ decay was faster in transgenics. TG myocytes showed a 15% and 12% decrease in the time required to reach 50% and 90% baseline fluorescence, respectively. Moreover, the time constant of Ca2+ decay measured by monoeXponential fitting of the data were reduced by 29% in TG myocytes (Figure 3D). Myocytes were then treated with caffeine to measure Ca2+ transients under the condition of maximum release of this cation from the SR. Caffeine abolishes the regulatory role of ryanodine receptor (RyR) channels in the release of Ca2+ from the SR. In the presence of caffeine, the amplitude of Ca2+ transients was 25% higher in TG than in WT (Figure 3E), indicating that Ca2+ loading of the SR was increased in TG myocytes. Therefore, Akt overexpression in myocyte nuclei does not enhance the amplitude of Ca2+ transients but appears to be coupled with a more efficient reuptake of Ca2+ by the SR during relengthening.

Stimulation Frequencies, Ca2+ Transients, and Sarcomere Mechanics
To determine whether TG myocytes possessed a better functioning SR than WT myocytes, Cr2+ handling and sarcomere shortening were measured simultaneously. Different rates of stimulation were used to assess the rate dependency of intracellular developed Ca2+ and myocyte contractility. TG and WT myocytes responded differently to an increase in pacing rate from 0.5 Hz to 1 Hz and 2 Hz (Figure 4A). Ca2+ transient amplitude and sarcomere shortening at 0.5 Hz were measured, and the corresponding values at 1 and 2 Hz were
normalized with respect to those detected at 0.5 Hz. This was done to have a uniform reference point for comparison and to express the data at higher frequency as a percent change of the results at 0.5 Hz. A negative percent change in Ca\textsuperscript{2+}/H\textsubscript{11001} transients was detected in WT myocytes when they were stimulated at 1 and 2 Hz. Conversely, a positive percent change in Ca\textsuperscript{2+}/H\textsubscript{11001} transients was seen in TG myocytes at 1 and 2 Hz (Figure 4B). Similarly, there was a consistent decrease in sarcomere shortening in WT myocytes and a consistent increase in sarcomere shortening in TG myocytes with increasing frequency of stimulation.

The distinct changes in Ca\textsuperscript{2+} transients of WT and TG myocytes with rates of stimulation can reflect different changes in the amount of Ca\textsuperscript{2+} available for release from the SR, alterations in the trigger of Ca\textsuperscript{2+} release, modifications in the process of Ca\textsuperscript{2+} release, or a combination of these.

**Figure 1.** Cardiac phenotype. A, Gross cardiac characteristics (WT, n=7; TG, n=10). RV indicates, right ventricular; Wt, weight. B, Optical sectioning by confocal microscopy of mononucleated (top), binucleated (middle), and tetrancleated (bottom) myocytes. Bars=20 μm. C, Volume distribution of myocytes (WT, n=659; TG, n=672). D, Average volume and number of LV myocytes (WT, n=7; TG, n=8). E and F, Expression of Akt and PI3K 110\textsubscript{α} in myocytes. Expression and phosphorylation levels for nuclear (Nuc) and cytoplasmic (Cyto) Akt in WT (n=4) and TG (n=4) myocytes. Data are mean±SEM. *P<0.05 vs WT. Mono indicates mononucleated; Bi, binucleated; Multi, multinucleated.
phenomena. These possibilities correspond, respectively, to 
Ca\(^{2+}\) loading of the SR, kinetics of L-type Ca\(^{2+}\) channels, and 
opening of RyR channels. For this purpose, the stimulation of 
myocytes at 2 Hz was followed by a test pulse that was 
applied after a 2-sec pause. This pause period relieves a 
potential inactivation of the L-type Ca\(^{2+}\) channels and/or RyR 
channels. In both WT and TG myocytes, Ca\(^{2+}\) transient 
amplitude and peak were comparable before and after the 
pause (Figure 4C), indicating that the release of Ca\(^{2+}\) at this 
pacing rate was not affected by the kinetics of L-type Ca\(^{2+}\) 
and RyR channels. Therefore, the differential response of WT 
and TG myocytes to the increased rates of stimulation is most 
likely dependent on the enhanced Ca\(^{2+}\) loading of the SR in 
TG myocytes.

**L-type Ca\(^{2+}\) Current and RyR Channels**

Ca\(^{2+}\) influx from the extracellular compartment to the inside 
of the cell via L-type channels plays a crucial role in 
excitation–contraction coupling by triggering the release of 
Ca\(^{2+}\) from the SR through the activation of the RyR chan-

nels. Although Ca\(^{2+}\) loading of the SR was greater in TG 
myocytes than in WT myocytes, Ca\(^{2+}\) transients were similar 
in the 2 groups of cells. The high level of Ca\(^{2+}\) in the SR of 
TG myocytes, in the absence of a corresponding increase in 
Ca\(^{2+}\) transients, raised the possibility that a defect may be 
present in the L-type Ca\(^{2+}\) current (\(I_{CaL}\)), which triggers the 
release of Ca\(^{2+}\) from its site of storage. Alternatively, the 
density and phosphorylation state of the RyR channels may 
be lower in TG myocytes attenuating Ca\(^{2+}\) flux from the SR 
and modulating Ca\(^{2+}\) flux from the SR to the cytoplasm are similar in TG and WT myocytes.

SERCA2a, Phospholamban, and 
Na\(^{+}\)–Ca\(^{2+}\) Exchange

SERCA2a and the Na\(^{+}\)–Ca\(^{2+}\) exchange (NCX) are the pre-
dominant contributors of the influx of Ca\(^{2+}\) into the SR and 
the extrusion of Ca\(^{2+}\) from the SR to the extracellular 
compartment, respectively. The mitochondrial Ca\(^{2+}\) uniporter 
is involved in a small reuptake of Ca\(^{2+}\) from the cytoplasm 
and the sarcolemmal Ca\(^{2+}\)-ATPase participates modestly in
the transport of Ca\(^{2+}\) outside of the cell.\(^{17}\) Phospholamban (PLB) regulates the function of SERCA2a, and the nonphosphorylated form of PLB inhibits SERCA2a and thereby the reuptake of Ca\(^{2+}\) by the SR. Conversely, the phosphorylated form of PLB promotes the role of SERCA2a and the transport of Ca\(^{2+}\) into the SR.\(^{21}\) Because of the major function that SERCA2a-PLB plays in the reuptake of Ca\(^{2+}\) into the SR (\(\approx 92\%\)) and NCX in the extrusion of Ca\(^{2+}\) to the extracellular space (\(\approx 7\%\)) during relengthening, these systems were characterized biochemically. This was done in an attempt to identify the molecular basis of the enhanced Ca\(^{2+}\) transient decay and increased Ca\(^{2+}\) loading of the SR in myocytes with nuclear-targeted Akt.

The quantity of SERCA2a was not different between TG and WT myocytes by Western blotting (Figure 6A) or immunocytochemistry (Figure 6B). Similarly, the amount of PLB was comparable in TG and WT myocytes (Figure 6C and 6D), but the level of phosphorylation of the monomeric and pentameric forms of PLB at Ser16 was increased in TG myocytes (Figure 6E and 6F). NCX protein increased in TG myocytes (Figure 6G and supplemental Figure II), but this effect on Ca\(^{2+}\) metabolism was blunted by enhanced PLB function. Together, these observations indicate that the faster decay of Ca\(^{2+}\) in TG myocytes is mediated by PLB phosphorylation and increased Ca\(^{2+}\) loading of the SR.

Phospholamban Function

PLB can be phosphorylated at Ser16 by cAMP-dependent protein kinase A (PKA),\(^{22}\) and at Thr17 by Ca\(^{2+}\)-calmodulin-dependent kinase (CaMKII).\(^{23}\) Dephosphorylation of PLB is mediated by protein phosphatase-1 (PP1) and protein phosphatase-2a (PP2a).\(^{24}\) The expression of phospho-PKA\(\alpha\) was higher in TG myocytes, whereas PKA\(\alpha\) and phospho-CaMKII were similar in the 2 groups of cells. Moreover, the level of PP1 but not PP2a was decreased in TG myocytes (Figure 7A). Protein kinase C \(\alpha\) (PKC\(\alpha\)) increases PP1 activity that, in turn, attenuates PLB phosphorylation.\(^{25}\) PKC\(\alpha\) protein was increased in TG myocytes, but the protein amount does not necessarily reflect upregulation of kinase activity (Figure 7A and supplemental Figure III). Therefore, the enhanced function of phospho-PKA\(\alpha\) may account for the reduction in PP1 phosphatase activity, which was implicated in the phosphorylation of PLB and the potentiation of SERCA2a.

Myofilament Proteins

TG myocytes had greater fractional shortening than WT myocytes in spite of similar Ca\(^{2+}\) transient amplitude. However, TG myocytes showed improved Ca\(^{2+}\) homeostasis, resulting in enhanced lusitropic function and shortening–frequency relationship and increased Ca\(^{2+}\) loading of the SR. In this regard, an increased myofilament Ca\(^{2+}\)-binding affinity was documented by plotting cytosolic Ca\(^{2+}\) and sarcomere length during steady-state contraction. The terminal portion of these hysteresis loops (Figure 7B), when Ca\(^{2+}\) decays slowly, is indicative of Ca\(^{2+}\)-binding affinity to the myofilaments. This relationship was shifted upwards in TG myocytes, pointing to increased sarcomere shortening for any given cytosolic Ca\(^{2+}\). These results support the notion that increases in myofilament Ca\(^{2+}\) responsiveness, together with an increased rate of SR Ca\(^{2+}\) uptake, promote a higher contractile function in TG myocytes. However, the lack of information on skinned fibers suggests caution in the interpretation of these results.

The state of phosphorylation of myosin light chain-2 (MLC-2) increases systolic function.\(^{26,27}\) Phospho–MLC-2 increases the force development of myocytes in response to intracellular [Ca\(^{2+}\)]. This positive inotropic effect of phospho–MLC-2 appears to be linked to an increase in cross-bridge–cycling kinetics that, in turn, increases the amount of force generation at a given intracellular [Ca\(^{2+}\)]. The protein level and state of phosphorylation of MLC-2 evaluated by Western blotting and immunocytochemistry were comparable in TG and WT myocytes (Figure 7C and
Therefore, enhanced myocyte shortening in TG cannot be accounted for by changes in phospho–MLC-2.

Discussion

The results of the current study indicate that the overexpression of Akt in myocyte nuclei had profound effects on the structure and function of the heart in the absence of cardiac hypertrophy. The ventricular myocardium was composed of a larger number of myocytes, which were smaller in size. Although myocardial mass and chamber volume were comparable in TG and WT, baseline ventricular hemodynamics was increased in TG and the enhanced cardiac performance involved both systolic and diastolic function. These positive changes at the organ level were sustained by corresponding positive changes in the mechanical behavior of ventricular myocytes. Peak shortening, velocity of shortening, velocity of relengthening and Ca\(^{2+}\)/H\(_{11001}\) handling were all improved in TG myocytes. These parameters of potentiated myocyte contractility were paralleled by an increased activity of SERCA2a mediated by an increased phosphorylation of PLB. Together, these cellular adaptations associated with the nuclear localization of Akt in myocytes demonstrate that targeted expression of this serine–threonine kinase may have important clinical implications for the diseased heart.

Nuclear-Targeted Akt and the Heart Phenotype

Akt is a downstream effector molecule initiated by the activation of PI3K that is part of the signaling pathway modulated by the insulin-like growth factor (IGF)-1/IGF-1 receptor system or insulin. Phospho-Akt is translocated to the nucleus where it phosphorylates transcription factors for genes that oppose cell death and promote cell growth. The cytoplasmic overexpression of Akt in the heart leads to cardiac hypertrophy that is fully accounted for by an increase in myocyte volume without an increase in myocyte number. Conversely, the forced expression of IGF-1 results in an increase in the number of cardiomyocytes and skeletal muscle cells because IGF-1 is a powerful inducer of cell proliferation and survival. With time, IGF-1 is associated with an increase in myocyte size, although myocyte multiplication remains the predominant cellular response. Therefore, similarities exist in the role of cytoplasmic-targeted Akt and IGF-1 in cell viability, but Akt and IGF-1 have a substantially different impact on the pattern of myocyte growth.

An important finding of the current study is that nuclear-targeted Akt resulted in a cardiac phenotype, which was superior to that obtained with the cytoplasmic localization of Akt and the overexpression of IGF-1. The ventricular muscle mass was constituted by an increased number of myocytes, which were smaller in volume preventing the development of cardiac hypertrophy. This novel form of myocardial assembly was characterized by enhanced cardiac function measured echocardiographically and hemodynamically. The potentiation of cardiac performance in nuclear-targeted Akt mice was not observed with cardiac restricted IGF-1 overexpression or with the cytoplasmic localization of Akt.

Rota et al. Akt and Myocyte Mechanics

Figure 4. Rate of stimulation, myocyte contraction, and Ca\(^{2+}\) transients. A, Simultaneous recording of Ca\(^{2+}\) transients and sarcomere shortening at 0.5-, 1-, and 2-Hz pacing rates. B, Relative changes in transient amplitude (ta) and sarcomere shortening (ss) at increasing rate of stimulation (WT, n=17; TG, n=20). C, Peak Ca\(^{2+}\) transients at 2-Hz pacing rate and after 2-sec pause (test) (WT, n=17; TG, n=11). Data are mean±SEM. *P<0.05 vs WT.
myocytes. In this regard, the infection of neonatal myocytes with an adenovirus carrying nuclear-targeted Akt does not result in an increased phosphorylation of cytoplasmic substrates of Akt, such as GSK3β and Bad. Under this setting, Akt function is exerted almost exclusively at the level of the nucleus. This explains, at least in part, why the molecular consequences of Akt activation in myocytes are dramatically different when excess Akt activity is present in the cytoplasm as opposed to the nucleus. Cytoplasmic-targeted Akt phosphorylates GSK3β that induces myocyte hypertrophy and interferes with the activation and commitment of cardiac progenitor cells. This is not the case in nuclear-targeted Akt, in which a significant increase in the generation of myocytes has been found by growth and differentiation of resident progenitor cells (M.S. and P.A., unpublished observations, 2005).

Nuclear-Targeted Akt and Myocyte Mechanics

Myocyte contraction and relaxation are under the control of the rise and decline in cytosolic Ca2+ levels. During the action potential, Ca2+ enters the cell via sarcolemmal L-type channels and I_{calc} triggers the release of Ca2+ from the SR by activating the RyR channels. The rise in intracellular free Ca2+ initiates contraction through the binding of Ca2+ to the myofilaments, whereas myocyte relaxation is promoted by the decrease in intracellular Ca2+ and dissociation of Ca2+ from the myofilaments. Lowering in cytosolic Ca2+ is accomplished mainly by Ca2+ sequestration into the SR by

Figure 5. Myocyte Ca2+ current. A, I_{calc}–voltage relationship in WT (n=3) and TG (n=6) myocytes. B, I_{calc} activation and inactivation curves in WT (n=13, n=14) and TG (n=16, n=10) myocytes. Fitting of the curves with Boltzmann equations indicated half-maximal activation potential of −14.19±1.20 mV for WT and −12.18±1.10 mV for TG myocytes. Potential giving 50% inactivation for I_{calc} was −24.09±1.36 mV for WT and −23.34±1.69 mV for TG. C, Biexponential fitting on I_{calc} inactivation using a step to 0 mV and average fast and slow time constants for WT (n=12) and TG (n=15) myocytes. D, I_{calc} recovery from inactivation for WT (n=12) and TG (n=14) myocytes. Data are mean±SEM. E, Localization of RyR (green fluorescence) in an isolated myocyte. Nuclei are stained by propidium iodide (blue). Bar=10 μm. The intensity of fluorescent signal for RyR was 88±4 arbitrary units (AU) in WT myocytes (n=52) and 79±3 AU in TG (n=63). F, Western blotting of RyR protein and phospho-RyR in WT (n=6) and TG (n=6). Optical density (O.D.) data were not statistically different.
SERCA2a and Ca\(^{2+}\) extrusion from the cell by the NCX.\(^{32}\) In its phosphorylated form, PLB enhances SERCA2a and Ca\(^{2+}\) transport into the SR, regulating the rate of cardiac relaxation and the amount of Ca\(^{2+}\) stored in the SR.\(^{21}\)

The ability of nuclear-targeted Akt to modify the mechanical behavior of myocytes in the absence of cellular hypertrophy has no precedent. The lack of changes in developed Ca\(^{2+}\), together with the increase in myofilament Ca\(^{2+}\) sensitivity, is consistent with the increase in fractional shortening and velocity of shortening and relengthening of TG myocytes. These properties differ somehow from the effects that IGF-1 overexpression has on myocyte contractility and myofilament Ca\(^{2+}\) sensitivity.\(^{33}\) Similarly, the consequences of nuclear-targeted Akt on myocyte performance markedly diverge from those associated with the cytoplasmic localization of this kinase.\(^{12}\) Conversely, nuclear-targeted Akt shows some of the properties of β-adrenergic stimulation,\(^{17}\) which potentiates myocyte mechanics by enhancing PKA and, thereby, the phosphorylation of PLB. Activation of β-receptors leads to myocyte apoptosis,\(^{34,35}\) whereas Akt protein has a potent antiapoptotic function. Because attenuation of cell death by β-blockers has been implicated in the

Figure 6. Expression of Ca\(^{2+}\) regulatory proteins. A, Western blotting of SERCA2a in WT (n=4) and TG (n=4) myocytes. B, SERCA2a localization (red fluorescence) in an isolated myocyte. Bar=10 μm. The intensity of fluorescent signal for SERCA2a was 62±3 arbitrary units (AU) in WT myocytes (n=60) and 55±4 AU in TG (n=39). C, Western blotting of PLB in WT (n=8) and TG (n=8) myocytes. D, PLB localization (yellow fluorescence) in an isolated myocyte. Bar=10 μm. The intensity of fluorescent signal for PLB was 77±5 AU in WT (n=42) and 67±4 AU in TG (n=51) myocytes. E, Western blotting of phospho-PLB in WT (n=8) and TG (n=8) myocytes. F, Phospho-PLB localization (magenta fluorescence) in an isolated myocyte. Bar=10 μm. The intensity of fluorescent signal for phospho-PLB was 21±1 AU in WT (n=48) and 60±3 AU in TG (P<0.001; n=64) myocytes. G, Western blotting of NCX in WT (n=8) and TG (n=8) myocytes. Optical density (O.D.) data for Western blotting are mean±SEM; in these cases, *P<0.05 vs WT.
favorable outcome of heart failure in patients, nuclear-targeted Akt possesses all the beneficial consequences of enhanced myocyte contraction present with β-adrenergic stimulation but, in contrast to β1 receptor activation, has a powerful positive effect on myocyte survival.1,8

The improved myocyte contractility with nuclear-targeted Akt is largely related to the influx of Ca2+ into its site of storage with increased loading of the SR. The increase in SERCA2a activity by PLB phosphorylation may represent a potential mechanism for increased SR function in TG. Phosphatase PP1, which dephosphorylates PLB, is downregulated in TG myocytes, providing an additional pathway for an effective reuptake of Ca2+ by the SR. Depressed myocyte mechanics and abnormal intracellular Ca2+ cycling are typical features of the failing human heart. A decrease in PLB phosphorylation and an increase in expression and activity of NCX have been implicated in the defects of Ca2+ loading of the SR in human heart failure.21,32,36,37 Impaired SR function results in an elevation in Ca2+ concentration in the cytoplasm, which, in turn, leads to the formation of reactive oxygen species and the initiation of the endogenous cell death pathway.38,39 The nuclear localization of Akt operates against these negative cellular processes and support the notion that nuclear targeting of Akt may be a candidate strategy for cardiac failure.

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Materials and Methods

Animals

Transgenic mice in which the nuclear-targeted Akt cDNA construct was subcloned downstream of the 5.5-kb mouse alpha-myosin heavy chain gene promoter were used. Genomic DNA was isolated from the tails and was analyzed by Southern blots. Experiments were performed in male transgenic mice (TG) and wild-type littermates (WT) at 3 months of age. Animals used in this study were maintained in accordance with the Guide for the Care and Use of Laboratory Animals and the experiments were approved by New York Medical College.

Myocyte volume and number

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 contained (in mmol/L) 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 mmol/L 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 mmol/L 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.

Isolated myocytes were fixed in 10% buffered formalin for the determination of myocyte volume. Myocyte nuclei were stained by propidium iodide (PI) and the cell cytoplasm by α-sarcomeric actin. Cell length and width were measured with a
computerized image analysis system. Since isolated cells assume a cross-sectional area that resembles a flattened ellipse, the ratio of the minor to the major axis of the ellipse was obtained by confocal microscopy. Cell volume was calculated assuming an elliptical cross-section with the major axis equivalent to cell width while the minor axis was computed from the measured ratios. Additionally, myocyte volume in each cell class was confirmed by three-dimensional optical section reconstruction by confocal microscopy. In each animal, 100-150 myocytes were measured in this manner. Sections of the LV were stained with hematoxylin and eosin and examined at X1,000 magnification with a morphometric reticle containing 42 sampling points to determine the volume fraction of myocytes in the myocardium. The total volume of myocytes in the LV was calculated from the product of LV volume and the volume percent of myocytes. The volume fraction of myocytes and the relative proportion of mononucleated, binucleated and multinucleated myocytes were utilized to compute the volume percent of each myocyte class in the tissue. The number of mononucleated, binucleated and multinucleated cells in the LV was computed from the quotient of their aggregate volume and the corresponding myocyte cell volume. These determinations were performed in 8 TG and 7 WT mice.

**Cardiac function**

Echocardiography was performed in conscious mice using a Sequoia 256c (Acuson, Mountain View, CA) equipped with a 13-MHz linear transducer (15L8). The anterior chest area was shaved and two-dimensional (2D) images and M-mode tracings were recorded from the parasternal short axis view at the level of the papillary muscles. Ejection fraction (EF) was calculated by the machine software algorithm, from the parasternal long axis view using the single plane area length method.

At sacrifice, mice were anesthetized with chloral hydrate (400 mg/kg body weight, i.p.) and a microtip pressure transducer (SPR-671, Millar Instruments, Houston, TX) connected to a chart recorder (Gould ES 2000, Gould, Inc., Oxnard, CA) was advanced into the LV and cavitory pressures and + and – dP/dt in the closed-chest preparation were obtained.
**Cardiac anatomy**

The abdominal aorta was cannulated with a polyethylene catheter and filled with phosphate buffer (0.2 M, pH 7.4) and heparin (100 IU/ml). In rapid succession, the heart was arrested in diastole by injection of 0.15 ml cadmium chloride (100 mmol/L) through the aortic catheter, the thorax was opened, perfusion with phosphate-buffered formalin was started, and the right atrium was cut to allow drainage. Perfusion pressure was adjusted to the mean arterial pressure. The left ventricular chamber was filled with fixative from a pressure reservoir set at a height equivalent to the in vivo-measured end-diastolic pressure for a 20-min fixation, a task accomplished by inserting a 25G3/4 Vacutainer (Becton Dickinson Co., Rutherford, NJ) into the left ventricle through the apex. At the end of the procedure, the left ventricle including the septum and the right ventricle were dissected and weighed. After measuring the major longitudinal intracavitary diameter, each left ventricle was serial-sectioned into three rings perpendicular to the major axis of the heart, after which the thickness of the free wall and septum and the transverse chamber diameter were measured by an image analyzer. At the mid-region, the minimal and maximal luminal diameters were used with the long axis to compute chamber volume. Systolic chamber radius and systolic wall thickness were calculated from the echocardiographic parameters. Measurements of wall thickness, chamber radius, and ventricular pressure were used to calculate diastolic and systolic wall stress.\(^3\,^6\)

**Cell shortening and Ca\(^{2+}\) transients**

Hearts were excised and left ventricular myocytes were enzymatically dissociated in manner slightly different from that employed for morphometric measurements and biochemical determinations. Briefly, perfusion of the myocardium was performed at 37° C with a Ca\(^{2+}\)-free solution gassed with 85% O\(_2\), 15% N\(_2\). After 5 min perfusion, 0.1 mmol/L CaCl\(_2\), 274 units/mL collagenase (type 2, Worthington Biochemical Corp, Lakewood, NJ) and 0.57 units/mL protease (type XIV, Sigma, St. Louis, MO) were added. Ca\(^{2+}\)-free solution contained (mmol/L): NaCl 126, KCl 4.4, MgCl\(_2\) 5, HEPES 5,
Glucose 22, Taurine 20, Creatine 5 Na Pyruvate 5 and NaH2PO4 5 (pH 7.4, adjusted with NaOH). After digestion, the heart was removed from the apparatus, the LV cut in small pieces and re-suspended in Ca2+ 0.1 mmol/L solution. Isolated LV myocytes were placed in a bath on the stage of an IX71 Olympus inverted microscope (Olympus, Melville, NY) for contractility, Ca2+ transients and patch-clamp measurements. Experiments were conducted at room temperature.

Cells were bathed continuously with a Tyrode solution containing (mmol/L): NaCl 140, KCl 4, MgCl2 1, HEPES 5, Glucose 5.5 and CaCl2 1.0 (pH 7.4, adjusted with NaOH). Measurements were performed in field-stimulated cells by using IonOptix fluorescence and contractility systems (IonOptix, Milton, MA) and by video edge detection (Crescent Electronics, Salt Lake City, UT). Contractions were elicited by rectangular depolarizing pulses, 2 ms in duration, and twice-diastolic threshold in intensity, by platinum electrodes.9,10 Changes in cell length were computed by edge track detection, while changes in mean sarcomere length were computed by determining the mean frequency of sarcomere spacing by fast Fourier transform and then frequency data were converted to length. Ca2+ transients were measured by epifluorescence after loading the myocytes with 10 μM Fluo-3 AM (Molecular Probes, Eugene, OR). Excitation length was 480 nm with emission collected at 535 nm using a 40x oil objective. Fluo-3 signals were expressed as normalized fluorescence (F/F0). Caffeine–induced Ca2+ transient was evoked by rapid application of 20 mmol/L caffeine to assess SR-Ca2+ load.11

**L-type Ca2+ current**

Data were acquired by means of the whole-cell patch clamp technique in voltage-clamp mode using a Multiclamp 700A amplifier (Axon Instruments, Union City, CA). Electrical signals were digitized using a 500 kHz 16-bit resolution A/D converter (Digidata 1322, Axon Instruments) and recorded using pCLAMP 9.0 software (Axon Instruments) with low-pass filtering at 2 kHz.10
L-type Ca\(^{2+}\) Current (\(I_{\text{CaL}}\)) properties were assessed using a Na\(^{+}\)-K\(^{+}\) free perfusing solution\(^{12}\) of the following composition (mmol/L): N-methyl-D-glucamine (NMDG) 140, CsCl 4, MgCl\(_2\) 1, HEPES 5, glucose 5.5, CaCl\(_2\) 1 and 4-aminopyridine 2 (pH 7.4 with CsOH). The composition of the pipette solution was (mmol/L): NMDG 10, CsCl 113, MgCl\(_2\) 0.5, Tris-ATP 5, glucose 5.5, HEPES 10, EGTA 5 and TEA-Cl 20 (pH 7.2 with CsOH). The pipettes were pulled by means of a Narishige PB-7 glass microelectrode puller (Narishige, Tokyo, Japan) and when filled had a resistance of 1-2 MΩ.

\(I_{\text{CaL}}\) current-voltage (I-V) relation and activation properties were determined applying depolarizing steps 300 ms in duration from holding potential (\(V_h\)) –70 mV in 5 mV increments. For inactivation properties, a 300 ms preconditioning step was applied within the range –70 to +60 mV in 10 mV prior to the 300 ms test step to 0 mV. The half maximal activation potential and the potential giving 50% inactivation for \(I_{\text{CaL}}\) was obtained by fitting the activation and inactivation curves with a Boltzmann equation.\(^{12}\) Inactivation time course of \(I_{\text{CaL}}\) was described by fitting the current traces between the inward peak and the end of the 300 ms pulse to 0 mV (from \(V_h\) –70 mV) with a biexponential function. Recovery from inactivation was studied by a double pulse protocol to 0 mV with variable interpulse duration.

**Western blotting**

Isolated myocytes were obtained from WT and TG hearts as described above. Whole myocyte proteins were obtained with a lysis buffer (50 mmol/L Tris-HCl, pH 7.4; 5 mmol/L EDTA; 250 mmol/L NaCl; and 0.1% Triton X-100) containing the protease inhibitors PMSF, 2 mmol/L, aprotinin, 1 µg/mL, DTT, 5 mmol/L, Na\(_3\)VO\(_4\), 1 mmol/L. Following 30 minutes in ice, lysates were centrifuged at 14,000 rpm for 10 minutes. Myocyte cytoplasmic and nuclear proteins were extracted by incubating the cells in hypotonic buffer and subsequently the nuclei in hypertonic buffer. Equivalents of 50 µg of proteins were separated by 6-15% SDS-PAGE, and transferred onto nitrocellulose filters. Blots for RyR, phospho-RyR, SERCA2α, PLB, phospho-PLB, NCX, PKAα, phospho-PKAα, phospho-CaMKII, PP1, PP2a, PKCa, MLC2, phospho-MLC2, Akt,
phospho-Akt and PI-3K were incubated overnight at 4°C with rabbit polyclonal anti-RyR and anti-phospho-RyR antibodies, mouse monoclonal anti-SERCA2a antibody (Abcam, Inc., Cambridge, MA), mouse monoclonal anti-PLB antibody (Upstate Biotechnology, Lake Placid, NY), rabbit polyclonal anti-phospho-PLB antibody (Upstate Biotechnology), mouse monoclonal anti-NCX antibody (Chemicon International, Inc., Temecula, CA), rabbit polyclonal anti-PKAα antibody (Santa Cruz Biotechnology, Santa Cruz, CA), goat polyclonal anti-phospho-PKAα antibody (Santa Cruz Biotechnology), goat polyclonal anti-phospho-CaMKII (Santa Cruz Biotechnology), mouse monoclonal anti-PP1 antibody (Santa Cruz Biotechnology), rabbit polyclonal anti-PP2a antibody (Santa Cruz Biotechnology), mouse monoclonal anti-PKCα antibody (Santa Cruz), rabbit polyclonal anti-MLC2 antibody (Abcam Inc.), goat polyclonal anti-Akt antibody (Santa Cruz Biotechnology) and rabbit polyclonal anti-phospho-Akt antibody (Upstate) in Tris-buffered saline containing 0.1% Tween 20 and 5% nonfat milk. Antibodies were used at concentrations suggested by manufacturers. HRP-conjugated IgG were used as secondary antibodies. Proteins were detected by chemiluminescence and optical density (OD) was measured with an image analyzer (Gel Doc 1000, Bio-Rad, Hercules, CA).

**Immunocytochemistry**

The distribution of RyR, SERCA2a, PLB, phospho-PLB and MLC2 was determined by immunocytochemistry. Freshly isolated myocytes were washed in PBS and fixed for 20 minutes in 4% paraformaldehyde. The same antibodies indicated under Western blotting were employed. Nuclei were stained with PI. Images were analyzed using Bio-Rad Radiance 2100 confocal microscope. Fluorescence intensity was measured utilizing ImagePro software.

**Data Analysis**

Data are presented as mean±S.E.M. Significance between two groups was determined by unpaired two-tailed Student’s t-test. P<0.05 was considered significant.
References


**Legends to Supplemental Figures**

**Figure S1.** Western blotting of RyR protein and phospho-RyR in WT and TG. For OD, n values and statistics, see Figure 5E.

**Figure S2.** Western blotting of phospho-PLB, NCX in WT and TG myocytes. For OD, n values and statistics see Figure 6.

**Figure S3.** Western blotting of phospho-PKAα and phospho-CaMKII. For OD, n values and statistics see Figure 7.
Figure S3

- p-PKAα
- actin
- p-CaMKII
- actin

WT TG