Phenotypic Spectrum Caused by Transgenic Overexpression of Activated Akt in the Heart

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Phenotypic Spectrum Caused by Transgenic Overexpression of Activated Akt in the Heart*

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The serine-threonine kinase, Akt, inhibits cardiomyocyte apoptosis acutely both in vitro and in vivo. However, the effects of chronic Akt activation in the heart are unknown. To address this issue, we generated transgenic mice (TG+) with cardiogenic-specific expression of a constitutively active mutant of Akt (myr-Akt) driven by the myosin heavy chain-α promoter. Three TG+ founders (9–19 weeks) died suddenly with massive cardiac dilatation. Two viable TG+ lines (TG564 and TG20) derived from independent founders demonstrated cardiac-specific transgene expression as well as activation of Akt and p70S6 kinase. TG564 (n = 19) showed cardiac hypertrophy with a heart/body weight ratio 2.3-fold greater than littermates (n = 17, p < 0.005). TG20 (n = 18) had less marked cardiac hypertrophy with a heart/body weight ratio 1.6-fold greater than littermates (n = 17, p < 0.005). Isolated TG564 myocytes were also hypertrophic with surface areas 1.7-fold greater than littermates (p < 0.000001). Echocardiograms in both lines demonstrated concentric hypertrophy and preserved systolic function. After ischemia-reperfusion, TG+ had a 50% reduction in infarct size versus TG− (17 ± 3% versus 34 ± 4%, p < 0.001). Thus, chronic Akt activation is sufficient to cause a spectrum of phenotypes from moderate cardiac hypertrophy with preserved systolic function and cardioprotection to massive cardiac dilatation and sudden death.

In most systems, Akt activation occurs downstream of the lipid kinase, PI 3-kinase, itself a powerful anti-apoptotic signal (1–8, 10). Signaling downstream of PI 3-kinase is complex and includes mitogen-activated protein kinases and p70S6 kinase, in addition to Akt, and appears to modulate many important cell processes including cell metabolism and growth (13, 14). In Drosophila, PI 3-kinase is a critical determinant of organ size and development (15), and recent work suggests it plays a similar role in the mammalian heart (16). The downstream pathways responsible for controlling cardiomyocyte size have not been identified.

Activation of PI 3-kinase leads to D3 phosphorylation of membrane phosphatidylinositol 4,5-bisphosphate, generating phosphatidylinositol 3,4,5-trisphosphate, some of which is converted to phosphatidylinositol 3,4-trisphosphate by an inositol phosphatase (17). Phosphatidylinositol 3,4-trisphosphate and phosphatidylinositol 3,4,5-trisphosphate accumulate in the cell membrane and recruit Akt and PDK1 to the cell membrane by binding to their pleckstrin homology domains, leading to phosphorylation and activation of Akt (18). Because PDK1 is constitutively active, movement of Akt to the sarcolemmal membrane is sufficient to lead to its activation. For this reason, incorporation of the src myristoylation signal creates a constitutively active Akt mutant (10). Downstream substrates of Akt mediate important effects on a broad range of cell functions including cell survival (19–21), inflammation (22–25), and metabolism (26, 27). The relative contribution of these downstream effectors in mediating the cardiac effects of Akt remains undefined.

To examine the effects of chronic Akt activation in the heart, we generated transgenic mice with cardiac overexpression of a constitutively active mutant of Akt (myr-Akt). Mice demonstrated a broad spectrum of phenotypes from sudden death with massive cardiac enlargement to cardiac hypertrophy with preserved systolic function and protection from ischemia-reperfusion injury. Two viable lines have been bred for five generations and characterized more fully. These lines should provide a valuable tool for investigating the effects of Akt activation in chronic models of cardiac disease.

EXPERIMENTAL PROCEDURES
Generation of Transgenic Mice

The cDNA encoding HA-tagged Akt with Src myristoylation (myr) signal (kindly provided by Dr. Thomas F. Franke, Columbia University) was subcloned downstream of the 5.5-kb murine α-myosin heavy chain

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1 The abbreviations used are: PI 3-kinase, phosphatidylinositol 3-kinase; HA, hemagglutinin; ERK, extracellular signal-regulated kinase; SAPK, stress-activated protein kinase; JNK, c-Jun NH2-terminal kinase; MOPS, 4-morpholinepropanesulfonic acid; Ab, antibody; GSK-3, glycogen synthase kinase-3.
FIG. 1. Overexpression of myr-Akt in the heart. A, cardiac overexpression of HA-tagged myr-Akt. Twenty µg of whole lysates from various organs in TG20 were separated by SDS-PAGE. Transgene expression was analyzed by Western blotting, using a monoclonal antibody to the HA epitope. The transgene was expressed in the heart but not in the lung, kidney, or skeletal muscle. Representative data from one of three independent experiments are shown. In TG564, slight expression of transgene was detected in the lung as well (data not shown). B, expression pattern in female and male transgenic mice. Immunoblotting was performed as above. Transgene expression in TG20 females was significantly less than in TG20 males. No difference was seen in transgene expression between female and male TG564 mice. C, Akt kinase activity. Akt kinase activity was examined with homogenates from wild type, TG−, and TG564+ hearts, using the GSK-3α/β fusion protein as substrate, as described under “Experimental Procedures.” The overall level of Akt expression was significantly increased in TG564+ mice (top), as was Akt kinase activity (bottom). Representative immunoblots of two separate experiments, total four independent TG+ mice, are shown.

Western Blotting

Hearts from 8–16-week old mice were removed from deeply anesthetized animals, snap frozen, and crushed in liquid nitrogen before tissue homogenization in cold lysis buffer (20 mM Tris-HCl (pH 7.6), 150 mM NaCl, 1% Triton X-100, 2 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM diithiothreitol, 1 mM sodium orthovanadate, 1 µg/ml leupeptin, 1 µg/ml aprotinin). Protein concentration was measured by the Bradford method (Bio-Rad). SDS-PAGE was performed under reducing conditions on 12% separation gels with a 4% stacking gel. Proteins were transferred to nitrocellulose membrane. Blots were incubated with primary antibodies to HA (12CA5, Roche Molecular Biochemicals), Akt (Cell Signaling), phospho-Akt (Ser-473, Cell Signaling), ERK1/2 (Santa Cruz), phospho-ERK1/2 (Cell Signaling), GSK-3β (Transduction Lab), phospho-GSK-3β (Cell Signaling), SAPK/JNK (Cell Signaling), p38 (Cell Signaling), phospho-p70S6 kinase (Cell Signaling), and p70S6 kinase (Cell Signaling) for 18–20 h at 4 °C. Blots were then incubated with horseradish peroxidase-conjugated secondary antibody and signal detected using enhanced chemiluminescence (Cell Signaling).

Kinase Assays

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

p70S6 Kinase Assay—Lysates were prepared as above and immunoprecipitated with Ab to p70S6 kinase (Cell Signaling). The immobilized immunoprecipitates were washed with HEPES-buffered saline (pH 7.5) containing 0.1% Triton X-100 and incubated for 30 min at 25 °C in reaction mixture containing 25 mM β-glycerophosphate, 20 mM MOPS (pH 7.2), 10 mM MgCl₂, 2 mM EGTA, 0.5 µM protein kinase inhibitor, 1 mM diithiothreitol, 50 µM unlabeled ATP, 5 µCi of [γ-32P]ATP, and 50 µM glycogen synthase peptide-2 (Upstate) as substrate. The mixtures were spotted onto Whatman P81 paper, washed with 0.5% phosphoric acid, and 32P measured by liquid scintillation spectroscopy. Kinase activity was reduced to background levels when 10 mM LiCl was included in the reaction mixture, suggesting the activity measured was GSK-3β (data not shown).

Histological Examination

Tissues were snap-frozen in OCT medium, and 10-µm sections were prepared for hematoxylin and eosin staining. Morphological analysis of hematoxylin and eosin-stained tissue was performed using microscopy and a SONY imaging system.

Cardiomyocyte Isolation

Left ventricular cardiomyocytes were isolated with the perfused-heart method described previously (30). Briefly, after deep anesthesia the mice, hearts were quickly excised, cannulated via the aorta, and perfused in the Langendorf mode with a constant perfusion pressure of 80 mm Hg. The hearts were first perfused for 5 min at 37 °C with 1.8 mM Ca²⁺-Tyrode (in mM: NaCl 137, KCl 5.4, CaCl₂ 1.8, MgCl₂ 0.5, HEPES 10, and glucose 10, pH 7.4), followed by Ca²⁺-free Tyrode for an additional 5 min. They were then perfused with a digestion solution containing 5 mg of collagenase D (Roche Molecular Biochemicals), 17.5 mg of collagenase B (Roche Molecular Biochemicals), and 1.5 mg of protease XIV (Sigma) in 35 ml of Ca²⁺-free Tyrode. After the hearts were palpably flaccid, the digestion solution was washed out with Ca²⁺-free Tyrode solution for 30 s. The left ventricle (including the septum) was cut into small pieces and gently agitated, allowing the myocytes to be dispersed in Ca²⁺-free Tyrode. The isolated myocytes were then fixed with 2% paraformaldehyde for 15 min and washed with phosphate-buffered saline. Using Cytospin2 (Shandon Inc., Pittsburgh),
myocytes suspended with phosphate-buffered saline were placed on glass slides and stained with anti-HA Ab. The Ab was detected using Vectastain ABC-Alkaline Phosphatase kit (VECTOR Lab). Images were digitally captured, and individual cells were traced and surface areas calculated using NIH image (version 1.60).

Echocardiography

Mice were anesthetized with ketamine (100 mg/kg intraperitoneal injection) and the anterior chest was shaved. Cardiac ultrasound imaging was performed in the left lateral decubitus position using a high-frequency 13.0 MHz linear transducer (Acuson Sequoia C256 with a 15L8 linear array) at a frame rate of 166 frames/s and imaging depth set at 10 mm. Estimated ejection fraction was calculated using a modified Simpson’s rule.

Ischemia-Reperfusion Model

TG20 male positive and negative littermates aged 9–12 weeks were subjected to ischemia-reperfusion as previously described (12). Briefly, seven animals from each group were anesthetized (Avertin), intubated, and ventilated. Left thoracotomy was performed and LAD ligated with 7-0 silk. 5 min into ischemia, 50 μl of fluorescent microspheres (10-μm Fluospheres, Molecular Probes) were injected into the LV cavity. After 30 min, the LAD ligature was released, and reperfusion visually confirmed. Mice were sacrificed 24 h after ischemia. Hearts were frozen in liquid N2, and sectioned from apex to base (Jung Frigocut 2800E, Leica) into four 2-mm sections. To delineate the infarct, sections were incubated in 5% (w/v) triphenyltetrazolium chloride (Sigma) in phosphate-buffered saline (pH 7.4) at 37 °C for 20 min. The area-at-risk delineated by fluorescent microspheres was visualized under UV light. For each section, the area-at-risk and infarct area were measured from enlarged digital micrographs using NIH image. %MI was calculated as the total infarction area divided by the total area-at-risk for that heart.

Statistical Analysis

Data are presented as the mean ± S.E. from at least three independent experiments and were compared using a two-tailed Student’s t test. The null hypothesis was rejected at p < 0.05.

RESULTS

Generation of myr-Akt Mice—Cardiac specific expression of constitutively active Akt (myr-Akt) was driven by the murine α-myocin heavy chain promoter, which produces predominantly postnatal ventricular transgene expression (28). Three transgene positive (TG+) founders (two females, one male) died suddenly with massive cardiac dilatation at ages 9–19 weeks. Two viable TG+ lines (TG564 and TG20) were derived from independent founders. Both lines exhibited Mendelian inheritance of the transgene consistent with autosomal (TG564) and X-linked (TG20) transmission stable over 5 generations (data not shown). Western blotting using a monoclonal antibody to the incorporated HA epitope confirmed cardiac-specific expression (Fig. 1A). Transgene expression was comparable in TG564 male and female mice. In TG20 mice, transgene expression was greater in male compared with female mice (Fig. 1B). Immunohistochemical staining in female TG20 mice revealed that not all cardiomyocytes expressed the transgene (data not shown) consistent with the expected inactivation of the transgene-encoding X-chromosome in some cardiomyocytes. Immunoblotting with monoclonal antibody to Akt demonstrated substantial overexpression of the transgene in comparison to the endogenous molecule (Fig. 1C). Akt activity was dramatically increased as measured by an in vitro kinase assay using a synthetic GSK-3α/β fusion protein as substrate (Fig. 1C, bottom).
Procedures. Both lines from 8 to 18 weeks to assess in vivo. Cardiomyocytes did not (Fig. 3B, C). Similarly, there was no difference between TG+ and TG− mice (Fig. 4B). We measured phosphorylation of p38 and p38 kinase activity with ATF-2 as substrate. Although occasional TG+ mice showed modestly enhanced phosphorylation of p38, most did not (data not shown). Moreover, p38 activity was similar between TG+ and TG− mice (Fig. 4C). GSK-3β is phosphorylated and inactivated by Akt (33). GSK-3β inactivation is required for cardiomyocyte hypertrophy in response to some stimuli in vitro (29). However, we found no difference in total or phosphorylated GSK-3β between TG+ and TG− mice (Fig. 4D, upper panel). Similarly, there was no difference in activity (Fig. 4D, lower panel). The ribosomal protein p70S6 kinase is another downstream target of Akt (34), which regulates translation initiation and has previously been reported to promote cellular growth (35, 36) and hypertrophy (37). Phosphorylation of Thr389 correlates well with p70S6 kinase activity (38, 39). In Akt transgenic mice, overall expression of p70S6 kinase was increased, as was phosphorylation at Thr389 as well as Thr421/431.

The abbreviations are: IVS, intraventricular septum; PW, posterior wall; EF, ejection fraction.

$p < 0.05 versus female negative animals.

$p < 0.05 versus TG20 female positive.

$p < 0.05 versus male TG20 positive.

$p = not significant.

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**TABLE II**

**Echocardiographic finding in TG mice**

$n = number of animals. Data are mean ± S.E.

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<td>mm</td>
<td></td>
<td>%</td>
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<td>Female negative (n = 10)</td>
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<td>0.64 ± 0.02</td>
<td>68.0 ± 2.4a</td>
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<td>Male negative (n = 15)</td>
<td>0.73 ± 0.04</td>
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<td>TG20 female positive (n = 13)</td>
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<td>0.84 ± 0.03b</td>
<td>63.8 ± 1.2a</td>
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<td>TG20 male positive (n = 10)</td>
<td>0.96 ± 0.04c</td>
<td>0.98 ± 0.04c</td>
<td>62.5 ± 2.8a</td>
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<tr>
<td>TG564 female positive (n = 9)</td>
<td>0.97 ± 0.04b</td>
<td>1.03 ± 0.03c</td>
<td>62.9 ± 1.9a</td>
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<td>TG564 male positive (n = 8)</td>
<td>0.94 ± 0.05b</td>
<td>0.92 ± 0.06b</td>
<td>62.0 ± 2.4a</td>
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**Fig. 4. Hypertrophic signaling pathways in Akt mice.** A, ERK activation. No change in phosphorylated or total ERK1/2 was seen in transgenic Akt mice. B, JNK activation. The activation of JNK was evaluated using c-Jun as substrate. The activity and level of expression of JNK were not changed. C, p38 activation. The activation of p38 was evaluated with phosphorylation of ATF-2, as described under “Experimental Procedures.” The activity and level of expression of p38 were not changed. D, GSK-3β activation. Similarly, no change in phosphorylated (Ser9) or total GSK-3β was seen in transgenic Akt mice (upper panel). The GSK-3β kinase activity in TG+ was not significantly different from that seen in littermate controls. Cumulative data from 12 animals (6 in each group) are shown (lower panel). All blots shown are from TG564 and represent at least three independent experiments.

**Transgenic Cardiac Akt Mice**

**Phenotypic Effects of Cardiac Akt Activation—Hearts from transgenic mice in both lines were substantially larger than transgene negative (TG−) littermates with evident gross cardiac hypertrophy (Fig. 2 and Table I). In TG564 mice, the HW/BW ratio was 2.5- and 2.1-fold greater than that seen in TG− female and male littermates (p < 0.005), respectively (Table I). TG20 had less marked cardiac hypertrophy with a HW/BW ratio 1.6- and 1.7-fold greater than that seen in TG− female and male littermates (p < 0.005), respectively.

To characterize the cardiac enlargement on a cellular level, we isolated cardiomyocytes from transgenic mice and their littermates. Cardiomyocyte surface area was measured from digitized micrographs of isolated cells. TG564 myocytes were substantially larger with a mean surface area 1.7-fold greater than that seen in TG− female and male littermates (p < 0.005), respectively (Table I). TG20 had less marked cardiac hypertrophy with a HW/BW ratio 1.6- and 1.7-fold greater than that seen in TG− female and male littermates (p < 0.005), respectively.

**Functional Analysis—**Echocardiograms were performed in both lines from 8 to 18 weeks to assess in vivo cardiac function as well as wall and chamber dimensions. TG20 mice demonstrated LV hypertrophy that was slightly more marked for male as compared with female TG+ mice (Table II). Wall thickness in TG564 mice was comparably increased in both males and females, and similar to that seen in male TG20 mice.

Systolic ventricular function (as indicated by ejection fraction) was normal in both lines (Table II).

**Signal Transduction in the myr-Akt Transgenic Mice—**Several signaling pathways relevant to cardiac hypertrophy were examined. We observed no change in the level of total extracellular regulated kinases (ERK)-1 and 2, or phosphorylated ERK-1 and -2 (Fig. 4A). Stress-activated protein kinase (SAPK/JNK) has also been implicated in cardiac hypertrophy both in vitro (31) and in vivo (32). Nevertheless, there was no difference between SAPK activity in TG+ and TG− mice (Fig. 4B). We measured phosphorylation of p38 and p38 kinase activity with ATF-2 as substrate. Although occasional TG+ mice showed modestly enhanced phosphorylation of p38, most did not (data not shown). Moreover, p38 activity was similar between TG+ and TG− mice (Fig. 4C). GSK-3β is phosphorylated and inactivated by Akt (33). GSK-3β inactivation is required for cardiomyocyte hypertrophy in response to some stimuli in vitro (29). However, we found no difference in total or phosphorylated GSK-3β between TG+ and TG− mice (Fig. 4D, upper panel). Similarly, there was no difference in activity (Fig. 4D, lower panel). The ribosomal protein p70S6 kinase is another downstream target of Akt (34), which regulates translation initiation and has previously been reported to promote cellular growth (35, 36) and hypertrophy (37). Phosphorylation of Thr389 correlates well with p70S6 kinase activity (38, 39). In Akt transgenic mice, overall expression of p70S6 kinase was increased, as was phosphorylation at Thr389 as well as Thr421/431.
Interest in Akt signal transduction has been heightened by the demonstration that Akt activation reduces injury in models of cardiac ischemia (10–12). However, all of these studies were performed using acute viral gene transfer to achieve transient Akt activation and documented benefits on relatively short-term endpoints. An understanding of the effects of chronic Akt activation both at baseline and in disease models may help determine the value of Akt signaling as a therapeutic target. In the current study, three transgenic founders (2F and 1M) with cardiac transgene expression (data not shown) died suddenly with massive cardiac enlargement at ages 9–19 weeks. While detailed quantitative analysis of these animals was not possible, this observation raises the possibility that chronic Akt activation can, under some circumstances, be detrimental. This toxicity may be related to the level of expression achievable with the α-mycin heavy chain promoter. In this context, however, it is worth noting that transgenic cardiac GFP expression has also been reported to induce dilated cardiomyopathy (40), and thus, this phenotype must be interpreted cautiously. On the other hand, two viable lines were produced which demonstrated marked Akt overexpression and dramatic increases in Akt activity that were well tolerated. Thus, while the level of Akt expression and activation are likely to be important considerations, there appears to be a broad range of Akt expression and activation that is well tolerated by the adult mammalian heart.

The construct used for these studies (myr-Akt) is rendered constitutively active by initial localization to the sarcolemma. We have previously shown that gene transfer at the same construct recapitulates many of the signaling and biological effects seen with insulin-like growth factor-I treatment (10). Moreover, after Ad.myr-Akt gene transfer to the heart in vivo, the HA-tagged construct is found not only at the sarcolemma but also in the cytosolic fraction (data not shown). Thus, to a large extent, myr-Akt produces a pattern of Akt activation resembling that seen with ligand activation of the endogenous molecule. However, the possibility that subtle differences in subcellular localization affect downstream signaling is worthy of further investigation.

Although PI 3-kinase activity is an important determinant of mammalian heart size (16), the downstream substrates mediating this effect have not been identified. Our data establish that Akt activation is sufficient to cause both cellular and gross hypertrophy and suggest that Akt may well mediate the effects of PI 3-kinase on cardiomyocyte size. The fortuitous finding of...
X-linked transmission in TG20 mice allows us to infer that cardiac Akt activation modulates cell size in a primary and cell autonomous manner, rather than secondary to hemodynamic changes or paracrine signals. The observed increase in cardiomyocyte surface area appears to quantitatively account for the entire increase in heart mass (assuming the increase in mass is proportional to volume and thus surface area$^{3/2}$). However, it is difficult to exclude the possibility that cardiomyocyte hyperplasia contributes to the observed increase in cardiac mass. Akt inhibits p21CIP1 (41, 42) and thus could affect cardiomyocyte proliferation (43). However, we found no evidence in bromodeoxyuridine incorporation studies for enhanced myocyte proliferation, although bromodeoxyuridine incorporation was evident in the small intestine from treated animals (data not shown). A dominant effect of Akt on cell size rather than cytoskeletal expression (29, 47). Interestingly, we did not observe enhancement of atrial natriuretic factor expression (29, 47). Interestingly, we did not observe enhanced atrial natriuretic factor in the myr-Akt mice (data not shown). In contrast, both total and phosphorylated p70S6 kinase were increased, as was kinase activity. p70S6 kinase (44) and a $\alpha$-Class Akt activation by Akt induces by Akt induces by Akt induces by Akt induces by Akt induces by Akt induces by Akt induces by Akt induces by Akt induces by Akt induces by Akt induces by Akt induces by Akt induces by Akt induces by Akt induces by Akt induces by Akt induces by Akt induces by Akt induces by Akt induces by Akt induces by Akt induces by Akt induces by Akt induces by Akt induces by Akt induces by Akt induces by Akt induces by Akt induces by Akt induces by Akt induces by Akt induces by Akt induces by Akt induces by Akt induces by Akt induces by Akt induces by Akt induces by Akt induces by Akt induces by Akt induces by Akt induces by Akt induces by Akt induces by Akt induces by Akt induces by Akt induces by Akt induces by Akt induces by Akt induces by Akt induces by Akt induces by Akt induces by Akt induces by Akt induces by Akt induces by Akt 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