Akt2 Regulates Cardiac Metabolism and Cardiomyocyte Survival*§

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The Akt family of serine-threonine kinases participates in diverse cellular processes, including the promotion of cell survival, glucose metabolism, and cellular protein synthesis. All three known Akt family members, Akt1, Akt2 and Akt3, are expressed in the myocardium, although Akt1 and Akt2 are most abundant. Previous studies demonstrated that Akt1 and Akt3 overexpression results in enhanced myocardial size and function. Yet, little is known about the role of Akt2 in modulating cardiac metabolism, survival, and growth. Here, we utilize transgenic mice with targeted disruption of the akt2 or the akt1 genes to demonstrate that Akt2, but not Akt1, is required for insulin-stimulated 2-[14C]deoxyglucose uptake and metabolism. In contrast, akt2−/− mice displayed normal cardiac growth responses to provocative stimulation, including ligand stimulation of cultured cardiomyocytes, pressure overload by transverse aortic constriction, and myocardial infarction. However, akt2−/− mice were found to be sensitized to cardiomyocyte apoptosis in response to ischemic injury, and apoptosis was significantly increased in the peri-infarct zone of akt2−/− hearts 7 days after occlusion of the left coronary artery. These results implicate Akt2 in the regulation of cardiomyocyte metabolism and survival.

Cardiac growth and metabolism are coordinated through the integration of a complex array of extracellular and intracellular signals. Much recent work suggests that the Akt family of intracellular serine-threonine kinases regulates both cardiac growth and metabolism (1–3). The Akt family of serine-threonine kinases consists of three isoforms, Akt1, Akt2, and Akt3, each encoded by distinct, highly conserved genes. All three isoforms are expressed in the myocardium, although Akt1 and Akt2 comprise the vast majority of total Akt protein in the heart (2). Examination of numerous experimental models implicates both Akt1 and Akt3 in regulating pathological and physiological hypertrophy (4–6). Indeed, the hypothesis that the phosphatidylinositol 3-kinase (PI3K) α-Akt1 cascade mediates physiological cardiac growth is now well founded (7). Cardiac-specific expression of constitutively active Akt1 (myristolated Akt1) in transgenic mice results in massive cardiac hypertrophy and fibrosis consistent with pathological hypertrophy (4), and nuclear localization of Akt1 was recently shown to augment ventricular function and contractility (8). A comparable phenotype was observed in response to cardiac overexpression of activated Akt3, whereas no observable cardiac growth defects were detectable in Akt3-deficient mice at baseline (6).

Akt family members are also key regulators of cellular metabolism. Indeed, GLUT4 translocation to the plasma membrane is a wortmannin-sensitive process (9), and Akt2-mediated phosphorylation of the syntaxin interacting protein (synip) results in docking and fusion of GLUT4-containing vesicles with the plasma membrane (10). Akt family members promote glycogen synthesis through phosphorylation and inhibition of glycogen synthase kinase 3 (GSK3), which itself inhibits glycogen synthesis (11). GSK3 phosphorylation results in the augmentation of glycogen synthesis, whereas Akt activation antagonizes the AMP-activated protein kinase (12), a key mediator of glycogenolysis and lipolysis. In addition, Akt kinases inhibit fatty acid metabolism by phosphorylating and inhibiting FOXO-1, a fork-head family transcription factor that positively modulates fatty acid oxidative gene expression (13).

Although the role of Akt family members in cardiac growth and metabolism has been widely studied, the role of Akt2 in the development of physiological and pathological cardiac hypertrophy is unknown. Additionally, the role of individual Akt family members in the regulation of cardiac metabolism remains unexplored. In the current study, an Akt2 loss-of-function murine model was utilized to assess the role of Akt2 in cardiac growth, metabolism, and cardiomyocyte survival. Here, we show that Akt2 is dispensable in the development of cardiac hypertrophy in response to a variety of physiological and pathological provocative stimuli. Conversely, we demonstrate that

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Akt2 is absolutely required for the maintenance of normal cardiac glucose metabolism and for cardiomyocyte survival in response to ischemic injury.

EXPERIMENTAL PROCEDURES

Maintenance of akt1−/− and akt2−/− Mice—Mice with targeted disruption of the akt1 or the akt2 genes were generated as previously described (14, 15). Mice were repeatedly backcrossed (more than six times) with WT C57Bl6 mice obtained from the Jackson Laboratory prior to experimentation. Progeny were screened by tail-prep PCR. All procedures were approved by the committee for the handling and care of laboratory animals prior to experimentation. Experimentation was performed in strict accordance with the committee for the handling and care of laboratory animals protocols (Washington University Approval #20030049).

Adult Mouse Cardiomyocyte Cultures—AMCMs were isolated according to standard procedures with minor modifications (16). Cultures were isolated in the presence of butanediol monoxime and were switched to culture medium containing 10 μM brefeldin (Sigma-Aldrich) prior to assay.

2-[3H]Deoxy-β-glucose Uptake Assays—Serum-deprived (16 h) adult male mouse cardiac myocyte cultures were incubated in mouse myocyte culture medium containing 1 μCi/ml 2DG (American Radiochemicals, Inc., St. Louis, MO) in the presence or absence of 2 nM insulin (Sigma-Aldrich) for 5’. 2DG uptake was determined to be linear with respect to time for up to 10-min 2DG pulse (data not shown). Non-cytoskeleton-mediated 2DG uptake was corrected by measuring 2DG uptake in AMCM cultures preincubated (15 min) with cytochalasin B (Sigma-Aldrich). Counts measured in these cultures were subtracted from experimental cultures as background. Uptake in each culture was normalized for total sample protein abundance by protein concentration determination using the Bradford Coomassie blue dye reagent. Results are expressed as cpm per μg of protein ± S.E. for n = 3 samples under each condition. Results are representative of a minimum of three experiments under each set of conditions.

Western Blotting—SDS-PAGE/immunoblotting were performed according to standard procedures as previously described. Total Akt2 antibodies and phospho-specific Akt, GSK3β, p70 S6 kinase (S6K), S6 ribosomal subunit, and

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ERK1/2 antibodies were obtained from Cell Signaling Technologies (Beverly, MA). Caspase-3, total ERK1/2, and total Akt1 antibodies were obtained from Santa Cruz Biotechnologies (Santa Cruz, CA). Total GLUT1 antiserum was a kind gift from the laboratory of Kelle Moley, Washington University School of Medicine. Phospho-specific Akt, GSK3β, and S6 antibodies were probed simultaneously. Thereafter, membranes were stripped of bound antibody using 0.2 M NaOH prior to reprobing.

**Isolated Working Mouse Heart Perfusion**—Isolated mouse working heart perfusions were based on a previously described procedure (17). Adult male mice (8 weeks old) were heparinized (100 units, intraperitoneal) 10 min prior to anesthesia. Animals were then deeply anesthetized with sodium pentobarbital (intraperitoneal). Hearts were excised and placed in an ice-cold Krebs-Henseleit bicarbonate (KHB) solution (118 mM NaCl, 25 mM NaHCO$_3$, 4.7 mM KCl, 0.4 mM KH$_2$PO$_4$, 2.5 mM CaCl$_2$, and 5.0 mM glucose, 30 microunits/ml insulin, pH 7.4). Hearts were cannulated first via the aorta and perfused retrogradely by the Langendorff method. Following left atrial cannulation, perfusion was switched to working heart perfusion with KHB solution containing 0.4 mM palmitate bound to 3% fatty acid-free bovine serum albumin with a preload pressure of 11.5 mmHg and an afterload pressure of 50 mmHg for 60 min with oxygenated buffer solution. To determine palmitate and glucose oxidation rates, trace amounts of [H]$^3$H]palmitate (0.1 μCi/ml) and [U-14C]glucose (0.1 μCi/ml) were used, respectively. Samples were collected every 10 min for 14CO$_2$ trapped in 1 M hyamine hydroxide solution as a result of glucose oxidation, $^3$H$_2$O was released into the buffer as a result of palmitate oxidation, and the radioactivity was counted. Functional measurements like cardiac output and aortic flows, peak systolic pressure, and heart rate were acquired every 10 min for 10 s using in-line flow probes (Transonic Systems, Inc.), the MP100 system from AcqKnowledge (BIOPAC Systems, Inc.), and a pressure transducer (TSD 104A, BIOPAC Systems, respectively). Coronary flow was calculated as the difference between cardiac output and aortic flows. Cardiac work was calculated as the product of peak systolic pressure and cardiac output. At the end of each perfusion, hearts were frozen immediately in liquid
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nitrogen. A small piece of heart tissue was also used for determining dry to wet weight ratio.

[^3H]Palmitate Uptake Assays—Male AMCMs were isolated and serum-starved for 1 h prior to incubation with up to 100 nM insulin for 20 h. Following the incubation period, a minimal essential medium containing 2 mM unlabeled palmitate complexed with 20% bovine serum albumin (3:1 complex ratio) and 1 μCi/ml[^3H]palmitate (PerkinElmer Life Sciences). Cultures were pulsed (5 min) and washed extensively in ice-cold 10 mM phloretin in phosphate-buffered saline solution. Cells were lysed in 0.1 N NaOH/0.2% SDS, and 80% of the lysate was counted in 5 ml of Ultima Gold liquid scintillation counting fluid. Uptake in each culture was normalized for total protein abundance by Bradford protein concentration determination.

Leucine Uptake Assays—Serum-deprived (16 h) adult mouse cardiac myocyte cultures were incubated in mouse myocyte culture medium containing 1 μCi/ml[^3H]leucine (Amersham Biosciences) in the presence or absence of 100 nM endothelin-1 (Sigma-Aldrich) or 10 nM IGF-1 for 16 h. Uptake was normalized for total protein amount as measured by Bradford protein concentration determination. Results are expressed as total cpm per μg of protein ± S.E.

Transverse Aortic Constriction—TAC operations were performed on anesthetized akt2−/− and WT mice to induce cardiac pressure overload (18). Sham-operated akt2−/− and their WT littermates were used as controls. The surgeon was blinded to the genetic status of the mice. Seven days after surgery, all groups were evaluated by echocardiography (18). Mice with a Doppler gradient between 3.5 and 5.0 m/s were included in subsequent analyses. Mice were sacrificed, and postmortem and histological studies were performed as described below.

Histology—Ventricular tissue was fixed in 10% formalin, paraffin-embedded, microtome-sectioned, and stained with hematoxylin & eosin. Myocyte cross-sectional areas from three to five randomly selected high power fields from three to five different mice in each group were calculated on a Carl Zeiss, Inc. Axioskop microscope using ImageJ (version 1.34S) software.

In some cases, myocardial tissue sections were evaluated by terminal deoxynucleotidyltransferase (TdT) nick end labeling assay (TUNEL). TUNEL was performed on 5-mm paraffin sections with a TdT-FragEL DNA fragmentation detection kit (Oncogene, Cambridge, MA). Sections were mounted on coverslips and evaluated by fluorescence microscopy.

Experimental Myocardial Infarction—Murine myocardial infarction surgery was performed as previously described (19). In brief, anesthetized and ventilated mice were subjected to thoracotomy, and a single 8-0 Prolene suture was tied around the proximal left coronary artery, ~1 mm distal to the atrioventricular junction. Successful occlusion was confirmed by the appearance of pallor of the anterior left ventricular wall. The incision was closed, and the animal was allowed to recover on a heating pad. The surgeon was blinded to the genetic status of the mice. At 1 day and at 7 days post-myocardial infarction (MI), mice were evaluated by echocardiography. The initial infarct size, indicated by the segmental wall motion score index (SWMSI), was determined as previously described (20). Only mice with a SWMSI between 0.2 and 0.5 were included in subsequent analyses. 7 days after surgery, mice were sacrificed and the hearts were dissected, weighed, and paraffin-embedded for histological examination.

In Vitro Apoptosis Assay—Serum-deprived (3 h) AMCMs were incubated in the presence of 25 μM H₂O₂ for 16 h prior to lysis and evaluation of apoptosis by the Cell Death Detection Apoptosis ELISA Assay Kit (Roche Applied Science, Basel, Switzerland) precisely according to manufacturer specifications.

Real-time Quantitative Reverse Transcription-PCR—Total RNA was isolated via TRIzol reagent from left ventricles obtained from sham- and MI-operated WT and akt2−/− mice 7

FIGURE 4. Reduced cardiac FOXO-1 phosphorylation is associated with enhanced cardiac palmitate uptake in Akt2-deficient mice. A, densitometric immunoblot quantification of FOXO-1 (Ser-256) phosphorylation in WT and akt2−/− AMCMs treated with 100 nM insulin for 15 min. The membrane was stripped and reprobed for actin to control for protein loading. B, [^3H]palmitate uptake in WT and akt2−/− AMCMs pretreated overnight with 100 nM insulin. Graphed are the mean cpm/μg of protein ± S.E. for each genotype. *, p < 0.05 versus WT. C, palmitate oxidation in WT and akt2−/− mice subjected to ex vivo working heart perfusion studies. The mean nanomoles of radiolabeled H₂O liberated per g of dry heart weight ± S.E. is graphed. The number of animals analyzed in each group (n) is indicated. *, p < 0.05 versus WT.
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Insulin-stimulated Signal Transduction and Glucose Transport Was Blocked in akt2−/− AMCMs—Although pharmacological studies supported the role of the PI3K-PDK1-Akt pathway in insulin-stimulated cardiomyocyte glucose uptake, they did not specify whether a specific Akt family member was required for this process. To address this issue, AMCMs derived from akt1−/− and akt2−/− mice were analyzed for insulin-stimulated signal transduction and glucose uptake.

Insulin-stimulated signaling via the canonical Akt pathway was impaired in akt1−/− and akt2−/− AMCMs incubated in 100 nM insulin for 5 min. Phosphorylation of Akt1–3 (Ser-473), GSK3β (Ser-9), p70 S6 kinase (Thr-389), and the S6 ribosomal subunit (Ser-235/236) was detected in WT AMCMs, but this response was reduced in both akt1−/− and akt2−/− cultures (Figs. 2A and 3A).

To determine whether reduced insulin signal transduction in AMCMs correlated with abnormal physiology, the ability of WT, Akt1-deficient, and Akt2-deficient AMCMs to transport glucose across the plasma membrane was examined. Insulin-stimulated cardiac glucose transport was measured in serum-deprived AMCMs stimulated with or without 2 nM insulin (5 min) in the presence of 1 μCi/ml 2DG. 2DG uptake in response to insulin was not impaired in akt1−/− AMCMs when compared with WT AMCMs (Fig. 2B). However, basal 2DG uptake was modestly elevated in akt1−/− AMCMs, and this may be due to increased GLUT1 protein levels observed in these cells (Fig. 2C).

In contrast to akt1−/− AMCMs, cardiomyocytes derived from akt2−/− mice displayed a marked defect in insulin-stimulated 2DG uptake (Fig. 3B). Indeed, 2DG uptake in WT AMCMs was stimulated by 139 ± 37% in response to insulin (Fig. 3B), but 2DG uptake in Akt2-deficient AMCMs increased by only 24% ± 8.9% after insulin treatment (p < 0.05 versus insulin-stimulated WT cultures). Basal 2DG uptake was normal in akt2−/− AMCMs, and this was associated with unchanged GLUT1 protein levels (supplemental Fig. S1). The glucose uptake defect in akt2−/− AMCMs was also observed in the presence of supraphysiologically concentrations of insulin (100 nM, supplemental Fig. S2). AMCMs isolated from mice haploinsufficient for akt2 also showed a

days post-operation. TaqMan real-time quantitative reverse transcription-PCR was subsequently performed as previously described (18).

Statistical Analysis—All statistical relationships were determined by two-tailed, two-sample homoscedastic t test with Bonferroni post-hoc correction for multiple comparisons. Results are shown as mean ± S.E.

RESULTS

Insulin-stimulated Glucose Uptake Depends on PI3K in Cultured Cardiomyocytes—Cardiomyocytes express two transmembrane glucose transporters, GLUT1 and GLUT4, and insulin-dependent glucose transport depends on the translocation of GLUT4 from intracellular vesicles to the plasma membrane (21). Previous work implicated the phosphatidylinositol 3-kinase (PI3K)-phosphoinositide-dependent kinase 1 (PDK1)-Akt pathway in cardiomyocyte glucose transport. Indeed, cultured cardiomyocytes lacking PDK1 are resistant to insulin-stimulated glucose uptake (22). However, other signaling pathways, including the p38 MAPK pathway, may be involved in insulin-stimulated glucose uptake.

To evaluate the role of various signaling pathways in cardiomyocyte metabolism, cultured AMCMs were stimulated with 2 nM insulin in the presence of various pharmacological agents, and 2DG uptake was evaluated. Treatment of cells with LY294002 (50 μM), an inhibitor of PI3K, completely blocked insulin-stimulated 2DG uptake (Fig. 1). However, treatment of cells with SB202190 (10 μM), a p38 MAPK inhibitor, did not affect 2DG uptake. Treatment of cells with PD98059 (50 μM), a MAPK kinase 1/2 (also called MEK) inhibitor, modestly reduced 2DG uptake in response to insulin, but 2DG uptake was significantly stimulated in comparison to cells treated with PD98059 alone.

Insulin-stimulated glucose uptake was normal in WT-stimulated cultures (three hypotheses): WT-stimulated versus WT control, p < 0.05 versus congenic control. Statistical Analysis—All statistical relationships were determined by two-tailed, two-sample homoscedastic t test with Bonferroni post-hoc correction for multiple comparisons. Results are shown as mean ± S.E.

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A.

| Vehicle (200nM, 15') | ET-1 (200nM, 15') |
|----------------------|-------------------|
| **WT**               | **akt2−/−**       |
| pAkt (S473)          | pAkt (S473)       |
| pp85 S6K (T412)     | pp85 S6K (T412)  |
| p70 S6K (T389)      | p70 S6K (T389)   |
| pGSTK3β (S9)        | pGSTK3β (S9)     |
| pS6 (S235/236)      | pS6 (S235/236)   |
| pERK1/2 (T202/Y204) | pERK1/2 (T202/Y204) |

B.

**FIGURE 6. ET1-stimulated growth is normal in akt2−/− AMCMs.** A, analysis of ET1-stimulated Akt pathway signaling in AMCMs. AMCM lysates from serum-deprived WT and akt2−/− cultures incubated in the presence or absence of 200 nM ET1 were analyzed by phospho-specific p70 S6K, GSTK3β, and S6 immunoblotting. Total ERK1/2 was probed as a loading control. B, quantification of immunoblots shown in A. *, p < 0.05 versus WT control; #, p < 0.01 versus WT-pretreated (t test with Bonferroni post-hoc correction, two hypotheses). C, analysis of leucine incorporation in AMCMs. Serum-deprived, cultured WT, and akt2−/− AMCMs were incubated with radiolabeled [3H]leucine in the presence or absence of 200 nM ET1 for 16 h. Mean trichloroacetic acid-precipitable counts/µg of protein ± S.E. are graphed. Data are representative of two experiments, n = 3 independent cultures per treatment condition. The following t tests were performed with Bonferroni post-hoc correction (three hypotheses): WT-stimulated versus WT control, akt2−/−-stimulated versus akt2−/− control, and akt2−/−-stimulated versus WT-stimulated. *, p < 0.05 versus WT control.

statistically significant defect in insulin-stimulated 2DG uptake (Fig. 3C), despite the fact that akt2+/− mice have normal total body glucose homeostasis (14).

To test whether the decreased transport of glucose into cultured akt2−/− cardiomyocytes was associated with decreased cardiac glucose oxidation, ex vivo working heart experiments were performed in the presence of insulin (30 microunits/ml) and radiolabeled glucose. Isolated working hearts from akt2−/− mice exhibited glucose oxidation rates that were reduced by 47.2% relative to WT littermate control hearts (Fig. 3D, p < 0.001 versus WT control). Hemodynamic parameters, including heart rate, peak systolic pressure, cardiac output, coronary flow, cardic work, stroke work, and cardiac power were also measured during working heart mode, and these were not statistically different in WT versus akt2−/− hearts (supplemental Fig. S3).

Akt2 Deficiency Results in Enhanced Palmitate Uptake and Oxidation—Akt negatively regulates mediators of fatty acid uptake and metabolism, including FOXO-1, a key transcription factor regulating the expression of fatty acid oxidation pathway genes (13). Phosphorylation of FOXO-1 by Akt at serine 256 reduces its transcriptional activity by promoting its exclusion from the nucleus. Insulin-stimulated phosphorylation of FOXO-1 was reduced in akt2−/− AMCMs when compared with WT AMCMs (Fig. 4A).

To examine whether reduced FOXO-1 phosphorylation was associated with enhanced fatty acid uptake, palmitate uptake assays were performed. Serum-starved WT and akt2−/− AMCMs were incubated with radiolabeled [3H]palmitate in the presence of chronic (20 h) 100 nM insulin stimulation. Palmitate uptake was increased by 26% ± 8.0% in akt2−/− AMCMs when compared with WT cultures (Fig. 4B, p < 0.01 versus WT).

To determine whether akt2−/− mice exhibited increased cardiac fatty acid oxidation, ex vivo working heart studies were performed. [3H]Palmitate oxidation was enhanced by 58.6% in Akt2-deficient mouse hearts when compared with WT controls (Fig. 4C, p = 0.013 versus WT).

Normal Ligand-induced Growth in akt2−/− Cardiomyocytes—Cardiac-specific overexpression of mutant forms of Akt1 or Akt3 promotes cardiac hypertrophy that may progress to congestive heart failure (4–6). Because the kinase domains of Akt1 and Akt3 are >80% homologous to the Akt2 kinase domain, we examined the possibility that Akt2 is also involved in cardiac growth.

The growth hormone-insulin-like growth factor-1 (IGF-1) axis is hypothesized to play a critical role in physiological cardiac hypertrophy secondary to exercise training (23, 24), and IGF-1 treatment of cultured cardiomyocytes is an in vitro model of physiological cardiac hypertrophy. IGF-1-stimulated signaling was evaluated in WT and akt2−/− AMCMs. Treatment of WT AMCMs with 10 nM IGF-1 resulted in the phos-
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A.

Echocardiographic Analysis of Mice after TAC or Sham Operation.

|                      | Sham Operation | TAC Operation |
|----------------------|----------------|--------------|
|                      | WT (n = 17)    | akt2−/− (n = 5) | WT (n = 17) | akt2−/− (n = 7) |
| HR (min⁻¹)           | 685 ± 37       | 642 ± 34      | 685 ± 37    | 646 ± 34        |
| LVFWd (mm)           | 0.65 ± 0.03    | 0.61 ± 0.04   | 0.67 ± 0.10 | 0.71 ± 0.08     |
| IVSd (mm)            | 0.69 ± 0.08    | 0.67 ± 0.04   | 0.70 ± 0.12 | 0.74 ± 0.07     |
| LVIDd (mm)           | 3.45 ± 0.14    | 3.38 ± 0.19   | 3.34 ± 0.17 | 3.24 ± 0.15     |
| LVMI (mg/g)          | 3.30 ± 0.34    | 3.07 ± 0.18   | 3.71 ± 0.62 | 3.79 ± 0.44     |
| LVd (mm)             | 1.52 ± 0.19    | 1.49 ± 0.19   | 1.49 ± 0.37 | 1.50 ± 0.29     |
| FS (%)               | 61.6 ± 4.2     | 57.1 ± 5.4    | 56.0 ± 0.0  | 53.7 ± 7.8      |
| Doppler vel. (m/s)   |                | 4.0 ± 0.8     | 3.9 ± 0.24  |               |

B.

Graphed are the mean cardiomyocyte cross-sectional area for each group. Numbers of mice analyzed in each group (n) are indicated below each bar.

C.

H&E

D.

Myocardial Cross-Sectional Area

FIGURE 7. Akt2−/− mice develop cardiac hypertrophy in response to pressure overload by TAC. A, echocardiographic analysis of mice after TAC or sham operation. 8-week-old mice underwent transthoracic echocardiography 7 days after TAC or sham operation. HR, heart rate; LVFWd, left ventricular posterior wall thickness in diastole; IVSd, intraventricular septal thickness in diastole; LVIDd, left ventricular internal dimension in systole; FS, fractional shortening. Doppler velocity was measured at site of transverse aortic constriction; *, p < 0.05 versus WT mice after sham operation. B, morphometric analysis of dissected LVs from WT and akt2−/− mice obtained 7 days after TAC or sham operation. Graphed are the mean LV weight-to-body weight ratios for each group. Numbers of mice analyzed in each group (n) are indicated below each bar. C, high powered photomicrographic analysis of myocyte cross-sectional area in homogenized and eosin-stained transverse cardiac sections obtained from mice analyzed in B. D, computerized cardiomyocyte cross-sectional area quantification in heart sections obtained from WT and akt2−/− mice subjected to sham or TAC operation. Graphed is the mean cardiomyocyte cross-sectional area ± S.E. Quantifications represent cardiomyocyte tracings of all cells in three to five distinct, random high powered fields per animal, obtained from three to five different animals in each treatment group.

To assess the ability of Akt2-deficient AMCMs to undergo physiological hypertrophy, serum-deprived AMCMs from 8-week-old WT and akt2−/− mice were incubated with [3H]leucine to a similar extent (p < 0.05) versus WT mice after sham operation. Akt2 differentially regulates pathological hypertrophy, serum-deprived AMCMs from 8-week-old WT and akt2−/− mice were incubated with [3H]leucine to a similar extent (p < 0.05) versus WT mice after sham operation.

Normal ET1-induced Protein Synthesis in akt2−/− Cardiac Myocytes—The normal hypertrophic response of akt2−/− AMCMs to IGF-1 stimulation does not exclude the possibility that Akt2 differentially regulates pathological hypertrophic signaling. Treatment of cultured cardiomyocytes with endothelin-1 (ET1), a G protein-coupled receptor agonist, is an in vitro model of pathological cardiac hypertrophy. ET1-stimulated signal transduction was evaluated in WT and akt2−/− AMCMs. Treatment of WT AMCMs with 200 nM ET1 resulted in the phosphorylation of p70 S6K, GSK3β, and S6 that was blocked in akt2−/− AMCMs (Fig. 6, A and B).

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Normal TAC-induced Cardiac Hypertrophic Response in akt2−/− Mice—To test the role of Akt2 in cardiomyocyte growth in an in vivo model of pathological cardiac hypertrophy, WT and akt2−/− mice were subjected to pressure overload by TAC. Baseline evaluation of 8-week-old akt2−/− mice by transthoracic echocardiography revealed that heart rate, left ventricular dimensions, and left ventricular fractional shortening

Phosphorylation of p70 S6K, GSK3β, and S6 that was reduced in akt2−/− AMCMs (Fig. 6, A and B). ERK activation in response to ET1 treatment was similar in both WT and akt2−/− AMCMs (Fig. 6, A and B).

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were all normal when compared with WT mice (supplemental Fig. S4).

TAC was performed on WT and akt2<sup>−/−</sup> mice, and the pressure gradient achieved was nearly identical in both groups, as determined by Doppler echocardiography (Fig. 7A). The echocardiographically determined LV mass index increased to a similar extent in WT and akt2<sup>−/−</sup> mice 7 days after TAC. Indeed, the LV mass index increased from 3.10 ± 0.34 to 3.71 ± 0.62 in WT mice, and the LV mass index increased from 3.07 ± 0.18 to 3.79 ± 0.44 in akt2<sup>−/−</sup> mice (Fig. 7A).

Morphometric analysis further demonstrated that TAC induced a similar degree of cardiac hypertrophy as measured by
the left ventricular weight-to-body weight ratio (LV/BW) in WT and akt2−/− mice (Fig. 7B). No statistical differences in LV/BW were detected in TAC-operated WT (LV/BW = 4.32 ± 0.3) versus TAC-operated akt2−/− mice (LV/BW = 4.19 ± 0.48). However, the mean baseline LV/BW was modestly greater in akt2−/− mice (Fig. 7B). Computerized photomicrography of hematoxylin and eosin-stained transverse cardiac sections demonstrated that the cardiomyocyte cross-sectional area increased to a similar extent in WT and akt2−/− mice 7 days after TAC (Fig. 7, C and D). Although akt2−/− mice exhibited an intact hypertrophic response to pressure overload by TAC, this was not due to a compensatory increase in Akt1 protein levels (supplemental Fig. S5).

Enhanced Apoptosis in the Peri-infarct Zone of akt2−/− Heart—Experimental MI by occlusion of the left coronary artery is also associated with pathological cardiac remodeling. In this model of myocardial infarction, an initial infarction stage occurs that is characterized mainly by necrotic cell death in the tissue directly supplied by the occluded coronary artery. A subsequent, often maladaptive phase of ventricular remodeling occurs in the days and weeks following MI surgery. Hallmarks of this chronic phase include persistent cardiomyocyte apoptosis, fibrosis, thinning of the ventricular wall at the infarct site, chamber dilatation, and cardiomyocyte hypertrophy in the uninvolved myocardium (19).

To determine whether Akt2 is an important component of this remodeling process, akt2−/− and WT mice were subjected to permanent ligation of the left coronary artery. Morphometric evaluation of mice 7 days after MI surgery revealed that cardiac hypertrophy, as measured by LV/BW, increased to a similar extent in WT and akt2−/− mice (Fig. 8A). β-Mycosin heavy chain and atrial natriuretic factor gene expression were subsequently measured by real-time quantitative reverse transcription-PCR as markers of LV stress. MI induced β-mycosin heavy chain to a similar extent in both WT (6.5- ± 1.4-fold) and in akt2−/− mice (7.5- ± 3.0-fold, supplemental Fig. S6A). MI also induced atrial natriuretic factor in both WT (10.4- ± 2.1-fold) and akt2−/− mice (22.9- ± 4.3-fold). Atrial natriuretic factor induction was significantly greater in MI-operated akt2−/− mice (p = 0.028), consistent with enhanced myocardial stress in Akt2-deficient hearts (supplemental Fig. S6B). Furthermore, a modest, but statistically significant, increase in cardiomyocyte area in unaffected myocardium was observed in akt2−/− mice when compared with WT mice 7 days after MI (Fig. 8, B and C).

The observation that atrial natriuretic factor and cardiomyocyte area increased out of proportion to LV/BW in akt2−/− mice after MI surgery may be explained by an increased rate of apoptosis. Previous work suggested that Akt proteins play an important anti-apoptotic function in many cell types (25, 26). Cardiomyocyte apoptosis was evaluated by TUNEL in the uninfarcted region of akt2−/− and WT myocardium 7 days after MI surgery. TUNEL positivity was significantly increased in akt2−/− mice when compared with that in WT mice (Fig. 8, D and E). The SWMSI was measured by echocardiographic analysis of mice 1 day post-MI to assess left ventricular wall movement during the acute stages of LV remodeling (supplemental Fig. S7) (19). The SWMSI was identical in MI-operated WT (0.34 ± 0.03) and akt2−/− (0.36 ± 0.05, p = 0.70) mice (0 is defined as normal wall motion and 1 is defined as akinesis). Therefore, the initial infarct region was similar in both WT and akt2−/− mice, and the increase in apoptosis in Akt2-deficient myocardium was not a result of more aggressive surgical intervention.

Despite comparable initial infarct sizes, a significantly larger infarct region was observed in akt2−/− mice 7 days after MI, as assessed by histological examination of transverse WT and akt2−/− cardiac sections stained with Masson’s trichrome (Fig. 8, F and G). In addition, echocardiographic evaluation showed LV chamber dilatation and reduced contractile function in akt2−/− mice 7 days after MI (supplemental Fig. S7).

The enhanced apoptotic phenotype in the akt2−/− heart was recapitulated in an in vitro model of oxidative stress-induced apoptosis. Serum-deprived akt2−/− AMCMs incubated in the presence of 25 μM H2O2 for 16 h exhibited greater apoptotic activity when compared with similarly treated WT AMCMs (Fig. 8H, p < 0.005), as assessed by an ELISA that measures histone-complexed DNA fragmentation.

DISCUSSION
Akt proteins regulate cell metabolism, growth and survival, and individual family members may have distinct biological roles in cardiac function.
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functions. We previously demonstrated that Akt1 is required for the physiological growth of the heart and that Akt1 antagonizes pathological cardiac hypertrophy (27). In this work we demonstrate that Akt2, but not Akt1, is required for insulin-stimulated glucose uptake, that Akt2 plays an anti-apoptotic role in heart, but that Akt2 does not significantly regulate physiological or pathological cardiac growth. The results described in this report are similar to the gross physiological analysis of both akt2−/− mouse models currently in the literature (14, 28). Both Akt2-deficient mouse model systems exhibit global insulin resistance. Cho et al. (14) report no discernible differences in body or organ size. However, the akt2−/− mice generated by Garofalo and co-workers (28) exhibited mild deficiencies in body weight and body length, lipoatrophy, and modestly decreased kidney, spleen, and thymus organ weight-to-body weight ratios.

Akt family members phosphorylate a variety of intracellular targets, including transcription factors, protein kinases, and translational factors. Our analysis of well known Akt effectors, including GSK3β and S6, revealed that activation of these proteins was reduced in akt2−/− AMCMs but that IGFl-1 and ET1-induced cell growth was normal. Therefore, these signaling intermediaries are not required for cardiomyocyte growth in response to these ligands. Recent work by McMullen and co-workers (29) demonstrated that S6K1 and S6K2 are sufficient, but are not necessary, to induce cardiac hypertrophy. Moreover, GSK3β phosphorylation by itself does not appear to be required for cardiac growth. Clearly, genetic murine models expressing a nonphosphorylatable form of GSK3β would help to elucidate the role of GSK3β in cardiac growth and metabolism. It is unclear at this time what downstream signaling molecules are required for ligand-induced growth in akt2−/− AMCMs, and it is possible that activation of calcineurin or the MAPKs may promote protein translation independent of GSK3β and S6 phosphorylation.

Our findings show that Akt2, but not Akt1, regulates insulin-stimulated glucose uptake in AMCMs downstream of the PI3Ks. However, insulin treatment activates both Akt family members in cardiomyocytes. Indeed, Fig. 3A shows that insulin treatment of akt2−/− AMCMs results in the phosphorylation and activation of Akt1. This implies that Akt2 has unique substrates that are not phosphorylated by Akt1 in the regulation of GLUT4 translocation to the plasma membrane. Recently, Akt2 was shown to phosphorylate and inactivate the intracellular protein synip, an inhibitor of GLUT4 vesicular docking with the plasma membrane (10). Akt1 was unable to phosphorylate synip in vitro. Experiments are currently ongoing to determine whether synip phosphorylation is blocked in insulin-stimulated akt2−/− AMCMs. Other unique substrates of Akt2 may be involved in GLUT4 vesicular translocation, docking, and fusion with the plasma membrane.

The current analysis of akt2−/− mice is limited by the possibility that defects in cardiac insulin action are due to a generalized tissue insulin resistance secondary to whole body hyperinsulinemia and not due to the specific absence of Akt2 in cardiomyocytes (14, 28). However, work by Cho indicates that soleus muscle insulin responsiveness, measured by 2DG uptake, is normalized at high doses (13.33 nm) of insulin stimulation (14). The impaired insulin-stimulated AMCM glucose uptake in response to supraphysiological insulin concentrations (100 nm insulin) suggests that the observed phenotype is due to an absolute, primary insulin signal transduction defect in the myocardium. This hypothesis is further supported by the fact that akt2 haploinsufficient AMCMs, derived from mice that maintain euglycemia, normal insulin levels, and peripheral insulin-sensitivity throughout their lifespan, exhibited impaired glucose uptake.

Another potential limitation of the current study is in the specific interpretation of the in vivo cardiomyocyte apoptosis data. Despite nearly identical acute infarct sizes, MI-operated Akt2-deficient mice developed pathological cardiac remodeling to a much greater extent than WT mice. We suspect that akt2−/− cardiomyocytes are uniquely sensitized to develop apoptosis in the infarct border zone, leading to enhanced infarct extension and exaggerated pathological remodeling in the days following MI. This model is supported by in vitro data suggesting that akt2−/− cardiomyocytes are intrinsically sensitized to oxidative stress-induced apoptosis in the absence of extrinsic hemodynamic, mechanical, or humoral factors (Fig. 8H). Given the current data, however, we cannot exclude the possibility that the larger infarct size in akt2−/− mice at 7 days is not a direct consequence of increased apoptosis, but instead is due to some other unidentified factor, and that this increased infarct size results in greater mechanical stress on the surviving myocytes, stimulating apoptosis at 7 days.

One clinical implication of these findings is that Akt2 may be a promising therapeutic target in the treatment of glucose metabolic cardiac disorders such as diabetic cardiomyopathy. Current mouse models of chronic cardiac Akt1 or Akt3 hyperactivity suggest that pathological heart growth exists as a possible side-effect of chronic, nonspecific Akt family hyperactivation. However, the current study suggests that Akt2 does not regulate post-developmental cardiac growth in response to a variety of physiological and pathological stimuli. Furthermore, the sensitization of akt2−/− mice to the development of cardiomyocyte apoptosis after myocardial infarction indicates that cardiac Akt2 activity promotes cardiomyocytes survival. Therefore, Akt2-specific activation in the heart is likely to correct the metabolic derangements in DCM without detrimentally affecting heart function. The global actions of Akt2 in regulating both glucose and fatty acid utilization in the heart therefore define Akt2 as a candidate for specific therapeutic intervention.

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