Glycogen Synthase Kinase-3α Reduces Cardiac Growth and Pressure Overload-induced Cardiac Hypertrophy by Inhibition of Extracellular Signal-regulated Kinases

Peiyong Zhai1, Shumin Gao1, Eric Holle1, Xianzhong Yu3, Atsuko Yatani4, Thomas Wagner5, and Junichi Sadoshima2

From the 4Cardiovascular Research Institute, Department of Cell Biology and Molecular Medicine, University of Medicine and Dentistry of New Jersey, New Jersey Medical School, Newark, New Jersey 07103 and 5Oncology Research Institute, Greenville, South Carolina 29605

Glycogen synthase kinase-3 (GSK-3) is a serine/threonine kinase having multiple functions and consisting of two isoforms, GSK-3α and GSK-3β. Pressure overload increases expression of GSK-3α but not GSK-3β. Despite our wealth of knowledge about GSK-3β, the function of GSK-3α in the heart is not well understood. To address this issue, we made cardiac-specific GSK-3α transgenic mice (Tg). Left ventricular weight and cardiac myocyte size were significantly smaller in Tg than in non-Tg (NTg) mice, indicating that GSK-3α inhibits cardiac growth. After 4 weeks of aortic banding (transverse aortic constriction (TAC)), increases in left ventricular weight and myocyte size were significantly smaller in Tg than in NTg, indicating that GSK-3α inhibits cardiac hypertrophy. More severe cardiac dysfunction developed in Tg after TAC. Increases in fibrosis and apoptosis were greater in Tg than in NTg after TAC. Among signaling molecules screened, ERK phosphorylation was decreased in Tg. Adenovirus-mediated overexpression of GSK-3α, but not GSK-3β, inhibited ERK in cultured cardiac myocytes. Knockdown of GSK-3α increased ERK phosphorylation, an effect that was inhibited by PD98059, rottlerin, and protein kinase Cε (PKCε) inhibitor peptide, suggesting that GSK-3α inhibits ERK through PKC-MEK-dependent mechanisms. Knockdown of GSK-3α increased protein content and reduced apoptosis, effects that were abolished by PD98059, indicating that inhibition of ERK plays a major role in the modulation of cardiac growth and apoptosis by GSK-3α. In conclusion, up-regulation of GSK-3α inhibits cardiac growth and pressure overload-induced cardiac hypertrophy but increases fibrosis and apoptosis in the heart. The anti-hypertrophic and pro-apoptotic effect of GSK-3α is mediated through inhibition of ERK.

GSK-33 is a proline-directed serine/threonine kinase that is ubiquitously expressed. It has versatile biological functions, including regulation of metabolism, cell growth/death, development, cytoskeletal organization, transcription, and protein translation (1–3). GSK-3 remains active at resting state and is inactivated by a variety of mitogens, many protein kinases including protein kinase B/Akt, and by the Wnt signaling pathway. Many targets of GSK-3 are negatively regulated by it, and inactivation of GSK-3 stimulates cellular functions by removing the repression (1–3).

GSK-3 has two isoforms, GSK-3α and GSK-3β. The molecular mass of GSK-3α is 51 kDa, and that of GSK-3β is 47 kDa (4). Structurally, the two isoforms have 97% sequence homology within their kinase domains, but GSK-3α has an extended N-terminal glycine-rich tail (2), and the two kinases share only 36% in the last 76 C-terminal residues (1). Although both isoforms share substrates, their expression patterns, substrate preferences, and cellular functions are not identical (for review, see Ref. 5). GSK-3α is not phosphorylated/inhibited by protein kinase C (PKC), whereas the activity of GSK-3β is decreased by about 50% when phosphorylated by some PKC isotypes, including PKC-α, -β1, and -γ (6). In neurons, GSK-3α, but not GSK-3β, is involved in regulating production of Alzheimer disease amyloid-β peptides (7). Genetic ablation of GSK-3β in mice results in an embryonic lethal phenotype (8), suggesting that GSK-3β and GSK-3α play different roles in development. Using constitutively active GSK-3β321A/321A/39A/39A knockin mice, it has recently been shown that inactivation of GSK-3β, rather than GSK-3α, is the major route by which insulin activates skeletal muscle glycogen synthase (9). GSK-3β, but not GSK-3α, regulates cardiomyocyte mitochondrial permeability transition in culture (10). Inhibition of GSK-3α more robustly enhances cAMP-response element- and NFκB-dependent transactivation than that of GSK-3β (11). Silencing of GSK-3α down-regulates the binding activity of early growth response-1 (EGR-1)
GSK-3α Reduces Cardiac Growth and Hypertrophy by Inhibiting ERK

TABLE 1
The differences between GSK-3α and GSK-3β reported in the literature

| CRE, cAMP-response element; EGR-1, early growth response-1. | GSK-3α | GSK-3β |
|------------------------------------------------------------|--------|--------|
| Molecular structure                                         | 51 kDa extended N-terminal glycine-rich tail | 47 kDa, 36% identity with GSK-3α in the last 76 C-terminal residues |
| Phosphorylation by PKC                                      | No     | Yes    |
| Regulates production of Alzheimer disease amyloid-β peptide | No     | No     |
| Phosphorylation of skeletal muscle glycogen synthase        | No     | No     |
| Regulates mitochondrial permeability transition             | No     | No     |
| CRE- and NFκB-dependent transactivation                      | No     | No     |
| EGR-1 transcriptional activity                              | Up-regulates | Down-regulates |
| Smad3/4-responsive transcription                             | Inhibits | Promotes |

FIGURE 1. A, expression of GSK-3α and phosphorylation (p-) level of GSK-3α in mouse hearts in response to 4 weeks of TAC. *, p < 0.05 versus SHAM. B, expression of GSK-3β and phosphorylation of GSK-3β in mouse hearts in response to TAC. C, expression of GSK-3α and its phosphorylation in areas remote from myocardial infarction (MI) 7 days after coronary ligation. *, p < 0.05 versus SHAM. D, expression of GSK-3β and its phosphorylation in areas remote from myocardial infarction 7 days after coronary ligation.

to EGR1-responsive element, whereas silencing of GSK-3β upregulates this activity (11). Smad3/4-responsive transcription is enhanced by GSK-3α small interfering RNA (siRNA) but slightly decreased by GSK-3β siRNA (11). Thus, GSK-3α is different from GSK-3β in many ways, despite many similarities (Table 1).

Both GSK-3α and GSK-3β exist in the heart (4). The function of GSK-3β has been studied in cultured cardiac myocytes in vitro and in transgenic animal models in vivo. GSK-3β negatively regulates β-adrenergic- and endothelin-induced cardiac hypertrophy in neonatal rat cardiac myocytes (NRCMs) (12–14). Cardiac-specific overexpression of constitutively active GSK-3β (GSK-3β(S9A)) in transgenic mice inhibits cardiac hypertrophy in response to pressure overload and isoproterenol infusion (15, 16). Cardiac-specific overexpression of wild type GSK-3β in mice caused reduced cardiac growth with diastolic dysfunction (17). These studies provided invaluable insights regarding the function of GSK-3β in cardiac hypertrophy. However, the function of GSK-3α in the heart remains to be elucidated. The present study was designed to study the function of GSK-3α in the heart and the underlying signaling mechanisms.

EXPERIMENTAL PROCEDURES

Primary Culture of Neonatal Rat Ventricular Myocytes—Primary cultures of ventricular cardiac myocytes were prepared from 1-day-old Crl: (WI) BR-Wistar rats (Charles River Laboratories, Wilmington, MA) as previously described (18). A cardiac myocyte-rich fraction was obtained by centrifugation through a discontinuous Percoll gradient.

Construction of Adenoviral Vectors—Recombinant adenovirus was constructed using an Adeno-X adenovirus construction kit (Clontech Laboratories Inc., Palo Alto, CA). We made replication-defective human adenovirus type 5 (devoid of E1 and E3) harboring GSK-3α (Ad-GSK-3α), GSK-3β (Ad-GSK-3β), and shRNA against GSK-3α (Ad-shRNA GSK-3α).

Transgenic Mice—A cDNA clone of GSK-3α was kindly provided by Dr. J. R. Woodgett. Using this clone and the mouse αMHC promoter (provided by Dr. J. Robbins), we made transgenic mice with cardiac-specific expression of GSK-3α.

GSK-3 Kinase Activity Assay—GSK-3α or GSK-3β was immunoprecipitated from tissue lysates with equal protein content. The immunoprecipitate was collected, washed, and resuspended in a reaction buffer containing 25 mM Tris-HCl, pH 7.5, 5 mM β-glycerol phosphate, 12 mM MgCl2, 2 mM dithiothreitol, 0.1 μM Na3VO4, and 200 μM ATP. One hundred ng of Tau was added as a substrate and mixed well. The reaction was carried out for 30 min at 37 °C. Phosphorylation of Tau by GSK-3 was detected by standard immunoblotting using a phospho-Tau antibody (Sigma).

Quantitative Reverse Transcription-PCR—Total RNA was prepared using the RNeasy fibrous tissue kit (Qiagen Inc., Valencia, CA), and first-strand cDNA was synthesized using the ThermoScript reverse transcription-PCR system (Invitrogen). Real-time PCR was then carried out on a DNA Engine Opticon 2 system (Bio-Rad) using the DynAmo HS SYBR Green qPCR kit (Bio-Rad). The specific oligonucleotide primers for atrial natriuretic factor and α-skeletal actin have been previously reported (18).

Echocardiography—Mice were anesthetized using 12 μl/g of body weight of 2.5% avertin (Sigma), and echocardiography was performed using ultrasonography (Acuson Sequoia C256, 33182 JOURNAL OF BIOLOGICAL CHEMISTRY VOLUME 282 • NUMBER 45 • NOVEMBER 9, 2007
Siemens Medical Solutions USA Inc., Malvern, PA) as previously described (18). A 13-MHz linear ultrasound transducer was used. Two-dimension guided M-mode measurements of LV internal diameter were taken from three or more beats and averaged. Left ventricular end-diastolic dimension was measured at the time of the apparent maximal LV diastolic dimension, whereas LV end-systolic dimension was measured at the time of the most anterior systolic excursion of the posterior wall.

**Histological Analysis**—Histological analyses of the heart sections were conducted as described previously (18). Heart specimens were fixed with 10% neutral-buffered formalin, embedded in paraffin, and sectioned at 6-μm thickness. Interstitial fibrosis was evaluated by picric acid Sirius red staining. The positively stained (red) fibrotic area was expressed as a percentage of total area. Total cardiac myocyte numbers were estimated using a published method (19). Briefly, the number of nuclei per unit area of myocardium \(N(n)_M\) was determined in 20 fields (40×). The average nuclear length \(D_n\) was determined in longitudinally oriented myocytes. The number of myocyte nuclei per unit volume of myocardium \(N(n)_V\) was calculated using the equation \(N(n)_V = N(n)_M / D_n\). The estimated total number of myocyte nuclei in the left ventricle was calculated as the product of the number of myocyte nuclei per unit volume, \(N(n)_V\), and the total ventricular volume, \(V_v\), which was derived from the left ventricular weight using the specific gravity of muscle tissue, 1.06 g/ml.

**Evaluation of Apoptosis in Tissue Sections**—DNA fragmentation was detected in situ using TUNEL as described (18). Briefly, deparaffinized sections were incubated with proteinase K, and DNA fragments were labeled with fluorescein-conjugated dUTP using TdT (Roche Applied Science). Nuclear density was determined by manual counting of 4′,6-diamidino-2-phenylindole-stained nuclei in 20 fields from each animal using the 40× objective and of TUNEL-positive nuclei in the same fields using the same power objective. Limiting counting of total nuclei and the TUNEL-positive nuclei to areas with a true cross-section of myo-
cytes made it possible to selectively count only those nuclei that clearly were within myocytes.

Transverse Aortic Constriction (TAC)—TAC was performed according to the method published previously (20). Permanent ligation of the left anterior descending coronary artery was conducted as described (21).

Immunoblotting Analysis—Cardiac tissue homogenates and cell lysates were made in CHAPS buffer (Sigma-Aldrich). We used anti-phospho-specific and corresponding non-phospho-specific antibodies against GSK-3α (S21), GSK-3β (S9), Akt (S473), p70 S6 kinase (T389), and extracellular signal-regulated kinases (ERKs) (T202/Y204) (Cell Signaling Technology) and anti-phospho-Tau antibody (Sigma-Aldrich).

Statistical Analysis—Data are reported as the mean ± S.E. Statistical analyses between groups were done by one-way analysis of variance, and when p values were significant, differences among group means were evaluated using the Bonferroni test. A p value less than 0.05 was considered significant.

RESULTS

Expression of GSK-3α Was Increased in Hypertrophied Myocardium Due to Chronic Pressure Overload or Myocardial Infarction—To examine the expression of GSK-3α under pathological conditions, the protein level of GSK-3α was measured in hearts subjected to pressure overload and in the remote area from myocardial infarction. The level of GSK-3α was increased by 2-fold, whereas phospho-GSK-3α was not significantly changed in the heart 4 weeks after TAC (Fig. 1A), indicating that GSK-3α was up-regulated during cardiac hypertrophy. In contrast, the expression of GSK-3β and the phosphorylation of GSK-3β were not changed in the heart 4 weeks after TAC (Fig. 1B). Similarly, GSK-3α expression was increased by 1.6-fold, and phosphorylation of GSK-3α was significantly decreased in myocardium remote from myocardial infarction 7 days after coronary ligation (Fig. 1C). In contrast, neither total expression nor phosphorylation of GSK-3β was significantly changed in the remote area of the infarcted heart (Fig. 1D).

Generation of GSK-3α Transgenic Mice—To study the effect of GSK-3α up-regulation on cardiac phenotype in vivo, two lines of transgenic mice with cardiac-specific overexpression of GSK-3α (Tg), line 13 and line 28, were established. Cardiac tissue transgene expression was assessed at the protein level using 2–3-month-old mice. The expression of GSK-3α in Tg mice was 4.3 and 3.7 times that of NTg mice in line 28 and line 13, respectively (Fig. 2A and supplemental Fig. 1A).
Interestingly, the expression of GSK-3β was down-regulated in Tg mice in both lines (Fig. 2B and supplemental Fig. 1B). The GSK-3α kinase activity in Tg was increased by 1.7-fold in line 28, and the GSK-3β activity was decreased by 40% (Fig. 2, C and D). We primarily characterized line 28 in this study.

### TABLE 3

| Hemodynamic measurements of GSK-3α Tg mice |
|--------------------------------------------|
| MAP, mean aortic pressure; LVSP, LV systolic pressure; N, number; HR, heart rate. |
|                                | 3-Month old | 7-Month old |
|                                | NTg         | Tg          | NTg         | Tg          |
| N                               | 6           | 6           | 5           | 4           |
| LVSP (mm Hg)                   | 101 ± 7     | 92 ± 5      | 102 ± 2     | 96 ± 3      |
| LVEDP (mm Hg)                  | 2.4 ± 0.4   | 3.0 ± 0.7   | 2.0 ± 0.0   | 2.7 ± 0.6   |
| +dP/dt (mm Hg/s)               | 9000 ± 764  | 7900 ± 983  | 9000 ± 500  | 9870 ± 331  |
| −dP/dt (mm Hg/s)               | 7500 ± 661  | 6625 ± 453  | 7500 ± 500  | 7000 ± 254  |
| MAP (mm Hg)                    | 74.9 ± 3.7  | 67.8 ± 6.8  | 78.7 ± 6.7  | 82.2 ± 6.2  |
| HR (bpm)                       | 485 ± 12    | 498 ± 15    | 558 ± 13    | 498 ± 48    |

**FIGURE 5.** The effect of 4 weeks of aortic banding on hypertrophy and fetal gene expression. A, LVW/BW. p < 0.001 (*) and p < 0.01 (#) are versus SHAM, p < 0.05 ($) and p < 0.01 ($) are versus the corresponding NTg, B, LVW/LT, p < 0.001 (*) and p < 0.01 (#) are versus SHAM, p < 0.05 ($) and p < 0.01 ($) are versus the corresponding NTg. C, wheat germ agglutinin–Texas Red staining of cardiac sections. D, myocyte cross-sectional area, p < 0.001 (*) and p < 0.01 (#) are versus SHAM, p < 0.05 ($) and p < 0.01 ($) are versus the corresponding NTg. E, atrial natriuretic factor (ANF) expression. p < 0.001 (*) and p < 0.01 (#) are versus SHAM. $, p < 0.05 versus NTg. GAPDH, glyceraldehyde-3-phosphate dehydrogenase. F, α-skeletal actin (ASA) expression. p < 0.001 (*) and p < 0.01 (#) are versus SHAM. $, p < 0.05 versus NTg.

GSK-3α Down-regulates Cardiac Physiological Growth—To examine the effect of GSK-3α on physiological growth of the heart, heart size, LV weight, and cardiac myocyte size were compared between NTg and Tg mice at both 3 and 7 months old. Tg mice had smaller hearts than NTg mice at both ages (Fig. 3A). The LV weight (LVW)/body weight (BW) and the LVW/tibia length (TL) in Tg mice were significantly lower than those in NTg mice at both ages (Fig. 3, B and C). The LV myocyte cross-sectional area was significantly smaller in Tg mice than in NTg mice at 3 and 7 months old (Fig. 3, D and E), indicating smaller myocyte size in Tg mice. To further examine the effect of GSK-3α on cardiac myocyte size, myocytes were isolated from 3-month-old mice. Cardiac myocyte capacitance, proportional to the cell surface area, was significantly decreased in Tg (Fig. 3F), indicating that the overall size of cardiac myocytes was smaller in Tg than in NTg. The estimated total number of cardiac myocytes was not significantly different between NTg and Tg at either 3 or 7 months old (supplemental Fig. II, A and B), suggesting that the smaller cardiac myocyte size rather than changes in the total number of myocytes is an important basis for the smaller heart in Tg. In the other line of Tg mice (line 13), LVW/BW and LVW/TL in Tg were significantly lower than those in littermate NTg (supplemental Fig. III, A and B). These data indicate that GSK-3α negatively regulates physiological growth of the heart.

GSK-3α Modestly Increases Apoptosis and Fibrosis but Does Not Cause Cardiac Dysfunction at Base Line—The percent fibrosis in Tg mouse hearts was slightly but significantly increased as compared with that in NTg at 3 and 7 months old (Fig. 4, A and B). Similarly, the TUNEL-positive nuclei were slightly but significantly increased in Tg mouse hearts at both ages (Fig. 4C). Despite the modest increase in apoptosis and fibrosis, Tg mice did not develop any signs of heart failure. The lung weight/BW and lung weight/TL were not significantly different between NTg and Tg mice (data not shown). The ejection fraction and fractional shortening (FS), determined by echocardiographic measurement, in Tg mice were not significantly different from those in NTg mice (Table 2). The +dP/dt, −dP/dt, and LV end-diastolic pressure (LVEDP), determined by...
hemodynamic analysis, were also not significantly different between NTg and Tg mice (Table 3). After up to 7 months of follow-up, no significant increase in premature mortality was found in either line 28 or line 13 Tg mice. These data indicate that Tg mice did not develop cardiac dysfunction at base line up to 7 months of age despite a modest increase in apoptosis and fibrosis.

**GSK-3α Reduces Cardiac Growth and Hypertrophy by Inhibiting ERK**

To examine the effect of GSK-3α on pressure overload-induced cardiac hypertrophy, 3-month-old Tg mice and NTg mice underwent TAC operation. Two to 4 weeks after TAC, Tg mice developed less severe cardiac hypertrophy than NTg mice. The increases in LVW/BW (2 weeks of banding, 37.5 versus 48.1%, $p < 0.01$; 4 weeks of banding, 48.7 versus 59.1%, $p < 0.01$) and LVW/TL (2 weeks of banding, 36.2 versus 55.3%, $p < 0.01$; 4 weeks of banding, 50 versus 65.9%, $p < 0.01$) were significantly smaller in Tg mice than in NTg mice (Fig. 5, A and B, supplemental Fig. IV), and the increases in cardiac myocyte cross-sectional area 4 weeks after TAC (21% versus 40%, $p < 0.01$) were also significantly smaller in Tg mice than in NTg mice (Fig. 5, C and D). Expression of fetal-type genes, such as atrial natriuretic factor and α-skeletal actin, was significantly less in Tg than in NTg mice (Fig. 5, A and B, supplemental Fig. IV), and the increases in heart attenuates pressure overload-induced cardiac hypertrophy. Interestingly, despite the inhibition of cardiac hypertrophy, Tg mice developed more severe cardiac dysfunction than NTg mice after TAC. Although no (of 11) NTg mice died after the operation, 2 (of 11) Tg mice died 3 days after TAC (Fig. 5, A and B, supplemental Fig. IV), and the increases in cardiac myocyte cross-sectional area 4 weeks after TAC (21% versus 40%, $p < 0.01$) were also significantly smaller in Tg mice than in NTg mice (Fig. 5, C and D). Expression of fetal-type genes, such as atrial natriuretic factor and α-skeletal actin, was significantly less in Tg than in NTg mice (Fig. 5, A and B, supplemental Fig. IV), and the increases in cardiac myocyte cross-sectional area 4 weeks after TAC (21% versus 40%, $p < 0.01$) were also significantly smaller in Tg mice than in NTg mice (Fig. 5, C and D). Expression of fetal-type genes, such as atrial natriuretic factor and α-skeletal actin, was significantly less in Tg than in NTg mice (Fig. 5, A and B, supplemental Fig. IV), and the increases in cardiac myocyte cross-sectional area 4 weeks after TAC (21% versus 40%, $p < 0.01$) were also significantly smaller in Tg mice than in NTg mice (Fig. 5, C and D). Expressions of fetal-type genes, such as atrial natriuretic factor and α-skeletal actin, was significantly less in Tg than in NTg mice (Fig. 5, A and B, supplemental Fig. IV), and the increases in heart attenuates pressure overload-induced cardiac hypertrophy. Interestingly, despite the inhibition of cardiac hypertrophy, Tg mice developed more severe cardiac dysfunction than NTg mice after TAC. Although no (of 11) NTg mice died after the operation, 2 (of 11) Tg mice died 3 days after TAC (Fig. 5, A and B, supplemental Fig. IV), and the increases in heart attenuates pressure overload-induced cardiac hypertrophy. Interestingly, despite the inhibition of cardiac hypertrophy, Tg mice developed more severe cardiac dysfunction than NTg mice after TAC. Although no (of 11) NTg mice died after the operation, 2 (of 11) Tg mice died 3 days after TAC (Fig. 5, A and B, supplemental Fig. IV), and the increases in heart attenuates pressure overload-induced cardiac hypertrophy. Interestingly, despite the inhibition of cardiac hypertrophy, Tg mice developed more severe cardiac dysfunction than NTg mice after TAC.
GSK-3α Reduces Cardiac Growth and Hypertrophy by Inhibiting ERK

GSK-3α Inhibits Phosphorylation of ERK through PKCε-dependent Mechanisms—To study the signaling mechanisms involved in down-regulation of cardiac growth by GSK-3α, activities of various signaling molecules were screened. Among them, phosphorylation of ERK was decreased by 40% in Tg hearts (Fig. 9A). After TAC, phosphorylation of ERK was increased in both NTg and Tg, but the increase in Tg (2-fold) was significantly smaller than that in NTg (2.7-fold) (Fig. 9B). In NRCMs in vitro, adenovirus-mediated overexpression of GSK-3α, but not GSK-3β, markedly reduced ERK phosphorylation (Fig. 9C), indicating that the effect on ERK phosphorylation may be specific to GSK-3α. On the other hand, knockdown of GSK-3α increased the phosphorylation of ERK in NRCMs (Fig. 9D). To study the signaling mechanism through which GSK-3α inhibits ERK phosphorylation, a MEK1 inhibitor and inhibitors for PKCβ and PKCε were applied to NRCMs transduced with the Ad-shRNA-GSK-3α. The selective and cell-permeable MEK inhibitor, PD98059, dose-dependently inhibited ERK phosphorylation caused by GSK-3α knockdown (Fig. 9E), indicating that GSK-3α negatively regulates ERK phosphorylation through MEK. Both the PKCβ inhibitor, rottlerin, and the PKCε inhibitor peptide also dose-dependently decreased ERK phosphorylation induced by GSK-3α silencing (Fig. 9, F and G), indicating that GSK-3α inhibits ERK phosphorylation through PKCβ/PKCε.

Response to Pressure Overload

Further Increases Cardiac Fibrosis and Apoptosis in Response to Pressure Overload—After TAC, cardiac fibrosis was increased in both NTg and Tg mice, with significantly more fibrosis in Tg mice (Fig. 7, A and B). The increase in percent fibrosis after 4 weeks of TAC was significantly greater in Tg than in NTg (1.52- versus 1.17-fold, p < 0.01). Similarly, TUNEL-positive nuclei were more numerous in both NTg and Tg mice 4 weeks after TAC, but Tg mouse hearts had significantly more positive nuclei than NTg (Fig. 7, C and D). The increase in percent TUNEL-positive nuclei was significantly greater in Tg than in NTg in response to 4 weeks of TAC (2.34-versus 1.72-fold, p < 0.01). The more severe fibrosis and apoptosis in Tg mouse hearts may, at least in part, explain more severe cardiac dysfunction in Tg mice after TAC.

GSK-3α Inhibits Growth and Promotes Apoptosis in Cardiac Myocytes in Vitro—To study whether GSK-3α directly acts on cardiac myocytes to inhibit cardiac growth and increase apoptosis, we performed in vitro experiments using cultured NRCMs. We made an adenovirus harboring GSK-3α (Ad-GSK-3α) and an adenovirus harboring a shRNA against GSK-3α (Ad-shRNA-GSK-3α). Ad-GSK-3α and Ad-shRNA-GSK-3α specifically increased and knocked down the expression of GSK-3α, respectively, without influencing the expression level of GSK-3β in NRCMs (Fig. 8A). Overexpression of GSK-3α decreased cardiac myocyte size, whereas knockdown of GSK-3α increased cardiac myocyte size (Fig. 8B). Protein content in cardiac myocytes, another indicator of cardiac myocyte growth, was significantly decreased in a dose-dependent manner in cells transduced with Ad-GSK-3α but was significantly increased dose-dependently in cells transduced with Ad-shRNA-GSK-3α (Fig. 8C). These results suggest that GSK-3α negatively regulates cardiac myocyte growth. To study whether GSK-3α inhibits agonist-induced hypertrophy, we treated GSK-3α-overexpressing cardiac myocytes with phenylephrine. Increased cell size and protein content caused by phenylephrine were abolished in cardiac myocytes overexpressing GSK-3α (Fig. 8, D and E). Cytosplasmic accumulation of mono- and oligonucleosomes, detected by Cell Death ELISA, was dose-dependently increased by overexpression of GSK-3α but was dose-dependently inhibited by knockdown of GSK-3α (Fig. 8F). These results suggest that GSK-3α promotes cardiac myocyte apoptosis.

after the operation, and postmortem pathological measurements revealed severe lung congestion (data not shown), indicating that the mice died due to heart failure. More severe lung congestion (higher lung weight/BW and lung weight/TL) was observed in Tg mice 2 and 4 weeks after TAC (Fig. 6, A and B, supplemental Fig. V, A and B). Echocardiographic measurements indicated that ejection fraction (EF) and fractional shortening (FS) in Tg mice were significantly lower than those in NTg mice 2 and 4 weeks after TAC (Fig. 6, C and D, supplemental Fig. V, C and D). The radius/wall thickness (r/w) ratio, an indicator of LV wall stress, was significantly higher in Tg than in NTg 4 weeks after TAC (Fig. 6E). LVEDP/EDD, an indicator of LV wall stiffness, was significantly greater in Tg than in NTg 4 weeks after TAC (Fig. 6F). Hemodynamic measurements indicated that, although LV end-systolic stress was not different between NTg and Tg either at base line or after TAC, LV end-diastolic stress was significantly higher in Tg than in NTg 4 weeks after TAC (Fig. 6G, H and I). LV +dP/dt and −dP/dt in Tg mice were significantly decreased compared with those in NTg mice, and the decreases were significantly greater in Tg than in NTg after 2 and 4 weeks of TAC (Table 4, supplemental Fig. VE).

GSK-3α Further Increases Cardiac Fibrosis and Apoptosis in Response to Pressure Overload—After TAC, cardiac fibrosis was increased in both NTg and Tg mice, with significantly more fibrosis in Tg mice (Fig. 7, A and B). The increase in percent fibrosis after 4 weeks of TAC was significantly greater in Tg than in NTg (1.52- versus 1.17-fold, p < 0.01). Similarly, TUNEL-positive nuclei were more numerous in both NTg and Tg mice 4 weeks after TAC, but Tg mouse hearts had significantly more positive nuclei than NTg (Fig. 7, C and D). The
GSK-3α Reduces Cardiac Growth and Hypertrophy by Inhibiting ERK

![Graphs and images]

**FIGURE 8.** A, expression of GSK-3α in cardiac myocytes transduced with a control adenovirus (LacZ, 30 m.o.i.), adenovirus harboring GSK-3α (GSK-3α, 30 m.o.i.) or adenovirus harboring a shRNA against GSK-3α (shRNA, 30 m.o.i.). B, cardiac myocyte size when myocytes were transduced with LacZ (30 m.o.i.), GSK-3α (30 m.o.i.), or shRNA (30 m.o.i.). *, p < 0.05 versus LacZ; n = 3. C, protein content in cardiac myocytes transduced with LacZ, GSK-3α, or shRNA at the indicated doses. The protein content of LacZ at 10 m.o.i. is designated as 1. p < 0.05 (*) and p < 0.01 ($) are versus LacZ; n = 3, D and E, cell size and protein content of cardiac myocytes transduced with LacZ (30 m.o.i.) or GSK-3α (30 m.o.i.) after phenylephrine (PE, 10 μM) stimulation. p < 0.05 (*) and p < 0.01 (#) are versus LacZ; n = 3. F, cytoplasmic accumulation of mono- and oligonucleosomes, a sensitive indicator of DNA fragmentation due to apoptosis, was quantitated by Cell Death ELISA Plus. Cardiac myocytes were transduced with LacZ, GSK-3α, or shRNA at the indicated doses. The apoptosis of LacZ at 10 m.o.i. is designated as 1. p < 0.05 (*) and p < 0.01 (#) are versus LacZ; n = 3.

GSK-3α Inhibits Cardiac Myocyte Growth and Promotes Apoptosis through Inhibition of ERK—We examined the mechanism through which down-regulation of GSK-3α induces cardiac hypertrophy. The MEK inhibitor, PD98059, significantly attenuated the effect of GSK-3α silencing on NRCM protein content (Fig. 10A), indicating that MEK-ERK-dependent mechanisms are the major pathway through which GSK-3α inhibits cardiac growth. Both the PKCδ inhibitor, rottlerin, and PKCe inhibitor peptide also significantly inhibited the increase in protein content due to GSK-3α silencing in NRCMs (Fig. 10B), indicating that GSK-3α may inhibit cardiac growth through PKCδ/PKCɛ-MEK-ERK-dependent mechanisms. Knockdown of GSK-3α significantly reduced apoptosis caused by serum starvation as expected (Fig. 10C). PD98059 significantly enhanced apoptosis caused by serum starvation and abolished the protective effects of GSK-3α silencing against serum starvation (Fig. 10C). These results suggest that endogenous GSK-3α stimulates apoptosis through inhibition of MEK-ERK-dependent mechanisms.

GSK-3α Inhibits p70 S6 Kinase through ERK-dependent Mechanisms—Because protein synthesis is regulated through Akt/mTOR signaling, we examined the impact of GSK-3α overexpression upon this signaling pathway both in vitro and in vivo. Although phospho-Akt and Akt were not different between NTg and Tg, phosphorylation of p70 S6 kinase, a downstream target of mTOR, was decreased in Tg (Fig. 11A). In cultured cardiac myocytes, overexpression of GSK-3α inhibited, whereas knockdown of GSK-3α increased, the phosphorylation of p70 S6 kinase (Fig. 11B). Furthermore, increased phosphorylation of p70 S6 kinase induced by GSK-3α knockdown was abolished by the MEK1 inhibitor PD98059 (Fig. 11C), suggesting that GSK-3α inhibits p70 S6 kinase through a MEK/ERK-dependent mechanism.

**DISCUSSION**

Our results suggest that GSK-3α negatively regulates cardiac growth and promotes apoptosis in cardiac myocytes and that GSK-3α is up-regulated by clinically important pathologic insults such as pressure overload and myocardial infarction. Cardiac-specific overexpression of GSK-3α in mice resulted in small hearts and cardiac myocyte size, increases in apoptosis and fibrosis, and normal cardiac function at base line. In response to pressure overload, GSK-3α transgenic mice exhibited less severe cardiac hypertrophy, enhanced apoptosis and fibrosis, and markedly reduced cardiac function with increased wall stress. Unlike GSK-3β transgenic mice, which also show inhibited cardiac growth (17), GSK-3α mice have normal base-line cardiac function up to 7 months of age and inhibition of ERK. In response to pressure overload, GSK-3α mice developed cardiac dysfunction, in contrast to GSK-3β (S9A) transgenic mice, which demonstrated well maintained cardiac function despite inhibition of hypertrophy (15).

In GSK-3α transgenic mice, expression of GSK-3β was significantly reduced. The molecular mechanism mediating the interplay between GSK-3α and GSK-3β is unknown at present.
We believe that the cardiac phenotype observed in Tg-GSK-3α is caused primarily by increases in GSK-3α rather than decreases in GSK-3β, because in transgenic mice with cardiac-specific overexpression of dominant negative GSK-3β, decreases in GSK-3β activity induce cardiac hypertrophy and improve cardiac function.4 We speculate that the GSK-3α dominant environment overrides any cardiac phenotype induced by the 40% reduction in GSK-3β activity in our transgenic mouse model.

Mitogen-activated protein kinases (MAPKs) consist of three distinct groups of kinases; ERKs, c-Jun N-terminal kinase, and p38 MAPKs. The ERKs are generally associated with cell growth and differentiation and are activated through phosphorylation of upstream MEK1. Specific activation of ERK in the heart by overexpression of activated MEK1 resulted in concentric LV hypertrophy (i.e. enhanced cardiac growth) (22). Our results show that activation of GSK-3α in the heart in vivo decreased ERK phosphorylation and attenuated cardiac physiological growth. Furthermore, our in vitro data demonstrated that the MEK1 inhibitor abolished the effect of GSK-3α silencing on protein synthesis. These results suggest that negative regulation of cardiac growth by GSK-3α is primarily mediated by the MEK1-ERK pathway, although GSK-3α could have many other potential down-stream signaling mechanisms (Fig. 12). The results of the present study suggest that inhibition of ERK by GSK-3α may lead to inhibition of cardiac growth. Abundant in vitro evidence shows that inhibition of MEK/ERK signaling attenuates cardiac myocyte hypertrophy induced by many agonists (23–27), suggesting that ERK activation is intimately involved in the development of cardiac hypertrophy. After aortic banding, an increase in ERK phosphorylation was associated with an increase in left ventricular mass (28). In response to pressure overload, in dominant negative Raf1 transgenic mice, ERK activity was reduced, and cardiac hypertrophy was attenuated (29). These observations support the notion that MEK/ERK signaling is required for the development of cardiac hypertrophy under pressure overload. Therefore, inhibition of ERK by GSK-3α may also, at least in part, account for decreased cardiac hypertrophy in Tg-GSK-3α mice in response to pressure overload. Because GSK-3α is up-regulated by TAC, GSK-3α serves as a negative feedback regulator of ERKs and hypertrophy in response to TAC.

The inhibition of ERK may be induced by GSK-3α but not by

4 Hirotani, S., Zhai, P., Tomita, H., Galeotti, J., Marquez, J. P., Gao, S., Hong, C., Yatani, A., Avila, J., and Sadoshima, J. Circ. Res. in press.
GSK-3α Reduces Cardiac Growth and Hypertrophy by Inhibiting ERK

GSK-3β. Overexpression of GSK-3β in the heart increased ERK phosphorylation in transgenic mice (17). In our GSK-3α transgenic mouse heart, ERK phosphorylation was inhibited. Furthermore, our results show that GSK-3β did not decrease the phosphorylation of ERK in cardiac myocytes but that GSK-3α did. It is speculated that the presence of ERK inactivation makes GSK-3α overexpression more prone to LV dysfunction in response to pressure overload compared with GSK-3β (S9A) overexpression mice, although this notion needs to be tested experimentally.

How, then, does GSK-3α inhibit the phosphorylation of ERK? Overexpression of PKCe in adult cardiac myocytes significantly increased PKCe activity and elevated ERK activity (30). In the PKCe knock out mouse left ventricle, ERK phosphorylation was attenuated both at baseline and after TAC operation (31). These data point to a possibility that PKCe activity is required for ERK phosphorylation. In our GSK-3α transgenic mouse hearts, PKCe activity was attenuated (data not shown). GSK-3α may inhibit ERK activation through down-regulation of PKCe activity. Our in vitro data showed that ERK phosphorylation caused by GSK-3α knockdown was inhibited by both PKCε and PKCe inhibitors, suggesting that endogenous GSK-3α inhibits ERK phosphorylation through PKCε-dependent mechanisms. It has been reported that PKCe activation is mediated through PKCε in ethanol-induced cardiac protection from ischemia (32). It is, therefore, possible that PKCε and PKCe are in the same pathway mediating ERK phosphorylation caused by GSK-3α silencing.

mTOR and its downstream targets, including p70 S6 kinase, play an important role in mediating increases in protein synthesis during cardiac hypertrophy (33, 34). Accumulating lines of evidence have suggested that the MEK/ERK pathway is involved in activation of mTOR signaling. For example, activation of protein synthesis in cardiac myocytes by the hypertrophic agonist phenylephrine requires ERK-dependent activation of mTOR signaling (24). The MEK/ERK pathway plays a major role in mediating activation of p70 S6 kinase during hypertrophic cardiac growth (35). The present study demonstrated a role of GSK-3α in inhibiting ERK-dependent p70 S6 kinase activation. MEK/ERK phosphorylates tuberous sclerosis complex 2 (TSC2) either directly or through p90RSK, thereby inducing dissociation of the TSC1-TSC2 complex, impairment of the ability of TSC2 to inhibit mTOR signaling, and subsequent increases in protein synthesis (36, 37). The precise mechanism by which the MEK/ERK pathway promotes mTOR signaling in cardiac myocytes, however, remains to be elucidated.

Cardiac hypertrophy is usually considered to be a compensatory mechanism, although prolonged presentation of hypertrophy often leads to heart failure. According to Laplace’s Law, an increase in wall thickness of the left ventricle leads to a decrease in wall stress and, consequently, in oxygen consumption. In this sense, cardiac hypertrophy may be an adaptive response to mechanical overload. In agreement with this notion, impairment of this compensatory process results in cardiac dysfunction in some animal models of cardiac hypertrophy. For example, cyclosporine A, an inhibitor of calcineurin, attenuated cardiac hypertrophy but enhanced cardiac dysfunction and heart failure due to pressure overload (38). RGS4, a promoter of heterotrimeric G protein deactivation, overexpressed in the heart reduced cardiac hypertrophy in response to pressure overload but increased mortality due to heart failure (39). On the other hand, attenuation of pressure overload-induced cardiac hypertrophy by the C-terminal peptide of Ga8,
thioredoxin, and GSK-3β (S9A) did not induce heart failure despite elevated wall stress (15, 40, 41). It is probable that the underlying signaling molecules altered by cardiac hypertrophic stimuli rather than the presence of hypertrophy alone are more important predictors of the prognosis of cardiac hypertrophy. In the case of GSK-3α activation, the present study shows that Tg-GSK-3α mice developed less cardiac hypertrophy but more apoptosis and interstitial fibrosis and more severe cardiac dysfunction in response to pressure overload. In the presence of GSK-3α overexpression, increased wall stress caused by lack of sufficient hypertrophy and increased apoptosis may stimulate one another, thereby facilitating heart failure.

In conclusion, the data presented here show that GSK-3α inhibits cardiac physiological growth, at least in part through inhibition of ERK activation. In response to pressure overload, GSK-3α transgenic mice developed less cardiac hypertrophy but more fibrosis and apoptosis along with more severe cardiac dysfunction. We propose that GSK-3α is a negative regulator of compensatory hypertrophy and a promoter of apoptosis. Thus, it is likely that stimulation of GSK-3α is detrimental during pressure overload-induced cardiac hypertrophy.

Acknowledgment—We thank Daniela Zablocki for critical reading of the manuscript.

REFERENCES

1. Doble, B. W., and Woodgett, J. R. (2003) J. Cell Sci. 116, 1175–1186
2. Frame, S., and Cohen, P. (2001) Biochem. J. 359, 1–16
3. Woodgett, J. R. (2001) Sci. STKE 2001, RE12
4. Woodgett, J. R. (1990) EMBO J. 9, 2431–2438
5. Hardt, S. E., and Sadoshami, J. (2004) Cardiov. Res. 63, 500–509
6. Goode, N., Hughes, K., Woodgett, J. R., and Parker, P. J. (1992) J. Biol. Chem. 267, 16878–16882
7. Phiel, C. J., Wilson, C. A., Lee, V. M., and Klein, P. S. (2003) Nature 423, 435–439
8. Hoeflich, K. P., Luo, J., Rubie, E. A., Tsao, M. S., Jin, O., and Woodgett, J. R. (2000) Nature 406, 86–90
9. McManus, E. J., Sakamoto, K., Armit, L. J., Ronaldson, L., Shpiro, N., Marquez, B. D., Wang, S., Ytrehus, K., Antos, C. L., Olson, E. N., and Sollott, S. J. (2004) J. Clin. Investig. 113, 1535–1549
10. Liang, M. H., and Chuang, D. M. (2006) J. Biol. Chem. 281, 30479–30484
11. Haq, S., Choukroun, G., Kang, Z. B., Ranu, H., Matsui, T., Rosenzweig, A., Molkentin, J. D., Alessandrini, A., Woodgett, J., Hajjar, R., Michael, A., and Force, T. (2000) J. Cell. Biol. 151, 117–130
12. Morisco, C., Seta, K., Hardt, S. E., Lee, Y., Vatner, D. F., and Sadoshami, J. (2001) J. Biol. Chem. 276, 28586–28597
13. Morisco, C., Zebrowski, D. C., Vatner, D. E., Vatner, S. F., and Sadoshami, J. (2001) J. Mol. Cell. Cardiol. 33, 561–573
14. Antos, C. L., McKinsey, T. A., Frey, N., Kutschke, W., McNally, J., Sheldon, J. M., Richardson, J. A., Hill, J. A., and Olson, E. N. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 907–912
15. Sanbe, A., Gullick, J., Hanks, M. C., Liang, Q., Osinska, H., and Robbins, J. (2003) Circ. Res. 92, 609–616
16. Michael, A., Haq, S., Chen, X., Hsich, E., Cui, L., Walters, B., Shao, Z., Bhattacharya, K., Kilter, H., Huggins, G., Andreucci, M., Periasamy, M., Solomon, R. N., Liao, R., Patten, R., Molkentin, J. D., and Force, T. (2004) J. Biol. Chem. 279, 21383–21393
17. Zhai, P., Yamamoto, M., Galeotti, J., Liu, J., Masurekar, M., Thaiss, J., Irie, K., Holle, E., Yu, X., Kupershmidt, S., Roden, D. M., Wagner, T., Yatani, A., Vatner, D. E., Vatner, S. F., and Sadoshami, J. (2005) J. Clin. Investig. 115, 3045–3056
18. Olivetti, G., Quaini, F., Lagrasta, C., Ricci, R., Tiberti, G., Capasso, J. M., and Anversa, P. (1992) Am. J. Pathol. 141, 227–239
19. Zhai, P., Galeotti, J., Liu, J., Holle, E., Yu, X., Wagner, T., and Sadoshami, J. (2006) Circ. Res. 99, 528–536
20. Odashima, M., Usui, S., Takagi, H., Hong, C., Liu, J., Yokota, M., and Sadoshami, J. (2007) Circ Res. 100, 1344–1352
21. Bueno, O. F., De Windt, L. J., Tymitz, K. M., Witt, S. A., Kimball, K. R., Kleivitsky, R., Hewett, T. E., Jones, S. P., Lefer, D. J., Peng, C. F., Kitsis, R. N., and Molkentin, J. D. (2000) EMBO J. 19, 6341–6350
22. Wang, L., and Proud, C. G. (2002) Circ. Res. 91, 821–829
23. Rolfe, M., McLeod, L. E., Pratt, P. F., and Proud, C. G. (2005) Biochem. J. 388, 973–984
24. Glennon, P. E., Kaddoura, S., Sale, E. M., Sale, G. J., Fuller, S. J., and Sugden, P. H. (1996) Circ. Res. 78, 954–961
25. Yue, T.-L., Gu, J.-L., Wang, C., Reith, A. D., Lee, J. C., Mirabile, R. C., Kreutz, R., Wang, Y., Maleeif, B., Parsons, A. A., and Ohlstein, E. H. (2000) J. Biol. Chem. 275, 37985–37990
26. Ueyama, T., Kawashima, S., Sakoda, T., Kikita, Y., Ishida, T., Kawai, M., Yamashita, T., Ishido, S., Hitota, H., and Yokoyama, M. (2000) J. Mol. Cell. Cardiol. 32, 947–960
27. Ingakki, K., and Mohly-Rosen, D. (2005) J. Mol. Cell. Cardiol. 39, 203–211
28. Morgan, H. E., and Beilchik, C. J. (1997) Mol Cell Biochem. 176, 145–151
29. Watson, P. A., Haneda, T., and Morgan, H. E. (1989) J. Biol. Chem. 265, 500–509
30. Inagaki, K., and Mochly-Rosen, D. (2005) Proc. Natl. Acad. Sci. U. S. A. 101, 13489–13494
31. Ma, L., Chen, Z., Erdjument-Bromage, H., Tempst, P., and Pandolfi, P. P. (2005) Cell 121, 179–193
32. Meguro, T., Hong, C., Asai, K., Takagi, G., McKinsey, T. A., Olson, E. N., and Vatner, S. F. (1999) Circ. Res. 84, 735–740
33. Rogers, J. H., Tamirisa, P., Kovacs, A., Weinheimer, C., Courtois, M., Blumer, K. J., Kelly, D. P., and Muslim, A. J. (1999) J. Clin. Investig. 104, 567–576
34. Esposito, G., Rapacciuolo, A., Naga Prasad, S. V., Takaoka, H., Thomas, S. A., Koch, W. J., and Rockman, H. A. (2002) Circulation 105, 85–92
35. Zhai, P., Yamamoto, M., Yang, G., Hong, C., Liu, J., Holle, E., Yu, X., Wagner, T., Vatner, S. F., and Sadoshami, J. (2003) J. Clin. Investig. 112, 1395–1406