Inhibition of Glycogen Synthase Kinase 3β During Heart Failure Is Protective

Shinichi Hirotsu,* Peiyong Zhai,* Hideharu Tomita, Jonathan Galeotti, Juan Pablo Marquez, Shumin Gao, Chull Hong, Atsuko Yatani, Jesús Avila, Junichi Sadashima

Abstract—Glycogen synthase kinase (GSK)-3, a negative regulator of cardiac hypertrophy, is inactivated in failing hearts. To examine the histopathological and functional consequence of the persistent inhibition of GSK-3β in the heart in vivo, we generated transgenic mice with cardiac-specific overexpression of dominant negative GSK-3β (Tg-GSK-3β-DN) and tetracycline-regulatable wild-type GSK-3β. GSK-3β-DN significantly reduced the kinase activity of endogenous GSK-3β, inhibited phosphorylation of eukaryotic translation initiation factor 2Be, and induced accumulation of β-catenin and myeloid cell leukemia-1, confirming that GSK-3β-DN acts as a dominant negative in vivo. Tg-GSK-3β-DN exhibited concentric hypertrophy at baseline, accompanied by upregulation of the α-myosin heavy chain gene and increase in cardiac function, as evidenced by a significantly greater E\textsubscript{max} after dobutamine infusion and percentage of contraction in isolated cardiac myocytes, indicating that inhibition of GSK-3β induces well-compensated hypertrophy. Although transverse aortic constriction induced a similar increase in hypertrophy in both Tg-GSK-3β-DN and nontransgenic mice, Tg-GSK-3β-DN exhibited better left ventricular function and less fibrosis and apoptosis than nontransgenic mice. Induction of the GSK-3β transgene in tetracycline-regulatable wild-type GSK-3β mice induced left ventricular dysfunction and premature death, accompanied by increases in apoptosis and fibrosis. Overexpression of GSK-3β-DN in cardiac myocytes inhibited tumor necrosis factor-α–induced apoptosis, and the antiapoptotic effect of GSK-3β-DN was abrogated in the absence of myeloid cell leukemia-1. These results suggest that persistent inhibition of GSK-3β induces compensatory hypertrophy, inhibits apoptosis and fibrosis, and increases cardiac contractility and that the antiapoptotic effect of GSK-3β inhibition is mediated by myeloid cell leukemia-1. Thus, downregulation of GSK-3β during heart failure could be compensatory. (Circ Res. 2007;101:1164-1174.)

Key Words: GSK-3β • heart failure • cardiac hypertrophy • apoptosis

GSK-3β is a ubiquitously expressed serine/threonine kinase that has versatile biological functions in cells, including regulation of metabolism, cell growth/death, and protein translation and transcription. Unlike most protein kinases, GSK-3β remains active in the resting state and is inactivated when cells are stimulated by mitogens, by other protein kinases, such as Akt, or by the Wnt pathway. In cardiac myocytes, GSK-3β phosphorlylates β-catenin, eukaryotic translation initiation factor (eIF)2Be, NFAT, GATA4, myocardin, and other proteins, thereby negatively regulating protein synthesis and gene expression. GSK-3β downregulates SERCA2a and enhances mitochondrial permeability transition, thereby leading to an inability to normalize cytosolic Ca\textsuperscript{2+} in diastole and reduced cell survival, respectively.

GSK-3β is an important negative regulator of cardiac hypertrophy. GSK-3β negatively regulates β-adrenergic and endothelin-induced cardiac hypertrophy in cultured neonatal myocytes in vitro. Cardiac specific expression of constitutively active GSK-3β (GSK-3β[S9A]) in transgenic mice inhibits cardiac hypertrophy in response to pressure overload and stimulation of the β-adrenergic receptors. Cardiac hypertrophy is mediated by β-catenin and nontransgenic mice. Conditional expression of GSK-3β[S9A] in transgenic mice reversed pressure overload–induced cardiac hypertrophy. Many hypertrophic stimuli inhibit GSK-3β, thereby removing its negative constraints on hypertrophy, which represents a unique mechanism of cardiac hypertrophy.

GSK-3β is phosphorylated at Ser9 and inactivated in heart failure patients. Considering the fact that GSK-3β is an important regulator of growth, death, and metabolism in other...
cell types, changes in the activity of GSK-3β should cause significant biological effects during heart failure. Constitutively active GSK-3β prevents pathological hypertrophy in transgenic mice and thus seems to be salutary.13,14 On the other hand, overexpression of wild-type GSK-3β prevents physiological growth of the heart and causes cardiac dysfunction.9 Expression of constitutively active GSK-3β in a genetic background of hypertrophic cardiomyopathy inhibits the development of hypertrophy but induces heart failure.16 Pharmacological (short-term) inhibition of GSK-3β mimics the protective effects of ischemic preconditioning.17 Inhibition of GSK-3β prevents mitochondrial permeability transition in cardiac myocytes.9 These studies appear to indicate that activation of GSK-3β is detrimental. Thus, whether GSK-3β is protective or detrimental for myocytes seems controversial.18 What previous studies have not addressed is whether or not long-term suppression of GSK-3β activity is protective or detrimental for the heart in vivo.

The goal of this investigation was to elucidate the effect of persistent inactivation of GSK-3β on cardiac phenotype at baseline and in response to pressure overload, using transgenic mice expressing a kinase inactive (dominant negative) form of GSK-3β.

Materials and Methods
For an expanded Materials and Methods section, please refer to the online data supplement at http://circres.ahajournals.org. All experiments involving animals were approved by the Institutional Animal Care and Use Committee at New Jersey Medical School. The data are expressed as means±SEM. Statistical analyses were performed using ANOVA and the Tukey post test procedure. Values of P<0.05 were considered significant.

Results
Generation of Tg-GSK-3β-DN
We generated transgenic mice with cardiac-specific expression of dominant negative GSK-3β (Tg-GSK-3β-DN) using the α myosin heavy chain (αMHC) promoter. We used a double-lysine mutant of GSK-3β, known to act as a dominant negative.19 Tg-GSK-3β-DN line no. 19 mice had 4.7±0.2-fold overexpression of GSK-3β (Figure 1A). Although GSK-3β immunoprecipitated from nontransgenic (NTg) mouse hearts phosphorylated Ser199 of tau, GSK-3β from Tg-GSK-3β-DN hearts induced little phosphorylation of tau (Figure 1B). GSK-3α immunoprecipitated from Tg-GSK-3β-DN hearts exhibited ~40% less phosphorylation of Ser199 tau than that from NTg (not shown). Thus, GSK-3β-DN almost fully suppressed the activity of GSK-3β and modestly reduced the activity of GSK-3α, thereby acting as a dominant negative primarily against GSK-3β. Consistently, basal phosphorylation of eIF2Be, a substrate of GSK-3β,4 was significantly reduced in Tg-GSK-3β-DN compared with NTg mice (Figure 1C). Furthermore, both β-catenin and myeloid cell leukemia (MCL)-1, the expressions of which are negatively regulated by GSK-3β,20 were significantly upregulated in Tg-GSK-3β-DN (Figure 1D). Collectively, the activity of GSK-3β is downregulated in Tg-GSK-3β-DN hearts.

Tg-GSK-3β-DN Develop Physiological Hypertrophy
Tg-GSK-3β-DN (line no. 19) showed a significantly greater left ventricular (LV) weight/body weight (LVW/BW) than NTg at 2.5 months and thereafter (Figure 2A). Tg-GSK-3β-DN (line no. 11, homozygous), with 3.2±0.9-fold overexpression, also exhibited a slightly but significantly greater heart weight (HW)/BW than NTg (4.35±0.05 versus 4.05±0.06, P<0.05) at 6 months. Thus, inhibition of GSK-3β enhanced cardiac growth. We primarily describe the phenotype of line no. 19. Individual myocytes isolated from Tg-GSK-3β-DN hearts at 2.5 to 3 months of age have significantly greater length and width, as well as electrophysiologically determined whole cell capacitance, proportional to the cell surface area, than those from NTg hearts (Figure 2B).

At 3 months, mRNA expression of atrial natriuretic factor was modestly upregulated in Tg-GSK-3β-DN hearts. mRNA expression of α-skeletal-actin in Tg-GSK-3β-DN hearts was not significantly different from that in NTg, whereas mRNA expression of βMHC was significantly downregulated in Tg-GSK-3β-DN hearts (Figure 2C). Interestingly, mRNA expression of αMHC was significantly upregulated in Tg-GSK-3β-DN hearts (Figure 2C). Thus, the expression pattern of hypertrophy-associated genes in Tg-GSK-3β-DN hearts mimics that of physiological hypertrophy.

Histological analyses conducted at 2.5 to 3 and 4 months of age indicated that the myocyte cross-sectional area was significantly greater in Tg-GSK-3β-DN hearts than in NTg hearts (Figure I in the online data supplement). No fibrosis was observed in Tg-GSK-3β-DN hearts (data not shown).

Tg-GSK-3β-DN Mice Have Elevated Maximum Cardiac Contractility
We did not observe significant increases in premature death in Tg-GSK-3β-DN up to 24 months of age. Echocardiographic measurement at baseline showed no significant differences in the chamber size. Although LV fractional shortening percentage (%FS) was slightly greater in Tg-GSK-3β-DN than in NTg (Table), the difference was not statistically significant.

Hemodynamic measurements indicated that Tg-GSK-3β-DN showed a slightly higher Emax and dP/dts than NTg at baseline. The difference in Emax and dP/dts between Tg-GSK-3β-DN and NTg became prominent after dobutamine injection (Figure 3A through 3C). Increases in cardiac output induced by dobutamine were significantly greater in Tg-GSK-3β-DN than in NTg (Figure 3D). The heart rate was not different between NTg and Tg-GSK-3β-DN after dobutamine administration (supplemental Figure II). These results suggest that the maximum contractility of Tg-GSK-3β-DN hearts is greater than that of NTg hearts.

The contractile function was also evaluated using isolated ventricular myocytes (3 to 4 months of age). Indices of isolated myocyte contraction, including percentage of contraction and +dL/dt, were greater in Tg-GSK-3β-DN than in NTg (supplemental Figure IIIA and IIIB). To examine the cellular mechanism for the enhanced myocyte shortening in Tg-GSK-3β-DN, we measured L-type Ca2+ channel currents (ICa). Myocytes from Tg-GSK-3β-DN exhibited a significantly greater ICa than those from NTg. Peak ICa (pA/pF),
plotted as a function of voltage, demonstrated no significant difference in the current–voltage relationships but showed a significantly greater peak $I_{Ca}$ density in myocytes from Tg-GSK-3β/DN than in those from NTg (supplemental Figure IIIC). Protein expression of L-type Ca$^{2+}$ channel was significantly elevated, whereas that of phospholamban and SERCA2a was not, in Tg-GSK-3β/DN hearts (supplemental Figure IV). These results suggest that increases in $I_{Ca}$ may contribute to the increased myocyte contractility in Tg-GSK-3β/DN.

The Effect of Transverse Aortic Constriction on LV Hypertrophy and Function in Tg-GSK-3β/DN
To examine whether inhibition of GSK-3β affects the development of heart failure under pathological stresses, we applied transverse aortic constriction (TAC) for 4, 6, and 8 weeks to Tg-GSK-3β/DN and NTg. To induce transition into decompensated hypertrophy, TAC was conducted using a 28-gauge needle. A comparable pressure gradient was applied to each group after 4 and 6 weeks, whereas the pressure gradient was 20% greater in Tg-GSK-3β/DN than in NTg at 8 weeks, possibly because of better cardiac function in Tg-GSK-3β/DN (supplemental Figure V). Kaplan–Meier survival analysis showed that there was no statistical difference in mortality after TAC between Tg-GSK-3β/DN and NTg. After TAC, HW/tibial length (TL) was greater in Tg-GSK-3β/DN than in NTg at all time points (Figure 4A). However, when the level of cardiac hypertrophy after TAC was normalized to that after sham operation, the TAC-induced fold increase in hypertrophy was similar between

Figure 1. Tg-GSK-3β/DN mice have reduced GSK-3β activity. A, Heart homogenates from Tg-GSK-3β/DN (TG) and NTg (NTG) mice were subjected to immunoblot analyses with anti–GSK-3β. B, Immune complex kinase assays of GSK-3β, using tau as a substrate. B (top), Immunoblot with Ser199 phospho-specific anti-tau antibody. B (bottom), Immunoblot with anti–phospho-specific (p-eIF2Bε) and total eIF2Bε antibodies. C, The levels of GSK-3β substrates, β-catenin, and MCL-1 were determined by immunoblot. Immunoblots with anti–α-actin antibody are shown as loading controls. In the bar graphs, the mean value of NTg was expressed as 1. Each column represents the mean of 5 animals.
Tg-GSK-3β-DN and NTg (supplemental Figure VI), suggesting that GSK-3β-DN primarily affects basal hypertrophy, but not TAC-induced hypertrophy.

Changes in LV systolic function during TAC were evaluated using echocardiography. LV %FS in NTg was significantly reduced at 6 and 8 weeks, whereas that in Tg-GSK-3β-DN was maintained up to 8 weeks and was significantly greater than that in NTg at 6 and 8 weeks (Figure 4B). When the relationship between HW/BW, representing the extent of LV hypertrophy, and lung weight (LW)/BW, representing the extent of LV decompensation, is plotted on x and y axes, respectively, the slope may indicate the extent of cardiac decompensation for a given extent of cardiac hypertrophy. Although the slope in Tg-GSK-3β-DN was significantly steeper than that in NTg at 4 weeks, the slope became less steep at 6 weeks and significantly less steep at 8 weeks than that in NTg (Figure 4C). These results suggest that Tg-GSK-3β-DN mice are more resistant to decompensation at a given extent of cardiac hypertrophy than NTg mice after 8 weeks of TAC.

Histological analyses of LV sections indicated that Tg-GSK-3β-DN mice after TAC have significantly fewer TUNEL-positive myocytes than NTg subjected to TAC (Figure 4D and supplemental Figure VII). Masson’s trichrome staining showed that less fibrosis was observed in Tg-GSK-3β-DN than in NTg mice after 8 weeks of TAC (Figure 4D). Taken together, long-term inactivation of GSK-3β decreases apoptosis/fibrosis and enhances functional compensation in response to pressure overload.

**Inhibition of GSK-3β Failed to Exert an Additive Effect on Exercise-Induced Cardiac Hypertrophy**

Because Tg-GSK-3β-DN developed well-compensated hypertrophy, we examined whether baseline cardiac hypertrophy, we examined whether baseline cardiac hypertro-
The heart-specific expression of the transgene was also increased in Tg-tetGSK-tTA (without Dox) than in Tg-tetGSK (Figure 5A). The progressive increase in premature death of Tg-tetGSK-tTA was also observed when Dox treatment was withdrawn at 10 weeks after birth (supplemental Figure XA), which eliminated contributions of transgene expression during fetal and neonatal development to the premature death of the mice. After Dox withdrawal, Tg-tetGSK-tTA exhibited progressive increases in LV end-diastolic dimension and decreases in %FS, as determined by echocardiographic measurement (supplemental Figure XB and Table III), suggesting that the postnatal conditional increase in GSK-3β expression induces cardiac dysfunction.

GSK-3β is inactivated during cardiac hypertrophy and heart failure. To examine the role of GSK-3β inactivation during heart failure, TAC was applied to Tg-tetGSK-tTA in the presence of Dox, and, after 4 weeks, Dox was withdrawn and the mice were followed for another 4 weeks (Figure 6A). Eight weeks of TAC significantly reduced the kinase activity of GSK-3β in Tg-tetGSK-tTA mice in the presence of Dox. However, Dox withdrawal induced transgene expression of GSK-3β, and the GSK-3β activity was no longer inhibited after 8 weeks of TAC (Figure 6B). Although the activity of GSK-3β after Dox withdrawal was significantly greater than in control mice (≈1.7-fold), it was not as high as in Dox-untreated Tg-tetGSK-tTA mice without TAC (≈5-fold shown in Figure 6A). In these experiments, all male Tg-tetGSK-tTA subjected to TAC (n=5) died after Dox withdrawal, but all female Tg-tetGSK-tTA mice survived. %FS was significantly reduced, whereas LV end-diastolic dimension was significantly increased in female Tg-tetGSK-tTA after Dox withdrawal compared with those treated with Dox (Figure 6C). Dox withdrawal significantly reduced septal and posterior wall thickness in Tg-tetGSK-tTA in the presence of TAC (supplemental Figure XI). At the time of euthanasia (8 weeks after TAC), Tg-tetGSK-tTA mice that underwent Dox withdrawal exhibited significantly greater LW/TL and HW/TL than Tg-tetGSK-tTA that were kept on Dox (Figure 6D). These results suggest that decreases in GSK-3β activity during heart failure are protective for the heart.

### Mechanisms of Apoptosis

We examined the molecular mechanism by which inhibition of GSK-3β protects cardiac myocytes from apoptosis.
We used adenovirus vector harboring GSK-3β-DN (Ad-GSK-3β-DN) to achieve inhibition of GSK-3β in a specific manner. Treatment of control virus (Ad-LacZ)-transduced cardiac myocytes with tumor necrosis factor (TNF)α, a pro-inflammatory cytokine known to induce apoptosis in cardiac myocytes, in the presence of cycloheximide significantly increased apoptosis, as determined by cytoplasmic accumulation of mono- and oligonucleosomes. Expression of GSK-3β-DN significantly reduced TNFα-induced cardiac myocyte apoptosis (Figure 7A). Expression of GSK-3β-DN induced significant accumulation of MCL-1, an antiapoptotic molecule (see Figure 1C), but not Bcl-2 nor Bcl-xL (supplemental Figure XII). To examine the role of MCL-1 in mediating the

Figure 3. A, Pressure–volume loops from NTg (left) and Tg-GSK-3β-DN (right) before and after dobutamine injection. The end-systolic pressure–volume relationships (ESPVR) before and after dobutamine injection are also depicted. Volumes are expressed in microliters after correction for parallel conductance. B (left), $E_{max}$ (the slope of ESPVR); *$P<0.05$, #$P<0.01$ vs baseline; $\$P<0.05$ vs NTg. B (right), Increases in $(\Delta E_{max})$ after dobutamine injection; *$P<0.05$ vs NTg. C (left), dP/dt; *$P<0.05$, #$P<0.01$ vs baseline, $\$P<0.05$ vs NTg. C (right), Changes in dP/dt after dobutamine injection; *$P<0.05$ vs NTg. D (left), Cardiac output; *$P<0.05$, #$P<0.01$ vs baseline, $\$P<0.05$ vs NTg. D (right), Increases in cardiac output after dobutamine injection; *$P<0.05$ vs NTg.
antiapoptotic effect of GSK-3β-DN, we conducted knockdown of MCL-1 using adenovirus harboring short hairpin (sh)RNA-MCL-1. shRNA-MCL-1, but not control shRNA, downregulated GSK-3β-DN–induced expression of MCL-1 in cardiac myocytes (Figure 7B). Downregulation of MCL-1 by shRNA-MCL-1, but not control shRNA, abolished the protective effects of GSK-3β-DN (Figure 7A). These results suggest that accumulation of MCL-1 plays an important role in mediating the antiapoptotic effect of GSK-3β inhibition.

Discussion

Our results suggest that persistent inhibition of GSK-3β induces cardiac hypertrophy in mouse hearts. Cardiac hypertrophy observed in Tg-GSK-3β-DN was well compensated. Hypertrophy induced by inhibition of GSK-3β appears to have properties of physiological hypertrophy for a number of reasons. First, hypertrophy in Tg-GSK-3β-DN mice is not accompanied by LV dilation. Second, the maximum contractility of the heart after dobutamine infusion and contractility
of individual myocytes isolated from Tg-GSK-3β-DN mice are enhanced. Third, αMHC expression was significantly higher in Tg-GSK-3β-DN. Increases in αMHC are frequently observed in physiological hypertrophy. Fourth, neither fibrosis nor apoptosis was increased in conjunction with cardiac hypertrophy in Tg-GSK-3β-DN. Fifth, GSK-3β-DN and swimming exercise failed to show completely additive effects on cardiac hypertrophy, suggesting that GSK-3β-DN and exercise induce hypertrophy partially through a common mechanism. These results collectively suggest that inhibition of GSK-3β mediates physiological growth of the heart. These observations are relevant to the mechanism of heart failure because physiological hypertrophy could negatively regulate the development of pathological hypertrophy.

GSK-3β phosphorylates GATA4 and eIF2B, thereby inhibiting cardiac hypertrophy through inhibition of either gene expression or protein translation. Because GATA4 protects the heart from load-induced heart failure, the
cardioprotective effects of GSK-3β inhibition may be mediated in part through GATA4. β-Catenin may also regulate physiological growth of cardiac myocytes through regulation of lymphocyte enhancer factor–dependent signaling mechanisms.28 The downstream signaling mechanism mediating physiological hypertrophy in Tg-GSK-3β-DN remains to be elucidated.

The activity of GSK-3β is gradually downregulated by pressure overload in humans and experimental animals.15 When the extent of hypertrophy after TAC was expressed as relative to that of sham-operated mice, there was no significant enhancement in the TAC-induced hypertrophy in Tg-GSK-3β-DN compared with NTg. This indicates either that further inhibition of GSK-3β does not have an additive effect on TAC-induced hypertrophy or that other signaling mechanisms are sufficient to induce TAC-induced hypertrophy.

Although chronic inhibition of GSK-3β failed to enhance TAC-induced hypertrophy, it maintains LV function against pressure overload and is therefore protective. Several lines of evidence support this notion. First, persistent inhibition of GSK-3β inhibits LV systolic dysfunction during pressure overload, as evidenced by preserved %FS at 6 and 8 weeks. Second, the level of lung congestion, as determined by LW/BW, is less in Tg-GSK-3β-DN than in NTg at a given level of cardiac hypertrophy, which indicates that Tg-GSK-3β-DN are more resistant to cardiac decompensation. Third, both apoptosis and fibrosis are less severe in Tg-GSK-3β-DN than in NTg in response to pressure overload. Collectively, these data indicate that inhibition of GSK-3β activates cardioprotective effects, thereby inhibiting apoptosis and fibrosis during cardiac stresses. Because increases in baseline contractility and L-type Ca2+ channel current were observed in single cardiac myocytes isolated from Tg-GSK-3β-DN, the direct effect of GSK-3β inhibition on cardiac contractility may also contribute to the preserved cardiac function during pressure overload in Tg-GSK-3β-DN. Other unknown mechanisms may also contribute to the protective effects of GSK-3β inhibition. Transgenic expression of GSK-3β-DN enhanced cardioprotective effects under TAC despite the fact that endogenous GSK-3β is partially inhibited during hypertrophy. Either nearly complete inhibition of GSK-3β activity or inhibition of GSK-3β before its inhibition by TAC in NTg may contribute to the enhancement of cardioprotection in Tg-GSK-3β-DN mice.

To further examine the role of GSK-3β in regulating heart failure, we generated a gain-of-function model using an inducible system. Our results suggest that stimulation of GSK-3β is detrimental in the adult heart, consistent with the previous report by Michael et al.8 The inducible system, however, allowed us to eliminate the influence of transgene expression during the fetal period. Furthermore, the
Inhibition of GSK-3β is cardioprotective

Hirotani et al

Our results suggest that accumulation of MCL-1 may play an important role in mediating the antiapoptotic effects of GSK-3β inhibition. GSK-3β induces proteasomal degradation of MCL-1 through direct phosphorylation. Inhibition of GSK-3β induced significant accumulation of MCL-1, and downregulation of MCL-1 abolished the antiapoptotic effects of GSK-3β inhibition on TNFα-induced apoptosis. Although GSK-3β regulates apoptosis of cardiac myocytes through interaction with the mitochondrial permeability transition pore complex, such interaction may be regulated through MCL-1. Inhibiting GSK-3β-mediated phosphorylation of MCL-1 could be a novel strategy for protecting cardiac myocytes from apoptosis during heart failure.

Another interesting observation in this report is that postnatal GSK-3β overexpression allows the heart to develop hypertrophy in the presence of heart failure. This may contradict the notion that GSK-3β is a negative regulator of cardiac hypertrophy. However, cardiac hypertrophy induced by postnatal overexpression of GSK-3β was accompanied by cardiac dilation, reduced contractility, and increases in apoptosis. Thus, GSK-3β may not inhibit pathological hypertrophy, which is presumably mediated through GSK-3β-insensitive mechanisms. Inhibition of physiological hypertrophy by GSK-3β could indirectly facilitate pathological hypertrophy.

In summary, inhibition of GSK-3β at baseline induces well-compensated cardiac hypertrophy similar to physiological hypertrophy and stimulates cardiac function. Inhibition of GSK-3β during pressure overload plays a protective role, possibly by inhibiting apoptosis and fibrosis, thereby preventing cardiac decompensation. GSK-3β inhibitors may be beneficial for heart failure treatment.

Acknowledgments

We thank Daniela Zablocki for critical reading of the manuscript.

Sources of Funding

Supported by United States Public Health Service grants HL 59139, HL67724, HL69020, AG23039, and AG28787; and American Heart Association Grant 0340123N. S.H. and H.T. were supported by...
Postdoctoral Fellowships from the American Heart Association Heritage Affiliate.

Disclosures
None.

References
Inhibition of Glycogen Synthase Kinase 3β During Heart Failure Is Protective
Shinichi Hirotani, Peiyong Zhai, Hideharu Tomita, Jonathan Galeotti, Juan Pablo Marquez, Shumin Gao, Chull Hong, Atsuko Yatani, Jesús Avila and Junichi Sadoshima

Circ Res. 2007;101:1164-1174; originally published online September 27, 2007;
doi: 10.1161/CIRCRESAHA.107.160614

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2007 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/101/11/1164

Data Supplement (unedited) at:
http://circres.ahajournals.org/content/suppl/2007/09/27/CIRCRESAHA.107.160614.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation Research can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation Research is online at:
http://circres.ahajournals.org//subscriptions/
Data supplement

Expanded methods

Materials

Tumor necrosis factor-α (TNFα) was purchased from R&D Systems.

Transgenic animals

Transgenic mice with cardiac specific expression of kinase inactive GSK-3β (GSK-3β-KI) (Tg-GSK-3β-KI) were generated on an FVB background using the α-myosin heavy chain promoter (courtesy of J. Robbins, University of Cincinnati) to achieve cardiac-specific expression. GSK-3β-KI was generated by mutating 85 Lys to Met and 86 Lys to Ile as described 1-3. Tetracycline-regulatable myc-GSK-3β (Tg-tetGSK) mice have been described 4 and are now available from NeuroPharma (Spain). In these mice the expression cassette for the transgene was generated using the tetracycline (tet)-OFF system, where the bi-directional tet-responsive promoter drives expression of GSK-3β in one direction and β-galactosidase in the other direction. Tg-tetGSK mice were crossed with cardiac specific tTA mice (Tg-tTA) 5 (purchased from Jackson laboratory). Tg-tetGSK and Tg-tTA were originally generated on a CBA x C57BL/6 background 4 and an FVB background 5, respectively. These mice were backcrossed with C57BL/6 background mice 6 times. Mice were fed with doxycycline (Dox) containing chow (#S481 Dox-diet sterile, Bio-Serv). In order to avoid expression of transgene during fetal stages, the Dox containing chow was given to mother mice throughout gestation and nursing periods. All experiments involving animals were approved by the Institutional Animal Care and Use Committee at the New Jersey Medical School.
**Immunoblot analyses**

Tissue homogenates were prepared using lysis buffer A containing 20 mmol/L Tris (pH 7.5), 10 mmol/L imidazole, 300 mmol/L sucrose, 25 mmol/L sodium fluoride, 10 mmol/L, β-glycerophosphate, 100 mmol/L NaCl, 1 mmol/L Na<sub>3</sub>VO₄, 1 mmol/L EGTA, 1 mmol/L EDTA, 2 µg/ml leupeptin, 1 µg/ml aprotinin, 1 mmol/L AEBSF, 0.5 mmol/L DTT. Anti-GSK-3β and β catenin antibodies were obtained from Transduction Laboratory. Anti-α-sarcomeric actin antibody was purchased from Sigma. Anti-phospho Ser 199 tau antibody was obtained from Sigma. Anti-MCL-1 antibody was obtained from Rockland Immunochemicals. Anti-phospho-eIF2Bε and total eIF2Bε antibodies were purchased from Bio Source and Santa Cruz Biotechnology, respectively. Anti-myc mouse monoclonal antibody (9E10) was obtained from Invitrogen. Anti-L type Ca<sup>2+</sup> channel (α1C) antibody was obtained from Santa Cruz Biotechnology. Anti-phospholamban and anti-SERCA2a antibodies were obtained from Affinity Bioreagents, Inc. Anti-Bcl-2 antibody was obtained from Cell Signaling Technology. Anti-Bcl-XL antibody was obtained from Santa Cruz Biotechnology.

**Protein kinase assays for GSK-3α/β**

The activity of GSK-3α/β was measured by immune complex kinase assay as previously described. Hearts were homogenized with 1 ml of lysis buffer A. GSK-3α and GSK-3β were immunoprecipitated with anti-GSK-3α antibody (Cell Signaling technology) and anti-GSK-3β antibody (Transduction laboratory), respectively. After washing the immune complex, the kinase activity was assayed, using 0.1 µg of tau (Sigma) as a substrate in a reaction buffer, containing 25 mmol/L Tris HCl (pH 7.5), 5 mmol/L β-glycerophosphate, 12 mmol/L MgCl₂, 2 mmol/L DTT, 0.1 mol/L Na<sub>3</sub>VO₄, 200 µmol/L...
ATP. After 30 min of incubation at 30 °C, the samples were subjected to immunoblot analyses, using anti-phospho Ser 199 tau antibody (Sigma). The extent of tau phosphorylation was quantitated by densitometric analysis.

**RT-PCR**

Total RNA was prepared using the RNeasy fibrous tissue kit (QIAGEN), and then first-strand cDNA was synthesized using the ThermoScript RT-PCR system (Invitrogen Corp.). Real-time PCR was then carried out on a DNA Engine Opticon 2 system (MJ Research Inc.) using the DyNAmo HS SYBR Green qPCR kit (Finnzymes). The following oligonucleotide primers were used in this study.

- **ANF**, sense 5’-ATGGGCTCCTTCTCCATCAC-3’ and antisense 5’-ATCTTCGGTACCGGAAGCTG-3’,
- **α-skeletal actin**, sense 5’-TATTCCTTCTGACCAGCTGAACGT-3’ and antisense 5’-CGCGAACGCAGACGCGAGTGCGC-3’,
- **αMHC** sense 5’-GGAAGAGTGAGCGGCGCATCAAGG-3’ and antisense 5’-CTGCTGGAGAGTTATTCTCTCG-3’,
- **βMHC** sense 5’-GCCAACACCAACCTGTCAAGGTTCC-3’ and antisense 5’-TGCAAAAGGCTCCAGTCTGAGGGC-3’, and
- **GAPDH**, sense 5’-TTCTTGTGCAGTGCCAGCCCTCGTC-3’ and antisense 5’-TAGGAACAGGAAGGCCATGCCCAG-3’.

**TAC**

The method for imposing pressure overload in mice has been described 7. Briefly, mice were anesthetized with a mixture of ketamine, xylazine, and acepromazine and mechanically ventilated. The left chest was opened at the second intercostal space. Aortic
constriction was performed by ligation of the transverse thoracic aorta with a 28-gauge
needle using a 7-0 braided polyester suture. Sham operation was performed without
constricting the aorta.

Swimming

Mice at 2 months of age were subjected to swimming exercise. The protocol was begun
with a 15 min training session twice daily, 6 days a week. The length of swimming was
increased by 10 min/day to a maximum of 90 min, and then the 90 min session was
applied twice a day for 6 weeks. Mice swam in tanks with a surface area of 225 cm² per
mouse with a water temperature of 30 to 35°C. Mice were monitored continuously to
ensure that they swam without interruption. All mice were subjected to
echocardiographic measurements before and after the swimming regimen.

Echocardiography

Mice were anesthetized using 12 μl/g body weight of 2.5% avertin (Sigma) and
echocardiography was performed using ultrasonography (Acuson Sequoia C256, Siemens
Medical Solutions USA Inc., Malvern, Pennsylvania, USA) as previously described. A
13-MHz linear ultrasound transducer was used. Two dimension guided M-mode
measurements of Left ventricular (LV) internal diameter were taken from at least three
beats and averaged. LV end-diastolic dimension (LVEDD) was measured at the time of
the apparent maximal LV diastolic dimension, while LV end-systolic dimension
(LVESD) was measured at the time of the most anterior systolic excursion of the
posterior wall.

Hemodynamic measurements
Mice were anesthetized as described above and a 1.4-French Millar catheter-tip micromanometer catheter was inserted through the right carotid artery into the aorta and then into the left ventricle, where pressures and left ventricular dP/dt were recorded. To measure arterial pressure gradients, LV end-diastolic pressure (LVEDP), and dP/dt, high-fidelity micromanometer catheters (1.4 French; Millar Instruments Inc., Houston, Texas, USA) were used as previously described.

**Pressure-volume loop analysis**

Mice were anesthetized with pentobarbital (60 mg/kg, ip). A 1.4-French pressure-conductance catheter (Millar Instruments, Houston, TX) was introduced into the left ventricle (LV) through the right carotid artery and the pressure-volume (P-V) relationship and hemodynamics were continuously recorded. After stabilization, the end-systolic P-V relationship and its slope, \( E_{\text{max}} \), were obtained by varying end-diastolic volume through intermittent occlusion of the inferior vena cava, both at baseline and after intraperitoneal bolus injection of dobutamine (10 μg/g body weight). On average, 10-20 loops were analyzed using the PVAN software from Millar Instruments.

**Histological analyses**

Histological analyses of the heart sections were conducted as described previously. Heart specimens were fixed with 10% neutral buffered formalin, embedded in paraffin, and sectioned at 6-μm thickness. Interstitial fibrosis was evaluated using Masson Trichrome staining as described previously.

**Whole heart X-gal staining**

Hearts were fixed with a buffer containing 0.1 mol/L phosphate buffer (pH 7.3) supplemented with 5 mmol/L EGTA, 2 mmol/L MgCl\(_2\) and 0.2% glutaraldehyde for 30
min, washed three times for 5 min with 0.1 mol/L phosphate buffer (pH 7.3) supplemented with 2 mmol/L MgCl₂, and stained with a buffer containing 0.1 mol/L phosphate buffer (pH 7.3) supplemented with 2 mmol/L MgCl₂, 5 mmol/L potassium ferrocyanide, 5 mmol/L potassium ferricyanide, 1 mg/ml X-gal over night.

**LV myocyte cross sectional area**

Myocyte cross sectional area was measured from images captured from silver-stained sections. Suitable cross sections were defined as having nearly circular capillary profiles and circular-to-oval myocyte sections. The outline of 100-200 myocytes was traced in each section, using Image-Pro Plus software (Media Cybernetics).

**Evaluation of apoptosis in tissue sections**

DNA fragmentation was detected in situ using TUNEL, as described. Briefly, deparaffinized sections were incubated with proteinase K, and DNA fragments were labeled with fluorescein-conjugated dUTP using TdT (Roche Molecular Biochemicals). Nuclear density was determined by manual counting of DAPI-stained nuclei in six fields of each animal using the 40x objective. TUNEL-positive nuclei in the entire section were identified and counted using the same power objective. Limiting counting of total nuclei and TUNEL-positive nuclei to areas with a true cross section of myocytes made it possible to selectively count only those nuclei that clearly were within myocytes.

**Cell cultures**

Primary cultures of cardiac ventricular myocytes from 1-day-old Crl:(WI) BR-Wistar rats (Charles River Laboratories) were prepared as described. Myocytes were cultured with serum for the initial 24 hrs and then in serum-free conditions for 48 hrs before experiments. Adenovirus was applied for 2 hrs at 24 hrs after cardiac myocyte isolation.
Adenovirus vectors

Adenovirus harboring shRNA against MCL-1 was generated using the Ad-Max system (Microbix). Briefly, the pSilencer 1.0-U6 expression vector was purchased from Ambion. The U6 RNA polymerase III promoter and the polylinker region were subcloned into the adenoviral shuttle vector pDC311 (Microbix). The hairpin forming oligos, corresponding to bases 849-867 (GGA CTG GCT TGT GAA ACA ATT CAA GAG ATT GTT TCA CAA GCC AGT CCT TTT TT) for shRNA-MCL-1 and to siCONTROL non-Targeting RNA #1 (Dharmacon) for control shRNA, and to their antisense sequences with ApaI and Hind III overhangs, were synthesized, annealed, and subcloned distal to the U6 promoter. The loop sequence is underlined. Recombinant adenoviruses were constructed, propagated and titered as previously described. Briefly, pBHGloxΔE1,3Cre (Microbix), including the ΔE1,3 adenoviral genome, was co-transfected with the pDC shuttle vector containing the gene of interest into 293 cells using Lipofectamine (Invitrogen). Through homologous recombination, the test genes were integrated into the E1,3-deleted adenoviral genome. Adenovirus constructs harboring GSK-3β−KI (Ad- GSK-3β−KI) and β-galactosidase (Ad-LacZ) have been described.

Analysis of DNA fragmentation by ELISA

Cytoplasmic accumulation of histone-associated DNA fragments were quantitated by the Cell Death Detection ELISA (Roche) as previously described. Briefly, isolated LV myocytes were perfused with Tyrode...
solution composed of 135 mmol/L NaCl, 1 mmol/L CaCl₂, 1 mmol/L MgCl₂, 5.4 mmol/L KCl, 10 mmol/L glucose, 5 mmol/L HEPES (pH 7.3) at 32 °C and then subjected to field stimulation at 1.0 Hz. Myocyte contractile function was measured using a video motion edge detector. Ca²⁺ currents (I₉Ca) were recorded using whole-cell patch-clamp techniques. The external solution was a K⁺ and Na⁺ free solution (to isolate I₉Ca from other ion channel currents as well as Na⁺/Ca²⁺ exchanger currents), containing 2 mmol/L CaCl₂, 1 mmol/L MgCl₂ 135 mmol/L TEA-Cl, 5 mmol/L 4-aminopyridine, 10 mmol/L glucose, 10 mmol/L HEPES (pH 7.3). The pipette solution contained 100 mmol/L Cs-aspartate, 20 mmol/L CsCl, 1 mmol/L MgCl₂, 2 mmol/L MgATP, 0.1 GTP mmol/L, 5 mmol/L EGTA, 5 mmol/L HEPES (pH 7.3). Cell capacitance was measured using voltage ramps of 0.8 V/s from a holding potential of -50 mV.

Statistics

All values are expressed as mean±SEM. Statistical analyses were performed using ANOVA and the Tukey post-test procedure with a p<0.05 considered significant. Comparison of heart weight/body weight (BW)-lung weight/BW regression lines of NTG and TG was performed by ANCOVA (analysis of covariance). Significant differences were determined by F test. Values of p<0.05 were considered significant.
Figure I

Left ventricular cardiac myocyte cross sectional area was determined as described in the Method section. Silver staining was conducted, using LV sections obtained from 2.5-3 month old Tg-GSK-3β-KI and NTg mice. Representative silver staining is shown. Bar = 20 μm. In the bar graph, each column represents the mean of 4 mice at 2.5-3 month old (left) and 4 month old (right).
Figure II

NTg and Tg-GSK-3β-DN (Tg) mice were subjected to dobutamine infusion. Heart rates before and after dobutamine injection are shown. *p<0.05 vs. baseline.
(AB) Myocyte contraction recorded in ventricular myocytes isolated from Tg-GSK-3β-KI (TG) and non-transgenic (NTG) mice at 2.5-3 months of age. (upper) Representative tracings of myocyte contraction. (lower left) Length changes were expressed as % of resting cell length. (lower right) The rate of contraction was expressed as $+dL/dt$. Data are mean ± S.E. Data points are from 12 TG and 8 NTG mice. (C) (upper) Whole-cell $I_{Ca}$ recorded in LV myocytes isolated from NTG (left) and TG (right) mice. Traces show currents elicited from a holding potential of -40 mV to the indicated potentials at 0.1 Hz. (lower) The I-V relationships of peak $I_{Ca}$. $I_{Ca}$ was normalized to the cell capacitance to give current densities (pA/pF). Data points are mean ± S.E. of NTG (n=31) and TG (n=19) cells.
Figure IV

Heart homogenates were prepared from Tg-GSK-3β-KI and NTg at 3 months old. (A) Immunoblotting of L-type calcium channel α1C and α-actin. *p<0.01 vs. NTg. (n=3,3) (B) Immunoblotting of phospholamban (PLB), SERCA2a, and α-actin. (n=2, 2).
Figure V

Tg-GSK-3β-KI and NTg mice were subjected to TAC. After 4, 6 or 8 weeks, the pressure gradient was measured as described in the Method section. Each column represents the mean of 7-13 mice. There was no significant difference in the pressure gradient between Tg-GSK-3β-KI and NTg after 4 or 6 weeks. After 8 weeks, the pressure gradient was significantly higher in Tg-GSK-3β-KI than in NTg. This might be due to the fact that LV dysfunction is more severe in NTg than in Tg-GSK-3β-KI.
Figure VI

Tg-GSK-3β-KI and NTg mice (3 month old males) were subjected to TAC for 4, 6, or 8 weeks. The extent of heart weight/tibial length (HW/TL) after TAC was expressed as relative to that of sham. See Figure 4A for details.
Non-transgenic (NTg) and Tg-GSK-3b-DN (TG) mice were subjected to TAC or sham operation for 6 weeks. TUNEL and DAPI staining was conducted. An arrow indicates a TUNEL positive cell in NTG.
Figure VIII

The effect of swimming exercise on HW/BW in TG (n=5) and NTG (n=5). Mice were subjected to the swimming protocol (sw), as described in the Method section, or sham (without exercise).
Figure IX

Postmortem analyses were conducted using Tg-tetGSK-tTA (tetGSK/tTA+, n=3) and Tg-tetGSK (tetGSK/tTA-, n=5) mice without Dox at an age of 30 weeks old. Lung weight/tibial length (Lung W/TL) (A) and Heart weight/TL (HW/TL) (B) are shown.
Figure X

(A) Kaplan-Meier survival analysis of Tg-tetGSK-tTA with or without Dox. Dox chow was withdrawn at 10 weeks. Echocardiographic analyses were conducted at time points indicated by arrows. (B) Time-dependent changes in LV end diastolic dimension (LVEDD) and % fractional shortening with or without Dox. * p<0.01 vs Dox treated group.
Figure XI

Conditional activation of GSK-3β during TAC. Tg-tetGSK-tTA mice were fed with Dox containing chow (Dox (+)) from the fetal period on and then subjected to TAC at 3-4 months old. After 4 weeks, Dox was withdrawn (Dox WD) from some mice. All mice were followed up for another 4 weeks. Note that transgene expression of GSK-3β remained suppressed in Dox (+) mice. Postmortem analyses were conducted. LV diastolic septal wall-wall thickness (DSEP WT) and LV diastolic posterior wall-wall thickness (DPW WT) are shown. Dox (+) n=5, Dox WD n=5.
Figure XII

Heart homogenates were prepared from Tg-GSK-3β-KI and NTg at 3 months old. There was no significant difference in protein expression of Bcl-2 or Bcl-xL between Tg-GSK-3β-KI and NTg. (A) Immunoblotting of Bcl-2 and α-actin. (n=3,3) (B) Immunoblotting of Bcl-xL and α-actin. (n=2, 2).
Supplemental Table I
Echocardiographic Analysis of tet-GSK/αMHC-tTA Bigenic Mice

<table>
<thead>
<tr>
<th></th>
<th>tet-GSK/αMHC-tTA (-)</th>
<th>tet-GSK/αMHC-tTA (+)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (weeks)</td>
<td>10</td>
<td>30</td>
</tr>
<tr>
<td>n</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>DSEP WT (mm)</td>
<td>0.65±0.02</td>
<td>0.87±0.01</td>
</tr>
<tr>
<td>LVEDD (mm)</td>
<td>3.71±0.03</td>
<td>4.10±0.29</td>
</tr>
<tr>
<td>DPW WT (mm)</td>
<td>0.68±0.01</td>
<td>0.89±0.03</td>
</tr>
<tr>
<td>%FS</td>
<td>34.5±0.6</td>
<td>29.4±0.4</td>
</tr>
<tr>
<td>HR (/min)</td>
<td>479±12</td>
<td>405±19</td>
</tr>
</tbody>
</table>

*p<0.05 vs tet-GSK/αMHC-tTA (-) (30 weeks)
Supplemental Table II
Organ Weights of tet-GSK/αMHC-tTA Bigenic Mice

<table>
<thead>
<tr>
<th>n</th>
<th>tet-GSK/αMHC-tTA (-)</th>
<th>tet-GSK/αMHC-tTA (+)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>BW (g)</td>
<td>28.4±0.6</td>
</tr>
<tr>
<td></td>
<td>TL (mm)</td>
<td>18.0±0.1</td>
</tr>
<tr>
<td></td>
<td>HW/BW (mg/g)</td>
<td>4.67±0.06</td>
</tr>
<tr>
<td></td>
<td>Lung/BW (mg/g)</td>
<td>4.42±0.20</td>
</tr>
<tr>
<td></td>
<td>Liver/BW (mg/g)</td>
<td>50.5±1.9</td>
</tr>
<tr>
<td></td>
<td>HW/TL (mg/mm)</td>
<td>7.96±0.17</td>
</tr>
<tr>
<td></td>
<td>Lung/TL (mg/mm)</td>
<td>6.96±0.22</td>
</tr>
<tr>
<td></td>
<td>Liver/TL (mg/mm)</td>
<td>77.8±3.17</td>
</tr>
</tbody>
</table>

*p<0.01 vs tet-GSK/αMHC-tTA(-)
Supplemental Table III
Echocardiographical Analysis of tet-GSK/αMHC-tTA Mice after Dox Withdrawal

<table>
<thead>
<tr>
<th></th>
<th>Dox (+)</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Age (weeks)</td>
<td>12</td>
<td>16</td>
</tr>
<tr>
<td></td>
<td>n</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>DSEP WT (mm)</td>
<td>0.65±0.02</td>
<td>0.71±0.02</td>
<td>0.86±0.05</td>
</tr>
<tr>
<td>LVEDD (mm)</td>
<td>3.71±0.03</td>
<td>3.81±0.03</td>
<td>4.05±0.06</td>
</tr>
<tr>
<td>DPW WT (mm)</td>
<td>0.68±0.01</td>
<td>0.72±0.02</td>
<td>0.83±0.05</td>
</tr>
<tr>
<td>%FS</td>
<td>34.5±0.6</td>
<td>32.5±0.5</td>
<td>30.8±23</td>
</tr>
<tr>
<td>HR (/min)</td>
<td>429±12</td>
<td>399±15</td>
<td>382±23</td>
</tr>
</tbody>
</table>

|                    | Dox withdrawal |        |        |
|                    | Age (weeks) | 12     | 16     | 24     |
|                    | n          | 6      | 4      | 4      |
| DSEP WT (mm)       | 0.66±0.02 | 0.80±0.03 | 0.93±0.07* |
| LVEDD (mm)         | 3.73±0.05 | 4.45±0.03* | 5.14±0.20* |
| DPW WT (mm)        | 0.68±0.02 | 0.75±0.02 | 0.83±0.06* |
| %FS                | 34.6±0.5  | 25.2±2.1* | 16.4±2.6* |
| HR (/min)          | 434±16   | 355±42   | 311±42* |

* p<0.05 vs 12 weeks (Dox withdrawal)
References


8. Kaplan KB, Bibbns KB, Swedlow JR, Arnaud M, Morgan DO, Varmus HE. Association of the amino-terminal half of c-Src with focal adhesions alters their properties and is regulated by phosphorylation of tyrosine 527. EMBO J. 1994;13:4745-4756.

