GSK-3β heterozygous knockout is cardioprotective in a knockin mouse model of familial dilated cardiomyopathy


1Department of Clinical Pharmacology, Graduate School of Medical Sciences, Kyushu University, Fukuoka, Japan; 2Department of Clinical Pharmacology, Faculty of Medicine, Zagazig University, Zagazig, Egypt; and 3Department of Pharmacology, Faculty of Pharmacy, Zagazig University, Zagazig, Egypt; and 4Department of Cardiac Physiology, National Cerebral and Cardiovascular Center Research Institute, Osaka, Japan

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Mohamed RM, Morimoto S, Ibrahim IA, Zhan D, Du C, Arioka M, Yoshihara T, Takahashi-Yanaga F, Sagaguri T. GSK-3β heterozygous knockout is cardioprotective in a knockin mouse model of familial dilated cardiomyopathy. Am J Physiol Heart Circ Physiol 310: H1808–H1815, 2016. First published April 22, 2016; doi:10.1152/ajpheart.00771.2015.—Glycogen synthesize kinase-3β (GSK-3β) plays a central role in both cardiac physiology and pathology. Herein we want to clarify the role of GSK-3β in familial dilated cardiomyopathy. We generated a mouse model carrying a heterozygous knockout mutation of GSK-3β (GSK-3K210KO), together with a K210 knockin mutation in cardiac troponin T (ΔK210 cTnT KI), which was proved to be one of the genetic causes of familial dilated cardiomyopathy (DCM). GSK-3β+/−KO also prevented cardiac enlargement, myocardial fibrosis, and cardiomyocyte apoptosis and markedly reduced the expression of cardiac β-myosin heavy chain isoform, indicative of HF, in DCM mice with homozygous ΔK210 cTnT KI mutation. GSK-3β+/−KO also extended the life span of these DCM mice. This study suggests that the inhibition of GSK-3β is cardioprotective in familial DCM associated with ΔK210 cTnT mutation.

dilated cardiomyopathy; glycogen synthase kinase-3β; myocardial remodeling; systolic function; survival

NEW & NOTEWORTHY

We explored the effect of glycogen synthase kinase-3β heterozygous knockout on a knockin mouse model with a mutation in cardiac troponin T, which is one of the genetic causes of familial dilated cardiomyopathy, and provided the first evidence that glycogen synthase kinase-3β inhibition may be beneficial for the treatment of this genetic dilated cardiomyopathy.

DEATHS DUE TO CARDIOVASCULAR disorders represented 30% of all global deaths, with 80% of those deaths taking place in low- and middle-income countries (24). Congestive heart failure (HF) is the end-stage of most cardiovascular disorders, a condition in which the heart cannot meet the body’s need of blood. Risk factors of congestive HF include hypertension, myocardial ischemia or infarction, valve diseases, and cardiomyopathies (21). Dilated cardiomyopathy (DCM) is a group of myocardial disorders characterized by ventricular dilation and systolic dysfunction and a major cause of congestive HF. It is the most common cardiomyopathy worldwide with various causes (e.g., gene mutation, toxin, ischemia, virus infection, and autoimmunity) (5, 10, 22). Many mutations in genes encoding for proteins in sarcomere, nuclear envelop, and cytoskeleton were proved to cause familial DCM and account for ~40% of all DCM cases (7). One of the sarcomeric protein mutations is caused by a deletion of three nucleotides AGA of cardiac troponin T (cTnT) gene, leading to elimination of one of four lysines residues encoded in tandem in exon 13 (designated as ΔK210) (13). The pathogenesis of DCM associated with this mutation has been demonstrated to be attributable to a decreased Ca2+ sensitivity of cardiac myofilaments, which should result in a decrease in myocardial force generation at both systole and diastole (23). A knockin (KI) mouse model of genetic DCM with this mutation develops progressive ventricular dilation and frequently dies of sudden cardiac death or HF death before reaching the age of 2 mo (7, 19), resembling affected members in human families with this mutation who have an increased risk of sudden cardiac death or HF death at a young age (13).

Glycogen synthase kinase-3 (GSK-3) is a serine/threonine protein kinase with two isoforms, α and β. Although the name is derived from its role in glycogen metabolism, recent studies have revealed that GSK-3 is a multifunctional enzyme regulating many biological processes in the heart, including gene expression, hypertrophy, development, proliferation, fibrosis, and apoptosis, being a therapeutic target for myocardial diseases (4, 17). Knockout (KO) of GSK-3β in the mouse whole body is embryonic lethal due to severe liver degeneration and obliteration of the ventricular cavity via cardiomyocyte hyperproliferation (14). However, GSK-3β conditional KO in the heart of mice improved cardiac function and prevented myocardial remodeling with evidence of cardiomyocyte proliferation in a postmyocardial infarction model (27). In a setting of pressure overload-induced HF, cardiac-specific overexpression of dominant negative GSK-3β in mice resulted in well-compensated cardiac hypertrophy with decreased apoptosis and fibrosis and increased contractility (9). Cardioprotective effect of ischemic/pharmacological pre- and postconditioning of the heart seems to converge on GSK-3β inhibition involved in the regulation of mitochondrial permeability transition pore (3, 11, 12). Furthermore, coxsackievirus B3 infection has been reported to induce cytopathic effects and apoptosis on cardiomyocytes, leading to DCM via tyrosin kinase-dependent “activation” of GSK-3β (28). It remains unknown whether the inhibition of GSK-3β is also beneficial for the treatment of familial...
DCM with this mutation. In this study, we generated a mouse model carrying both a heterozygous KO mutation of GSK-3β (GSK-3β<sup>+/−</sup> KO) and a ΔK210 Ki mutation in cTnT (ΔK210 cTnT Ki) on the genetic background of C57Bl/6 to clarify the effect of GSK-3β inhibition on the pathogenesis and sudden cardiac death in familial DCM with this mutation.

MATERIALS AND METHODS

Animals. A Ki mouse model of DCM, in which 3 base pairs encoding for Lys210 (K210) in cTnT were deleted from the endogenous gene Tnnt2 in C57BL/6 mice, was developed as described elsewhere (7). GSK-3β<sup>+/−</sup> KO mice were generated as described before (15). DCM mice with heterozygous K210 cTnT Ki mutation (DCM HT) were crossed with GSK-3β<sup>+/−</sup> KO mice to obtain GSK-3β<sup>+/−</sup> DCM HT mice, which in turn were crossed with DCM HT mice to obtain GSK-3β<sup>+/−</sup> DCM HM. Wild-type (WT), DCM HT, and DCM HM groups were used as control against GSK-3β<sup>+/−</sup> KO, GSK-3β<sup>+/−</sup> DCM HT, and GSK-3β<sup>+/−</sup> DCM HM groups, respectively. Each group contained more than three mice of about equally mixed sex.

The investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication no. 85-23, revised 2011). The experimental protocol was reviewed and approved by the Committee of Ethics on Animal Experiments of the Faculty of Medicine, Kyushu University, Japan. This study was performed according to the Guidelines for Animal Experiments of the Faculty of Medicine, Kyushu University, and The Law (No. 105) and Notification (No. 6) of the Japanese Government.

Western blot analysis. Mice (n = 3–8/group) received intraperitoneal injection of 300 μl heparin (300 U/mouse). After 10 min, the hearts were excised from mice under 3% isoflurane inhalation anesthesia; the depth of anesthesia was checked by ensuring that noxious pinch stimulation of the hind paw, the fore paw, and the ear with blunt forceps did not evoke any motor reflexes. The isolated hearts were perfused with oxygenated Krebs-Henseleit solution (in mM; 118 NaCl, 4.7 KCl, 1.2 MgSO₄, 1.2 KH₂PO₄, 25 NaHCO₃, 2.5 CaCl₂, 0.5 EDTA-Na₂, HEPES, 11 d-glucose containing 50 2,3-butanedione monoxime) in a Langendorff mode. About 100 mg of the left ventricle (LV) was dissected from the heart and homogenized in 600 μl Laemmli’s sample buffer containing 6 μl protease inhibitor and then kept in 80°C. At time of use, samples were subjected to two cycles of thawing and freezing (15 min each), followed by heating at 95°C for 4 min. Supernatant was separated by centrifugation at 10,000 rpm for 2 min at 4°C (protein concentration was measured using BIO-RAD protein assay) and subjected to Western blot analysis, as described previously (25). Total GSK-3β expression level was measured using monoclonal anti-GSK-3β antibody (BD Biosciences, San Jose, CA); relative phosphorylation level of phospho-GSK-3β (pGSK-3β; Ser9) was obtained by normalizing the signals probed by polyclonal anti-pGSK-3β antibody (Cell Signaling, Danvers, MA) to those probed with anti-GSK-3β after stripping. Total β-catenin expression level was measured using monoclonal anti-β-catenin antibody...
(BD Bioscience, San Jose, CA), relative phosphorylation level of β-catenin (Ser33/Thr41) was obtained by normalizing the signals probed by polyclonal anti-phospho-β-catenin antibody (Cell Signaling, Danvers, MA) to those probed with anti-β-catenin. Relative phosphorylation level of Akt (Ser473) was obtained by normalizing the signals probed by polyclonal anti-phospho-Akt antibody to those probed with anti-Akt (Cell Signaling, Danvers, MA). Expression levels of brain natriuretic peptide (BNP) were determined using an anti-pro-BNP polyclonal antibody (Abcam, Cambridge, MA). GAPDH was used as the protein loading control detected by monoclonal anti-GAPDH antibody (Abcam, Cambridge, MA). Data were analyzed using Scion image for windows software (Scion, Meyer Instruments, Houston, TX).

**Echocardiography.** Transthoracic echocardiography (M-mode) was measured under isoflurane inhalation anesthesia. Induction was performed in an isolation chamber with 2% isoflurane in 100% O2, and anesthesia was maintained at 1–1.5% in 100% O2 at a flow rate of 1 l/min via a small nose cone. The duration of exposure to anesthesia was ~10 min for each mouse (n = 5–15 mice/group) using a 14-MHz linear array probe with a diagnostic ultrasound system (Nemio SSA-550A) (Toshiba Medical Systems, Ohtawara, Tochigi, Japan).

**Histochemistry.** The isolated hearts (n = 3–6 mice/group) were perfused with oxygenated Krebs-Henseleit solution in a Langendorff mode and fixed in a 10% formalin neutral buffered solution, as described previously (7). Fixed hearts were cut transversely at the midventricular level, embedded in paraffin, sectioned at 5 μm, and stained with Masson trichrome. For measurement of cardiac fibrosis, photos of Masson trichrome-stained sections were taken (using ×20 lens magnification) in microscope Keyence BZ-9000 (Keyence, Japan) and quantified using the Image J program from the NIH.

**Detection of apoptosis in the LV myocardium.** Apoptosis in the LV was detected by terminal deoxynucleotidyl transferase dUTP nick-end labeling staining using the CardioTacs staining kit ( Trevigen, Gaithersburg, MD) in sections collected from 2-mo-old mice (6 cardiac sections from different mice). Apoptotic nuclei were stained blue after incorporation of biotinylated dUTP by terminal deoxynucleotidyl transferase to 3'-OH residues in fragmented DNA and subsequent detection using streptavidin-conjugated horseradish peroxidase reaction with TACS blue label. Tissues were counterstained with nuclear fast red. The number of apoptotic cardiomyocytes was normalized to the total number of cardiomyocytes in a cardiac section.

**Analysis of myosin heavy chain isoform content.** Myosin heavy chain (MHC) protein of the LV myocardium lysate (n = 4–5 mice/...
group) was separated on 6.5% SDS-PAGE and stained with Coomasie brilliant blue dye. Relative β-isof orm expression (percentage of total MHC) was determined as described previously (7). Data were analyzed using an optical densitometric scan using Phoretix gel analysis software (Phoretix International).

Statistics. Data are presented as means ± SE. Statistical significance was determined by two-way ANOVA, followed by a post hoc Bonferroni test for selected pairs of data, or log-rank (Mantel-Cox) test for survival curve analysis using GraphPad Prism version 5 (GraphPad Software). P values < 0.05 are considered significant.

RESULTS

GSK-3β expression, activity, and Akt activation in the LV myocardium of DCM mice with GSK-3β−/− KO. GSK-3β protein expression level in the LV myocardium was not significantly different between WT and DCM mice. GSK-3β−/− KO was confirmed to reduce the protein expression level of GSK-3β by ~50% in DCM mice, as well as in WT mice (Fig. 1A). At the age of 2 mo, the fraction of GSK-3β inactivated by Ser9 phosphorylation in total GSK-3β was significantly smaller in DCM mice than in WT mice, indicating that GSK-3β would be activated to some extent in DCM mice (Fig. 1B). GSK-3β−/− KO had no significant effect on the fraction of inactivated GSK-3β in DCM mice, so that the absolute amount of active GSK-3β should be decreased to ~50% by GSK-3β−/− KO in DCM mice. Consistent with these findings, the phosphorylation levels of β-catenin, a direct substrate of GSK-3β, were significantly higher in DCM mice compared with WT mice and significantly decreased by GSK-3β−/− KO in DCM mice (Fig. 1C). Moreover, the phosphorylation (i.e., activation) levels of Akt, an upstream kinase of GSK-3β, were significantly lower in DCM mice compared with WT mice, suggesting that GSK-3β is activated in DCM mice by down-regulation of phosphatidylinositol 3-kinase/Akt pathway (Fig. 1D). At the age of 1 mo, GSK-3β activity in DCM mice hearts estimated from the phosphorylation of GSK-3β and β-catenin was not significantly different from that in WT mice (data not shown).

Effects of GSK-3β−/− KO on LV systolic function, dimensions, wall thickness, and survival in DCM mice. Figure 2A shows representative M-mode echocardiographic images of LV of mice with various genotypes at 1 and 2 mo old. Summarized echocardiographic data showed progressive deterioration in LV systolic function assessed by ejection fraction or fractional shortening and LV end-systolic dimension, along with progressive LV dilation assessed by LV end-diastolic dimension in DCM but not in WT mice (Fig. 2, B–G). GSK-3β−/− KO prevented the progressive deterioration of LV function in DCM mice (Fig. 2G).

Fig. 3. Effects of GSK-3β−/− KO on body (BW), heart (HW), and lung weight (LW) of 2-mo-old DCM mice (top) and sex differences (bottom). A: BW. B: HW/BW. C: LW/BW. Values are means ± SE; n = 6–9 mice/group. **P < 0.01. ***P < 0.001. D: gross morphology of hearts.
systolic function in DCM mice, although it did not prevent the progression of LV dilation. GSK-3β+/− KO had no significant effects on the LV systolic function, dimension, and wall thickness in non-DCM mice and on the LV wall thickness (LV posterior wall thickness at diastole and Interventricular septum thickness at diastole) in DCM mice.

Log-rank test on Kaplan-Meier survival curves demonstrated that GSK-3β+/− KO slightly, but significantly (P = 0.0049), extend the life span of DCM mice with homozygous mutation, which frequently suffer sudden death due to fatal arrhythmia around the age of 8 wk (Fig. 2H).

**Effects of GSK-3β+/− KO on heart and lung weight of DCM mice.** Mice in all genotype groups had similar body weight (BW) at the age of 2 mo, with male having significantly higher BW than female in the DCM HM group (Fig. 3A). Mice in WT, GSK-3β+/−, DCM HT, and GSK-3β+/− DCM HT groups also had similar heart weight (HW/BW) and lung weight (LW/BW) (Fig. 3, B and C). DCM HM and GSK-3β+/− DCM HM mice had significantly higher HW than WT mice (Fig. 3B). GSK-3β+/− DCM HM mice had a significantly lower HW and smaller hearts than DCM HM mice, indicating that GSK-3β+/− KO prevents cardiac enlargement in DCM mice (Fig. 3, B and D). DCM HM mice also had a significantly higher LW than WT mice, indicating congestive HF (Fig. 3C). However, GSK-3β+/− DCM HM mice had a significantly lower LW than DCM HM mice, which was not significantly different from the LW of WT mice, indicating that GSK-3β+/− KO prevents the rapid progression of HF by preserving cardiac pump function in DCM HM mice.

**Effects of GSK-3β+/− KO on cardiac β-MHC and BNP expression in DCM mice.** The expression of β-MHC, indicative of HF, was markedly increased in DCM HM mice compared with WT mice. GSK-3β+/− KO reduced the expression of β-MHC in DCM HM mice to a level that was not significantly different from that in WT mice (Fig. 5, A and B).

The reexpression of another fetal gene BNP was also significantly increased in DCM mice compared with WT mice,
and GSK-3β+/− KO reduced the expression of BNP in DCM mice (Fig. 5, C and D).

**Long-term effects of GSK-3β+/− KO on DCM HT mice.**

Long-term follow-up showed that GSK-3β+/− KO prevented the progressive deterioration in LV systolic function, LV dilation, and thinning of LV wall in DCM HT mice, at least until the age of 5 mo (Fig. 6). We also noted that GSK-3β+/− KO prevented the age-related deterioration in LV systolic function and thinning of LV wall in non-DCM mice, at least until the age of 8 mo. At the age of 1 yr, no significant differences were observed in the BW, HW, and LW among all groups.

**DISCUSSION**

The principal finding of this study is that GSK-3β+/− KO in the whole body is cardioprotective in the case of familial DCM with ΔK210 cTnT mutation, in accordance with the previous studies showing that cardiac-specific KO or inhibition of GSK-3β is beneficial in the postmyocardial infarction and pressure overload-induced HF model of mice (9, 27). In this study, we demonstrated that GSK-3β+/− KO significantly improved the LV systolic function and reduced cardiac enlargement, myocardial fibrosis, cardiomyocyte apoptosis, and β-MHC and BNP expression using the KI mouse model, which closely recapitulates the clinical phenotype of the human patients with this mutation (7). In this study, we generated for the first time a mouse model carrying both GSK-3β+/−/− KO mutation and a heterozygous or homozygous ΔK210 KI mutation in cTnT, which was proved to be one of the genetic causes of familial DCM (13). GSK-3β+/− KO caused ∼50% reduction in total GSK-3β protein expression in these DCM mice, as demonstrated by Western blot analysis. GSK-3β activity was significantly higher in DCM mice compared with WT mice, as evidenced by Western blot analysis using anti-phospho-GSK-3β Ser9 antibody, which detects the inhibitory phosphorylation of GSK-3β (3). DCM mice also showed higher levels of phospho-β-catenin (Ser33/37/Thr41) compared with WT mice, which could reflect higher kinase activity of GSK-3β in DCM mice (4, 18), and phosphorylation (i.e., activation) levels of Akt, an upstream inhibitor of GSK-3β, was also significantly lower in DCM. Decreased cardiac function might lead to an activation of GSK-3β indirectly through compensatory activation of sympathetic nervous system, because catecholamines could directly inhibit the secretion of insulin, which inhibits GSK-3β through activating the phosphatidylinositol 3-kinase/Akt signaling pathway (6, 8, 20). In DCM HM mice, LV systolic function was rapidly deteriorated at 2 mo old, with myocardial fibrosis, β-MHC and BNP expression, HW and LW being significantly higher compared with that in WT mice. However, the increase in LW was only ∼30% compared with that in WT mice, excluding the possibility of severe congestive HF, which is usually associated with 300–400% increase in LW. Most DCM HM mice died suddenly around the age of 2 mo, probably due to lethal arrhythmia (7). GSK-3β−/− KO significantly improved the LV systolic function and reduced myocardial fibrosis, cardiomyocyte apoptosis, and the LW to normal levels in DCM mice.

The cardioprotective effect of GSK-3β+/− KO in DCM mice is manifested in the reduced expression of β-MHC in the LV. Cardiac MHC is the molecular motor in the sarcomeric contractile apparatus involving force development in the heart (26). There are two MHC isoforms, α and β, with ∼93% amino acid identity. β-MHC has slower sliding velocity and produces lower power output because of its lower ATPase activity compared with α-MHC (1, 16). Relative expression levels of these isoforms are affected by disease states such as cardiac failure or hypertrophy. α-MHC is a predominant form in normal mice hearts, while β-MHC expression level is significantly increased in failing mice hearts (7). A small increase in the relative expression of α-MHC has been shown to augment cardiomyocyte power output (26). It remains un-
clear whether GSK-3β KO increases the relative expression of α-MHC directly or indirectly in DCM mice.

Cardiomyocyte apoptosis is a prominent pathological feature of familial DCM (7). GSK-3β knockdown has been shown to prevent TNF-α-induced cardiomyocytes apoptosis by up-regulating the anti-apoptotic factor myeloid cell leukemia-1 (9). In ischemia-reperfusion injury, the cardioprotective effect of ischemic/pharmacological pre- and postconditioning converges at inhibition of GSK-3β, which prevents the opening of mitochondrial permeability transition pore and subsequent cardiomyocyte apoptosis (3, 11, 12). Consistent with these previous studies, we found that GSK-3β KO reduced cardiomyocyte apoptosis in our DCM mouse model, suggesting that prevention of cardiomyocyte apoptosis may be involved in the cardioprotective effects of GSK-3β KO on DCM mice.

Inhibition of GSK-3 by SB216763 or small interfering RNA has also been shown to prevent transforming growth factor-β1-induced human lung fibroblasts differentiation into myofibroblasts, suggesting a direct regulatory role of GSK-3β on fibrosis (2). Further studies are warranted to clarify whether prevention of fibroblast differentiation is also involved in the cardioprotective effects of GSK-3β KO in our DCM mice.

Finally, the present study demonstrated that GSK-3β KO in the whole body is cardioprotective and beneficial for survival in the KI mouse model of DCM with K210 cTnT mutation, which closely recapitulates the clinical phenotype of the human patients with the same mutation. The present study cannot discriminate whether the cardioprotective effects are mediated by cardiomyocytes or noncardiomyocytes. Furthermore, the mechanism should also be considered taking into account the non-cardiac-specific GSK-3β KO. Nevertheless, the results of the present study suggest that GSK-3β inhibitors may hold promise for treating familial DCM with this mutation.

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DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

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