Blocking cardiac growth in hypertrophic cardiomyopathy induces cardiac dysfunction and decreased survival only in males

Stephen W. Luckey,1 Jason Mansoori,1 Kelly Fair,1 Christopher L. Antos,2 Eric N. Olson,3 and Leslie A. Leinwand1

1Department of Molecular, Cellular, and Developmental Biology, University of Colorado, Boulder, Colorado; 2Max-Planck Institut fuer Entwicklungbiologie, Tuebingen, Germany; and
3Department of Molecular Biology, University of Texas Southwestern Medical Center, Dallas, Texas

Submitted 9 June 2006; accepted in final form 21 September 2006


Hypertrophic cardiomyopathy (HCM) is an autosomal dominant disease caused by mutations in sarcomeric proteins (21). The disease is relatively common with an incidence estimated at 1 in 500 people, and individuals exhibit clinical heterogeneity even with mutations in the same protein (21). Mutations in human β-myosin heavy chain (β-MyHC) gene are typically associated with cardiac myocyte hypertrophy, myofibrillar disarray, small vessel coronary artery disease, arrhythmias, and sometimes sudden death (21).

To better understand the pathogenesis of HCM, transgenic mouse models expressing mutant alleles of the α-MHC in the heart have been generated in our laboratory (40) and others (10). The HCM transgenic mouse under study here expresses a missense mutation in codon 403 with an additional deletion in the actin-binding domain of the murine α-MyHC protein (40). It is important to note that this is not an overexpression model, since transgenic sarcomeric proteins replace endogenous ones (32). Cardiac-specific expression of this mutant protein in mice models several aspects of HCM, including myocyte hypertrophy, myofibrillar disarray, fibrosis, and elevated atrial natriuretic factor (ANF), β-MyHC, and α-skeletal actin (7, 39, 40).

Furthermore, HCM mice display age- and sex-dependent differences in heart morphology and function (7, 26). Interestingly, dietary modification from a soy-based diet to a casein-based diet in this HCM model improved cardiac function and reduced disease markers such as β-MyHC and activation of caspase (37).

Although numerous signaling pathways have been identified in the progression of cardiac hypertrophy, those involved in the progression of HCM remain largely ill defined. One pathway that seems logical to investigate is glycogen synthase kinase-3β (GSK-3β). GSK-3β has been demonstrated to be critical in pathological hypertrophic growth (as reviewed in Ref. 14) (1, 13). For example, transgenic mice expressing caGSK-3β in the heart have reduced myocardial growth in response to chronic calcineurin activation, β-adrenergic stimulation by isoproterenol, and pressure overload mediated by thoracic aortic banding (1). Inducible expression of caGSK-3β also blocked pressure overload-mediated hypertrophy as well as partially reversing established hypertrophy (35). Whereas no adverse consequences of caGSK-3β overexpression have been reported, a transgenic mouse overexpressing a cardiac-specific wild-type GSK-3β had limited postnatal growth with significant diastolic dysfunction and increased left ventricular pressures (23).

Because transgenic expression of caGSK-3β has been shown to antagonize several pathological hypertrophic stimuli, we hypothesized that the caGSK-3β transgene would attenuate the hypertrophic phenotype in our HCM mouse model. These experiments would also address the critical question of whether blocking hypertrophy in a genetic model of HCM is beneficial or detrimental. The data presented here demonstrate that caGSK-3β prevents hypertrophic growth mediated by a mutant MyHC gene. When compared with HCM transgenic littermates, HCM/GSK-3β males displayed reduced cardiac function, decreased sarcoplasmic reticulum Ca2+-ATPase (SERCA) expression, increased ANF expression, and increased mortality, whereas there was no apparent deleterious

Address for reprint requests and other correspondence: L. A. Leinwand, Dept. of Molecular, Cellular, and Developmental Biology, Univ. of Colorado, Boulder, Campus Box 347, Boulder, Colorado 80309-0347 (e-mail: leslie.leinwand@colorado.edu).

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
effect of limiting hypertrophic growth of the heart in the doubly transgenic females. The beneficial effects of a casein-based diet were also demonstrated by improved survival in the HCM/GSK males.

METHODS

Mice. A mouse model representing HCM was used in this study. The HCM mice expressed a mutant MyHC transgene (R403Q missense mutation with an additional deletion of amino acids 468-527) driven by a rat α-MyHC promoter as previously described (7, 26, 40). HCM mice were crossed with transgenic mice expressing a cardiispecific, constitutively active form of GSK-3β that contains a serine-9-to-alanine mutation (1). The offspring generated included nontransgenic (NTG) littermate controls, HCM, caGSK-3β, and double transgenic animals (HCM/GSK-3β). Mice had a genetic background of C57/BL6, and the presence of the each individual transgene was detected by polymerase chain reaction (PCR). All mice were fed a soy diet ad libitum except when a casein diet is indicated. All research involving the use of mice was performed in strict accordance to approved protocols by the Institutional Animal Use and Care Committee at the University of Colorado.

Immunoblotting. Left ventricular tissue was prepared and homogenized in standard lysis buffer as previously described (17). Total protein (25 μg) was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Immunoblotting utilized antibodies reactive to anti-GSK-3β (Santa Cruz Biotechnology), anti-phospho-GSK-3β (Santa Cruz Biotechnology), anti-phospholamban (PLB) (Affinity Bioreagents), anti-phospho-PLB (Upstate), or anti-SERCA (Bethyl).

Histological analysis. For histological analysis, hearts were rapidly excised, rinsed in cold phosphate-buffered saline, and weighed. The whole heart was fixed in 10% phosphate-buffered formalin for 24 h at 4°C and then placed into 70% ethanol until utilized. All sections were processed, embedded in paraffin, sectioned, and stained with either hematoxylin-eosin or Masson’s trichrome stain by Premier Histology (Boulder, CO). Sections were then analyzed by light microscopy. Fibrosis was evaluated by blinded observers and scored as a percentage of total microscopic area from at least 8–10 fields per heart.

Ribonuclease protection assay. Total RNA was isolated from frozen left ventricular tissue by using TRIZOL Reagent (Invitrogen). Ribonuclease protection assay (RPA) was performed using the RPA III kit (Ambion). The specific genes with the corresponding protected nucleotide lengths are as follows: ANF (208), β-MyHC (243), SERCA (341), α-skeletal actin (155), and GAPDH (132). The gel was exposed to Phospholmage (Molecular Dynamics, Amersham), and the corresponding bands were quantified by ImageQuant 5.1 (Molecular Dynamics). GAPDH was used as an internal control.

Echocardiography. Contractile function was evaluated by echocardiography. Briefly, mice were lightly sedated with Avertin (1.25%, 13–16 μl/kg) and were positioned with their backs down. Echocardiography was performed by using a Sonos 5500 (Hewlett-Packard) equipped with a 15-MHz linear-array transducer. Two-dimensional directed M-mode images were obtained in both parasternal long-axis and short-axis views and used for the measurement of left ventricular wall thickness and dimensions. All the measurements were performed according to the American Society of Echocardiography recommended guidelines (34).

Data and statistical analysis. Comparison of survival rates was performed by Kaplan-Meier analysis. Results are presented as means ± SE. Statistical analysis of group differences was performed by one-way ANOVA. If significance was achieved (P < 0.05), Tukey’s post hoc comparison was performed.

RESULTS

Increased phosphorylation of GSK-3β in HCM mice. GSK-3β activity has been shown to be inactivated by phosphorylation in response to several hypertrophic stimuli (14). As previously shown, HCM mice of both sexes exhibit cardiac hypertrophy (40). To determine whether phosphorylation of serine-9 in GSK-3β is associated with cardiac hypertrophy in these HCM mice, we examined the phosphorylation state of GSK-3β in the left ventricles of HCM mice. Whereas total GSK-3β protein content in the heart was unchanged, phosphorylation of GSK-3β was significantly elevated (1.7-fold) in HCM males when compared with that of NTG mice (Fig. 1). A similar increase in the phosphorylation of GSK-3β in the hearts of HCM females at the same age as well as in both sexes of HCM animals at 8 mo of age was previously observed (37). These results indicate that suppression of GSK-3β activity by phosphorylation is one of the signaling events associated with the hypertrophic growth in HCM mice.

caGSK-3β expression attenuated cardiac growth in HCM mice. To directly test the hypothesis that increased phosphorylation of GSK-3β was important in the hypertrophic growth in the HCM mice, we cross-bred animals expressing caGSK-3β with the HCM transgenic mouse model. HCM/GSK-3β doubly transgenic mice were generated along with parental littermate controls. HCM mice developed cardiac hypertrophy at 4 mo of age, which was maintained at 10 mo as assessed by macroscopic appearance (Fig. 2A) and by heart weight-to-body weight ratios (HW:BW) (Fig. 3), consistent with previous reports (39, 40). Both male and female mice expressing caGSK-3β had reduced heart sizes at 4 and 10 mo of age (Figs. 2 and 3). Crossing the caGSK-3β transgene into HCM mice reduced the cardiac growth normally observed in the HCM mice at 4 and 10 mo of age (Figs. 2 and 3). The HW:BW values

Fig. 1. Increased phosphorylation of glycogen synthase kinase-3β (GSK-3β) in hypertrophic cardiomyopathy (HCM) male mice at 4 mo of age. A: representative Western blots are shown using antibodies against total GSK-3β (bottom) and phosphorylated-GSK-3β (top). The relative intensity of each band was measured and calculated. B: quantitation of phospho-GSK-3β to total GSK-3β levels in nontransgenic (NTG, open bar) and HCM (solid bars) hearts at 4 mo of age. Values are means ± SE of 5 individual hearts. ^P < 0.05 vs. NTG.
for the HCM/GSK-3β mice were ~34% and 28% smaller than those of HCM animals at 10 mo of age in male and female mice, respectively. However, there was a gender difference in younger animals in that the HW:BW values of female HCM/GSK-3β mice were significantly larger than those of doubly transgenic males at 4 mo of age (P < 0.05), but there was no significant difference at 10 mo of age.

caGSK-3β did not affect the histopathological phenotype in HCM. Hearts of HCM male mice had been previously shown to exhibit fibrosis and myocellular disarray (8), and as expected, left ventricular tissue from HCM males at 4 and 10 mo exhibited pronounced abnormal cardiac architecture containing disordered myocytes and progressive interstitial fibrosis (Fig. 2A and C). In males, collagen content was ~3.9% in HCM and 4.9% in HCM/GSK-3β mice compared with that of NTG counterparts at 10 mo of age (Fig. 2C). Whereas myocyte hypertrophy and disarray were observed in the hearts of 10-mo-old HCM females, fibrosis was less than those in HCM males (Fig. 2B and C). The degree of myocellular disarray and interstitial fibrosis was indistinguishable between HCM/
GSK-3β and HCM males or between HCM/GSK-3β and HCM females.

caGSK-3β impaired contractile function in HCM males. To determine the functional impact of blocking hypertrophy in the HCM animals, left ventricular chamber size and contractile function were assessed by serial echocardiography in 4-mo- and 10-mo-old male and female caGSK-3β, HCM, and doubly transgenic mice. Four-month-old HCM males had a decrease in fractional shortening compared with that of NTG littermates (Fig. 4, A and B). By 10 mo of age, reduced fractional shortening accompanied increased end-systolic dimensions (ESD) in both male and female HCM animals (Fig. 4, A and B). Although the reduced cardiac function in 10-mo-old females is different from that previously published (26), the method of cardiac function evaluation was different (isovolumetric heart preparation vs. echocardiography) as were the heart rates at which the data were acquired (300 vs. 575 beats/min, respectively).

Male HCM/GSK-3β mice displayed a marked increase in left ventricular chamber size at 4 mo of age compared with that of NTG and HCM that increased over time (Fig. 4). End-diastolic dimensions (EDD) and ESD of the HCM-GSK-3β hearts were increased compared with those of the 10-mo-old HCM males (3.8 ± 0.2 vs. 3.21 ± 0.1 mm and 2.5 ± 0.1 vs. 1.87 ± 0.1 mm, respectively; Fig. 4A). Enlargement of left ventricular chamber dimensions in the HCM/GSK-3β males were not accompanied by statistically significant wall thinning compared with HCM males (0.96 ± 0.01 vs. 1.0 ± 0.01 mm, respectively). Activation of GSK-3β in male HCM mice also reduced percent fractional shortening and ejection fraction compared with those of HCM mice at 10 mo of age (Fig. 4C). In female HCM/GSK-3β, left ventricular dimensions and contractile function at 10 mo of age were no different between HCM and HCM/GSK-3β female littermates (Fig. 4, B and D). Collectively, these results indicate that blocking hypertrophy by active GSK-3β in HCM male hearts was accompanied by increased left ventricular chamber dimensions and reduced contractile function, whereas there was no pronounced functional effect of blocking hypertrophy on the hearts of HCM females.

caGSK-3β altered expression of hypertrophic markers in HCM mice. It is well established that the progression of cardiac hypertrophy is accompanied by changes in the levels of expression of several genes (15). Given the effects of caGSK-3β on the progression of disease in the HCM mice, the expression levels of β-MyHC, ANF, GAPDH, SERCA, and α-skeletal actin mRNA were measured in left ventricular tissue of 4-mo- and 10-mo-old mice using a RNase protection assay (Fig. 5A). Transcripts for β-MyHC, ANF, and α-skeletal actin were upregulated in the hearts of male and female HCM animals (Fig. 5, B and C, respectively) as expected (8, 39). Whereas caGSK-3β resulted in elevated ANF expression in males as previously described and in females (Fig. 5) (1), expression of both the caGSK-3β and mutant MyHC transgenes in 4-mo- and 10-mo-old animals profoundly elevated ANF mRNA levels in both sexes, and it significantly reduced SERCA levels in males (Fig. 5). Interestingly, there was a sex difference between HCM/GSK-3β males and females at both ages, with higher induction of ANF mRNA in the males compared with female mice.

Modification of calcium handling proteins in HCM mice expressing caGSK-3β. In support of the mRNA expression levels, SERCA protein levels were also decreased in HCM/ GSK-3β males (Fig. 6). In contrast, there were no observable differences in the expression of SERCA between HCM/ GSK-3β and HCM females. Furthermore, there were no significant differences in the protein level of PLB in either sex. Site-specific phospho-antibodies were used to evaluate protein kinase A-dependent phosphorylation (serine-16) of PLB (19). The level of phosphorylation was elevated in both HCM males and females compared with that in NTG controls, but this increase was reduced by expression of caGSK-3β transgene in both male and female HCM mice. Taken together, the relative ratio of PLB to SERCA was elevated in HCM/GSK-3β males, and phosphorylation of PLB is reduced in both HCM/GSK-3β male and female mice compared with the HCM parental genotype.

Decreased survival of male HCM/GSK-3β mice. One critical question arising from the observation that caGSK-3β prevented hypertrophy in the HCM mice is whether it has a beneficial or harmful impact on animal survival. Kaplan-Meier analysis indicated that male HCM/GSK-3β double transgenic animals had significantly increased mortality; ~60% survival at 12 mo and 25% survival at 18 mo (Fig. 7A). There was no difference in survival in the female HCM/GSK-3β animals compared with that of other genotypes of female littermates.
To ensure that these survival and phenotypic differences between males and females were not due to differential expression of either transgene, Western blot and semiquantitative RT-PCR were used to assess the caGSK3-β/H9252 and MyHC transgenes, respectively. Equivalent expression levels of caGSK-3β protein and mutant MyHC RNA were seen between males and females (data not shown).

Recent data have demonstrated that modifications from a traditional soy-based diet to a casein-based diet dramatically improved cardiac morphology and function in HCM mice (37). To investigate whether a casein-based diet would improve the survival of the HCM/GSK-3β males, the diet of these animals was changed from a soy-based diet to a casein-based diet. In a manner consistent with the previous study, dietary alteration improved survival in the HCM/GSK males (Fig. 7B). Survival proportions at 18 mo of age in HCM/GSK males improved from 25% on the soy diet to 55% on the casein diet.

**DISCUSSION**

It is clear that caGSK-3β can inhibit cardiac hypertrophy in several in vivo models of hypertrophy (1, 35), and our findings here indicate that activation of GSK-3β is sufficient to limit cardiac growth in this model of HCM. This is the first study to demonstrate the anti-hypertrophic effects of caGSK-3β in HCM animals. The data also indicate that blocking cardiac growth in HCM males by active GSK-3β is detrimental. In contrast, suppression of cardiac growth by caGSK-3β in HCM females had no detrimental or beneficial impact on cardiac function or survival. Finally, survival of HCM/GSK-3β males was improved by feeding the mice a casein diet, adding further support to the link between diet and cardiomyopathy.

These results definitively demonstrated that active GSK-3β in HCM animals blocks cardiac growth consistent with previous investigations (1, 35). Interestingly, these earlier investigations also demonstrated elevated hypertrophic markers such as ANF and brain natriuretic peptide above either parental genotype when caGSK-3β was expressed in transgenic mice with cardiac-specific expression of constitutively active calcineurin (1). ANF expression is similarly elevated in both HCM/GSK-3β males and females (Fig. 5). Cardiac function, levels of fibrosis, and long-term survival were not measured in the earlier studies (1, 35). Moreover, these investigations indicate that active GSK-3β can dissociate cardiac growth from gene expression, histopathology, and dysfunction.

Whereas studies have implicated many different signaling molecules, including GSK-3β, in pressure overload or agonist-stimulated pathological cardiac hypertrophy, less is known about signaling pathways in HCM (7, 8, 36). Earlier studies in male mice from the same transgenic HCM model used in the current report showed that myocardial levels and activity of G protein-coupled β-adrenergic receptor kinase 1 protein were increased (7), as well as increased protein kinase A-mediated phosphorylation of phospholamban (Fig. 6). Subsequent studies demonstrated that high-level overexpression of the β2-adrenergic receptor (AR) caused rapid progressive cardiac failure with elevated mortality by 1 yr (8). In contrast, expres-
sion of a peptide inhibitor of the \(\beta\)-AR kinase 1 prevented cardiac remodeling, hypertrophic gene expression, and contractile dysfunction (8). Clearly, HCM mice have elevated adrenergic signaling, and blocking this signaling pathway improves the cardiac phenotype in HCM mice. Since active GSK-3\(\beta\) prevented short-term hypertrophic growth mediated by \(\beta\)-adrenergic stimulation (1), we cannot exclude that disruption of adrenergic signaling by GSK-3\(\beta\) downstream of the adrenergic receptor complex in HCM mice may be involved in inhibiting cardiac growth with no effect on histopathology, gene expression, nor cardiac function. In support of this hypothesis, \(\beta\)-blockade has been demonstrated to reduce pressure overload-mediated cardiac growth, whereas treatment had no impact on fibrosis (20, 27, 29, 30). However, clinical data suggests that \(\beta\)-blockers mostly affect heart failure-related symptoms and may slightly improve survival but with no impact on cardiac hypertrophy (11, 28, 31). More comprehensive studies are needed to determine the effect of \(\beta\)-receptor signaling in the HCM/GSK-3\(\beta\) mice.

This investigation supports the number of sex-related differences identified in cardiac phenotypes (5, 18). It is not known whether these results are specific to active GSK-3\(\beta\) or whether the sexual dimorphism seen in the HCM/GSK-3\(\beta\) mice would also be observed using a different anti-hypertrophic model with the HCM mice. It should be noted that there were no typical signs of heart failure or distress in the HCM/GSK-3\(\beta\) males.

Fig. 5. RNase protection analysis of atrial natriuretic peptide (ANF), \(\beta\)-myosin heavy chain (MyHC), sarcoplasmic reticulum Ca\(^{2+}\)-ATPase (SERCA), \(\alpha\)-skeletal actin, and GAPDH. A: representative RNA protection assay of hearts from 10-mo-old male and female mice. Lane 1, NTG; lane 2, HCM; lane 3, caGSK-3\(\beta\); lane 4, HCM/GSK-3\(\beta\). Quantitative analysis of hypertrophic-associated genes from the left ventricle of male (B) and female (C) transgenic littermates at 4 (open bar) and 10 mo (solid bar) of age. The relative intensities of the resultant bands were quantified in their linear range by automated computer densitometry, and each respective gene was normalized to GAPDH. The bar graph shows means \(\pm\) SE of 4–6 individual hearts.

\*Significantly different (\(P < 0.05\)) from NTG; \*significantly different from HCM; \#significantly different from caGSK-3\(\beta\). GSK, caGSK-3\(\beta\); \(\alpha\)-skActin, \(\alpha\)-skeletal actin.

Fig. 6. Western blot analysis for calcium handling proteins. A: left ventricular homogenates of 10-mo-old NTG, HCM, caGSK-3\(\beta\), and HCM/GSK-3\(\beta\) mice were probed with antibodies that recognize SERCA, phospholamban (PLB), and phosphorylation of phospholamban at serine-16. Representative immunoblots from 4 independent animals of each transgenic group are shown. Coomassie staining served as protein loading control. B: relative intensities of the resultant bands were quantitated for male (open bars) and females (solid bars). Mean values \(\pm\) SE of 4 individual hearts. \#\#P < 0.05 vs. NTG; \#\#significantly different from HCM.
and we hypothesize that these animals die of sudden cardiac death. Furthermore, a small number of HCM/GSK-3β females were maintained after 18 mo of age and demonstrated no delay in lethality (data not shown). Interestingly, reduced survival in HCM/GSK-3β males is consistent with other studies that have described similar sex-differences in survival (4, 9, 12, 16, 25). The etiology of the sex-specific differences presented in this study will be addressed in future studies.

These data demonstrate that blocking cardiac growth by active GSK-3β in males accelerates the cardiac dysfunction and increased mortality. Other studies have similarly demonstrated that blocking hypertrophy was associated with cardiac dysfunction, left ventricular dilatation, and increased mortality (2, 3, 22, 33). In contrast, clinical and experimental investigations have concluded that cardiac hypertrophy is an independent risk factor that leads to heart failure (6, 24, 38). It is interesting that blocking cardiac growth by active GSK-3β has a negative effect on males, whereas female cardiac physiology remains relatively unaffected. This sexually dimorphic effect of active GSK-3β suggests that there may be other potential modifiers in males that detrimentally impact cardiac physiology.

Finally, simply changing the diet of the mouse reduced the mortality in the HCM/GSK-3β males (Fig. 7). A recent study from our laboratory demonstrated that modifying the diet of the HCM mice from a soy-based diet to a casein-based diet favorably altered cardiac function and morphology (37). The previous investigation proposed that phytoestrogens in standard soy-based laboratory diet promote these changes most profoundly benefiting HCM males. Stauffer et al. (37) also observed that phosphorylated GSK-3β is reduced in HCM males on the casein diet. These preliminary data are interesting and warrant further investigations.

In summary, we have shown that activation of GSK-3β significantly attenuates cardiac hypertrophy in both sexes of a mouse model of HCM. However, reduced myocardial size was associated with poor contractile function, altered gene expression, and increased mortality only in HCM/GSK-3β males, whereas smaller heart size in HCM females had no obvious detrimental effects. The present findings may have important implications in that blocking hypertrophy through chronic activation of GSK-3β in HCM may not be uniformly beneficial. It may be that the extent and timing of caGSK-3β expression during pathogenesis could potentially be beneficial therapeutically. Future experiments will also be aimed at understanding the basis for the sex differences as well as the mechanism of premature death in the HCM/GSK-3β males.

ACKNOWLEDGMENTS

We are grateful to Silke Maass and to Karin Nunley for aid with the RNase protection assays and Ping Yue for the echocardiographic measurements. We also thank Gail Ackerman and Margaret Isenhart for care of the mice. We thank Premier Histology (Boulder, CO) for help with the histological staining.

GRANTS

This work was supported by National Heart, Lung, and Blood Institute Grant HL-56510 to L. A. Leinwand and by a National Heart, Lung, and Blood Institute National Research Service Award F32 HL-72565 to S. W. Luckey.

REFERENCES

Izumo S, Nadal-Ginard B, Mahdavi V.

Hardt SE, Sadoshima J.

MacLennan DH, Kranias EG.

Konhilas JP, Maass AH, Luckey SW, Ikeda K, Stauffer BL, Olson EN, Gradman AH, Alfayoumi F.


23.

22.

21.

20.

19.

18.

17.

16.

15.

14.

13.

12.

11.

10.

9.

8.

7.

6.

5.

4.

3.

2.

1.


J Cell Biol hypertrophic myocyte hypertrophy.

Nat Rev Mol Cell Biol cardiac contractility.


Br J Pharma cardiovmyocyte hypertrophy.


