We report that dietary modification from a soy-based diet to a casein-based diet radically improves disease indicators and cardiac function in a transgenic mouse model of hypertrophic cardiomyopathy. On a soy diet, males with a mutation in the α-myosin heavy chain gene progress to dilation and heart failure. However, males fed a casein diet no longer deteriorate to severe, dilated cardiomyopathy. Remarkably, their LV size and contractile function are preserved. Further, this diet prevents a number of pathologic indicators in males, including fibrosis, induction of β-myosin heavy chain, inactivation of glycogen synthase kinase 3β (GSK3β), and caspase-3 activation.

Introduction
The promise of nutritional genomics in considering the pathogenesis and treatment of disease is beginning to be recognized (1–5). For example, soy-rich diets are perceived to be generally beneficial from a health standpoint, particularly in the context of the cardiovascular system (6). This is exemplified by the fact that American consumers will spend $4.7 billion on soy foods and dietary supplements. However, there has not been a systematic experimental evaluation of how individuals with defined genetics respond to different diets and how sex might modify this interaction. One starting point to evaluate the effect of a particular diet such as soy is the laboratory rodent, where the standard diet is soy-based. Potent physiologic effects of soy diets have been demonstrated in both laboratory and clinical settings. Some of these effects have been reported to be beneficial and some may have adverse consequences. Among the beneficial properties are the prevention of cancer and a lowering of cholesterol (7). Among the potentially adverse effects are an increase in androgen levels and a decrease in thyroid peroxidase (8, 9). Many of the physiologic effects of a soy diet have been attributed to the soy isoflavones or phytoestrogens, and there are many experimental studies that have studied the effects of genistein and daidzein, the 2 most prominent isoflavones in soy (10). Indeed, soy isoflavones improve hyperlipidemia and cardiovascular disease associated with abnormalities in lipid metabolism via activation of PPARα (11, 12). In human and rodent heart failure, PPARα activity is attenuated (13, 14). However, a recent large epidemiologic study revealed no beneficial effect of dietary phytoestrogens on the incidence of clinical coronary or cerebrovascular events in women (15).

Despite this focus on phytoestrogens, soy has many complex nutrients and is consumed as a major part of the diet in many cultures. Further, decades of literature on experimental laboratory rodents have been in the context of a soy-based diet (rather than a diet supplemented by dietary phytoestrogens), and thus it is of great interest to examine the interaction of the soy diet and genetic models of disease. In the current study, we have asked what the impact of diet is on a genetic mouse model of cardiac hypertrophy and how sex might modify such a proposed interaction.

Myocardial hypertrophy in response to a disease stimulus consists initially of compensatory myocellular enlargement. However, the heart ultimately reaches a point where the stress overwhelms compensatory processes, and ventricular chamber enlargement, wall thinning, and impaired contractile function ensue, leading to heart failure. The myocardial mechanisms underlying this transition are thus far unknown.

Sex-specific variation in myocardial hypertrophy and progression to heart failure have been clearly documented over the past several decades (16, 17). In response to a disease stimulus (hypertension, ref. 18; valvular disease, ref. 17; sarcomeric mutations, ref. 19; and aging, refs. 20, 21), both sexes initially develop LV hypertrophy. However, men subsequently develop heart failure with chamber dilation, wall thinning, and impaired contractile function. Women develop heart failure with preserved LV contractile function. The sex difference in cardiac function favors the survival of women with heart failure (22, 23).

While these sex-differences in clinical cardiac disease are reasonably well characterized, there is a dearth of mechanistic information about the triggers of increased mortality. Several studies have evaluated the effects of sex hormones (24, 25) and phytoestrogens (15) on the cardiovascular system in humans, predominantly investigating effects on heart attack or stroke, disorders more associated with abnormalities of blood vessels. While estrogen and testosterone receptors have been identified in the myocardium (21, 26, 27), less is known about the influence of sex hormones on the myocardium.

We have developed a genetic mouse model of hypertrophic cardiac disease that exhibits the sex-dependent phenotypic characteristics documented in clinical populations (28). That is, while females preserve cardiac contractile function and continue to increase their cardiac mass, male mice develop thin ventricular walls and have poorly contractile hearts. This hypertrophic cardiomyopathic (HCM) mouse expresses a mutant myosin heavy chain (MyHC) transgene in the heart, and all of the characterization has been performed on animals fed a soy diet (29). We hypothesized that phytoestrogens influence cardiac growth and that the standard rodent diet plays an influential role in the development of this dilated, poorly contractile phenotype in male mice. We tested this hypothesis by comparing HCM mice and WT littermate controls of both sexes consuming a standard soy-based diet, a casein-based diet (30, 31), or a casein diet supplemented with daidzein and genistein. Further, we determined the effect of diet on several aspects of known hypertrophic signaling.

Nonstandard abbreviations used: BW, body weight; ER, estrogen receptor; GSK3β, glycogen synthase kinase 3β; HCM, hypertrophic cardiomyopathy; HW, heart weight; MyHC, myosin heavy chain; TL, tibia length; VW, ventricular weight.

Conflict of interest: The authors have declared that no conflict of interest exists.


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Results

Diet and cardiac growth. Diet was found to significantly affect cardiac mass. Ventricular weight (VW) (RV and LV) was normalized to tibia length (TL) (Figure 1A). As expected, the HCM animals had higher VW/TL than their sex-matched WT controls fed the same diet (P < 0.005 for all 4 groups). Also, as expected, male animals had larger hearts than female animals (P < 0.0001 for all 4 groups). However, HCM animals had significantly more hypertrophy on the casein diet than on the soy diet (P < 0.05 each). The male HCM animals fed a casein-based diet had greater cardiac hypertrophy (17%) than the HCM males fed a soy diet (9%) when compared with control mice. In contrast, diet did not have an effect on hypertrophy in female HCM animals (Figure 1B). As might be expected, diet also influenced body weight (BW). All mice consuming the casein diet were significantly heavier than those on the soy diet. Body composition, evaluated by dual-energy x-ray absorptiometry (DEXA), demonstrated no significant diet-dependent differences in lean BW; however, the animals consuming the casein diet had higher body fat than those consuming the soy-based diet. Importantly, normalization of VW for BW did not influence the differences between the transgenic groups noted above.

Dietary estrogens and cardiac growth. There are several components that are different between the soy and casein diets. We investigated the possibility that phytoestrogens contribute to differences in cardiac growth between the diets. The phytoestrogens daidzein and genistein were added to the casein diet. This supplemented diet was fed to male and female WT and HCM mice and compared with mice consuming the standard soy and casein diets. There was no significant effect of diet on heart weight/BW (HW/BW) ratios in either the male or female WT mice at 2 months of age. However, there was a significant effect of diet on HW/BW ratio in the male and female HCM animals at this early time point (P < 0.01). Moreover, the influence of diet was observed in a sex-dependent fashion (sex × diet interaction, P < 0.05). The influence of adding phytoestrogen to the casein diet was greater in the male mice, augmenting the HW/BW ratio by approximately 6%, while this addition attenuated cardiac growth in the female HCM mice by 6% (Figure 2).

Endogenous sex steroids and cardiac growth. The influence of the dietary compounds may occur through estrogenic effects on endogenous sex steroid receptors. These receptors have been implicated in cardiac growth in other models (32, 33). We investigated the possibility that diet contributes to the phenotype via a sex steroid receptor–mediated mechanism. Male and female HCM mice on the casein diet underwent prepubertal surgical gonadectomy with subsequent placebo, estrogen, or testosterone supplementation or sham operation. Gonadectomy with placebo supplementation (absence of all sex steroids) resulted in lower cardiac mass regardless of sex compared with sham-operated animals (males: 3.37 ± 0.19 mg/g BW versus 4.16 ± 0.37 mg/g; females: 2.93 ± 0.21 mg/g versus 3.45 ± 0.90 mg/g). Testosterone supplementation of the females (4.47 ± 0.10 mg/g) and estrogen supplementation of the males (3.93 ± 0.27 mg/g) caused cardiac mass to be similar to the opposite sex sham-operated animals. For comparative numbers, see those noted above. In summary, lack of sex hormones results in lower HW/BW ratios in both sexes, and testosterone and estrogen appear to be primary determinants of normalized heart mass.

Diet and in vivo cardiac function. Cardiac contractile function evaluated by echocardiography was profoundly affected by diet in male HCM animals (Figure 3). At 8 months of age, HCM males fed a soy diet had significantly depressed contractile function (P < 0.0001) while animals fed a casein diet were indistinguishable from WT.
The decrease in contractile function (Figure 3A) was accompanied by significant wall thinning (Figure 3B) and LV chamber dilation. Thus, a soy-based diet has a very significant detrimental effect on cardiac function in this model of hypertrophic disease.

**Diet and blood pressure in vivo.** Estrogen is associated with improvements in endothelium-mediated arterial vasodilation, a marker of vascular health. Oral genistein supplementation has also been shown to improve endothelial function in humans (5). Alterations in endothelial and arterial function may lead to alterations in blood pressure that influence the development of cardiac hypertrophy. We therefore sought to determine whether the sex- and diet-dependent differences were due to differences in systemic blood pressure. In vivo hemodynamics with Millar catheterization revealed no significant differences in mean, systolic, or diastolic arterial pressure (data not shown). The lack of influence of sex or diet on blood pressure suggests a primary myocardial influence of diet on the differences observed in cardiac function.

**Diet and myocardial fibrosis and myocellular disarray.** The effect of diet on histopathology as evidenced by collagen deposition and myofilament disorganization was evaluated at 8 months of age using picrosirius red staining. Fibrosis and myocellular disarray can be visualized under polarization light microscopy by yellow/orange birefringence and lack of green sarcomeric birefringence, respectively. Highly ordered sarcomeres have light-green birefringent properties (Figure 3). Increased collagen deposition causes increased myocardial stiffness and is associated with myocellular disarray. Both of these features have been implicated in myocardial contractile dysfunction (34–36). HCM animals display an increase in collagen deposition and myocellular disarray compared with WT (Figure 4). This was evidenced by a transition from predominantly thin collagen fibers (green birefringence) in the WT controls to predominantly thick collagen fibers (yellow/orange birefringence) in the HCM male and female mice (Figure 4 and data not shown). The lack of uniform myofilament birefringence in the HCM sections is also indicative of an increase in myocellular disarray. Most importantly, there was a distinctly worse histopathologic phenotype in the soy HCM males when compared with the casein HCM males. We observed no significant difference in fibrosis and disarray among diets in the female HCM mice. Moreover, the histopathologic phenotype was indistinguishable between the sexes on the casein diet. The increased fibrosis and myocellular disarray observed in the male HCM mice consuming the soy-based laboratory diet seem likely to contribute to the contractile dysfunction in this experimental group.

**Diet and β-MyHC protein synthesis.** Heart failure in rodents and humans is associated with increased levels of the slower, more energy efficient myosin motor protein, β-MyHC. α-MyHC, the predominant isoform in murine hearts, declines with worsening heart failure and is accompanied by increases in β-MyHC (37). An increase in β-MyHC protein content acts in a dominant fashion and is one of the mechanisms underlying myocardial contractile dysfunction (38, 39). Additionally, β-MyHC content can be used as an objective assessment of disease severity. We measured the β-MyHC content of the LV at 8 months of age (Figure 5, A and B). Because contractile function was abnormal in the male HCM mice consuming the soy diet, we hypothesized that more β-MyHC protein would be observed in this group. Indeed, male HCM mice fed the soy diet expressed more β-MyHC than the female HCM mice, consistent with the more severe disease state in males. In addition, there was strikingly lower β-MyHC content in the male HCM mice consuming the casein diet. This is of particular interest since the absolute and normalized heart weights were greater in the casein-fed mice. This suggests that the growth that occurred in the HCM hearts due to the casein diet is a beneficial (or physiologic) hypertrophy. The increased β-MyHC protein expression may be a mechanism contributing to the impairment in systolic function in the male HCM mice on the soy diet.

**Diet and the IGF-1/Akt/glycogen synthase kinase 3 protein kinase cascade.** Since IGF-1 treatment of cardiac myocytes in culture increases β-MyHC synthesis (40, 41), we explored the IGF-1/Akt/glycogen synthase kinase 3 (IGF-1/Akt/GSK3) cascade. Sex-dependent differences
In the IGF-1/Akt/GSK3β pathway, there has been observed in humans and animal models of cardiac disease (42). Several animal models have indicated that this pathway is involved in cardiac hypertrophy via phosphorylation of Akt (protein kinase B; activation) and GSK3β (inactivation) (43, 44). Increased nuclear localization of phospho-Akt has been observed in the myocardium of premenopausal women as well as in response to estrogen stimulation in cultured cardiomyocytes (42). IGF-1 has been identified as one of several potential ligands responsible for estrogen-independent activation of estrogen receptors (ERs) (45, 46). Based on this information, we evaluated GSK3β, a terminal protein in this cascade, for changes in activity as a potential protein kinase leading to the sex and diet differences in this disease model. GSK3β in the active, unphosphorylated form suppresses protein synthesis by inhibiting translation initiation factor eIF2α. Phosphorylation of GSK3β allows translation initiation and promotes hypertrophy in cardiac myocytes (47).

We hypothesized that the soy diet increases activity of the IGF-1/Akt/GSK3β cascade, which is then permissive of β-MyHC protein synthesis. Therefore, the phosphorylation state of GSK3β as a fraction of total GSK3β was measured in LV homogenates. As predicted, the HCM mice exhibited a 2- to 3-fold higher ratio of phosphorylated (inactive) to total GSK3β compared with WT controls (Figure 5C). However, phosphorylated GSK3β was over 3-fold higher in the male HCM mice fed a soy diet compared with male HCM mice fed a casein diet. This elevation correlates well with the significant increase in β-MyHC protein. While we expected to observe a higher ratio of phospho- to total GSK3β in the HCM mice, the diet difference in the HCM males is an unexpected and novel finding. Significantly higher phosphorylation states as observed in the HCM males consuming the soy diet may be a mechanism contributing to the transition to the decompensated state.

**Diet and apoptosis.** One mechanism underlying cardiac dilation is myocardial apoptosis. Sex differences in apoptotic myocardial cell loss have been identified (20). Importantly, genistein induces apoptosis via caspase-3 in a number of settings (48). For example, in human prostate cancer cell lines, genistein induces apoptosis through expression and activation of caspase-3 (49). Caspase-3 has been shown to be a proapoptotic factor in cardiac myocytes by initiating mitochondrial protein dissociation (50). An increase in programmed myocardial death therefore could underlie the development of the dilated phenotype in the male HCM mice on a soy diet. We measured caspase-3 activity in the hearts at 8 months of age. There is a marked elevation in caspase-3 activation in the male HCM mice consuming the soy-based diet, which is reversed in male HCM mice consuming the casein-based diet (Figure 6).

**Discussion**

The salient findings from the current study are as follows: (a) Diet significantly alters cardiac structure and function in WT and HCM animals; (b) The diet-dependent phenotype differs significantly between sexes at the macroscopic and cellular levels; (c) The phytoestrogens daidzein and genistein influence cardiac growth in a sex-dependent fashion; (d) Diet-dependent alterations in the
IGF-1/Akt/GSK3β pathway, β-MyHC content, and caspase-3 activation are potential molecular mechanisms responsible for these changes in phenotype. To our knowledge, this is the first report of significant differences in cardiac muscle adaptation due to dietary manipulation. These data strongly suggest further investigation into a link between diet and cardiomyopathy.

A major difference between the 2 diets in the current study is the protein source. The standard laboratory diet is soy based, containing significant amounts of phytoestrogens, which are major steroid receptor ligands and have been shown to have multiple biological effects. Although the relative estrogenic activity is 10^-2 to 10^-3-fold less than estradiol or estrone, the principal circulating estrogens in most mammals (4), there are several characteristics that promote biological activity. These estrogenic compounds have less nonspecific binding to proteins in blood, which enhances the number of molecules available for receptor binding (51). In addition, serum concentrations of phytoestrogens with modest dietary intake have been observed to be 10^-3 to 10^-4-fold higher than endogenous estradiol concentrations (4). Phytoestrogens also have a preference for the ERβ and several non-ER related actions (i.e., protein tyrosine kinase inhibitor and antioxidant). Perhaps most importantly, these pleiotropic effects have been observed to occur within an individual organ or cell, consistent with steroid action in general.

We and others (52–56) have demonstrated that sex steroids influence cardiac growth. The exact mechanism of action is unclear, but additional investigation is ongoing. These results establish the biological plausibility of the influence of phytoestrogens on the heart to elicit cardiac growth via a sex steroid–receptor mechanism. While there are several reasonable theories regarding the influence of phytoestrogens on sex hormone receptors in the heart, we would propose that it is a difference in the effective estrogen dose between the sexes that may be responsible for the results of our investigation.

Since sex-dependent alterations in myocardial ERs have not been described, we would propose that the augmented estrogenic exposure in the male mice contributes to the dilated phenotype. We speculate that, because female animals have higher endogenous estrogen levels, the proportional increase in estrogenic compounds via diet is less in females compared with males, who are chronically exposed to significantly lower levels of estrogenic compounds. For example, it has been estimated that basal female 17β-estradiol levels are 5–30 pg/ml while males have negligible levels (57–60). Serum phytoestrogen levels have been reported in the 1000–2000 ng/ml range on several standard rodent diets independent of sex (61).

Differences in affinity and estrogenic activity between phytoestrogens and endogenous estrogens make it difficult to assess the proportion of biological activity due to each individual compound in female animals. In males this is not the case since endogenous estrogenic compounds are present only in negligible quantities; therefore the full effect of the phytoestrogens would be observed. To support this argument further, Boettger-Tong et al. have demonstrated a failure of the usual biochemical and morphological uterine responses to exogenously administered estradiol in animals that were fed a rodent diet with soy meal and alfalfa (62). This occurred when the rodent diet had been reformulated without any investigator notification. Since, as noted above, isoflavone levels have not been demonstrated to be different between males and females on identical diets, the absolute influence of these compounds should be substantially greater in the male animals that have virtually no basal estrogenic stimulation. Moreover, there is in vivo evidence that genistein acts as an agonist at ERs when administered alone and as an antagonist when coadministered with 17β-estradiol (63). It is plausible that a similar mechanism is behind the sex difference in cardiac phenotype.

Indeed, our results demonstrate that phytoestrogen supplementation augments cardiac growth in the HCM males and attenuates growth in the HCM females. The current results do not make any assumptions regarding the molecular and functional phenotype of the HCM mice on the supplemented diet. Importantly, adding phytoestrogens to the casein diet does not recapitulate the entire cardiac phenotype of the young animals consuming the soy diet. This is not surprising given that the dietary milieu is complex and unregulated in the soy diet. There are clearly other components in the diet that influence cardiac growth. This fact accentuates the importance of dietary intake in other experimental models. However, it is beyond the scope of the current study to differentiate the other components of the soy diet that may contribute to the sex difference in phenotype.

Phytoestrogens also activate PPARα, which is downregulated in heart failure in vivo. In our genetic model, we observed increased cardiac growth in animals consuming a soy-free diet, suggesting that PPARα is not contributing to the improvement in the phenotype in our model. However, the casein diet was also associated with an improvement in cardiac function assessed by echocardiography in the absence of a change in systemic blood pressure. Importantly, the increased growth was accompanied by improved cellular markers of disease including lower β-MyHC expression and improved cellular architecture (less thick collagen deposition and less myocardial disarray).

The soy-based laboratory diet was associated with increased caspase-3 activity and increased activity through the IGF-1/Akt/GSK3β pathway in the diseased animals relative to WT. IGF-1 stimulation is associated with increased β-MyHC synthesis in rat models (64). Augmentation of this pathway may lead directly to the systolic impairment observed in the male HCM mice on the soy diet. Alternatively, the increased activity along this pathway may be a secondary phenomenon in response to the differences in cardiac growth. Future directions will include defining the role of this pathway by crossbreeding the HCM model with a constitutively active GSK transgenic mouse (44) and identification of a common upstream activator. The increase in caspase-3 activity in HCM hearts on a soy diet indicates elevated levels of myocardial death, or apoptosis. Preliminary data from our laboratory (data not shown) demonstrate that the increase in caspase-3 activity was associated with decreased levels of Bcl-2 (an inhibitor of apoptosis) and pro–caspase-9 (a precursor of effector caspases). These additional preliminary results further support the conclusion that there is augmented apoptosis in the HCM mice on a soy diet. Genistein has been shown to induce apoptosis via activated caspase-3 in a number of settings (48, 49). An increase in apoptosis is likely an important contributor to the adverse remodeling observed in dilated cardiomyopathy in human clinical populations and may be responsible in part for the transition from the compensated hypertrophic to the decompensated dilated state in the mouse model.

Our results suggest that the dilated phenotype observed in the male HCM mice on the soy diet is a result of an augmented growth pathway and an augmented programmed cell death pathway. It is likely that the decompensated phenotype results from a transition from a balance to an imbalance of cell growth and cell death.
Components of the soy diet have been associated with both myocellular hypertrophy and apoptosis in several other model systems and may be modifiers of the balance between these 2 processes in vivo. Investigation of functional and molecular changes occurring in the hearts of mice on the phytoestrogen-supplemented diet will further elucidate the influence of phytoestrogens on cardiac disease. Additional investigation into other specific components of the soy diet as well as identification of characteristics of these pathways during the compensated state are necessary to fully elucidate these mechanistically.

Taken together these data show an increase in nonpathologic, or physiologic, cardiac growth in this HCM mouse model on a soy-free diet. The modifiers of caspase activity and the IGF-1/Akt/GSK3β hypertrophic pathway are unknown but deserve additional attention. This discovery may lead to novel dietary modifications that prevent progression or formation of some hypertrophic cardiomyopathies. Future studies will be directed against identifying specific genetic modifiers that affect the response of the heart to diet. These will include testing the effect of diet on different strains of inbred mice. There is a large literature of human studies in which correlations have been made between certain diets and diseases, particularly cancer (3). And, in some cases, the results have been inconclusive. The kind of study reported here on genetically identical groups of mice of both sexes should address the issue of genetic variability in the human studies and begin to define the role that genes play in dietary responses.

Methods

Animals. The HCM mouse model used in this study expressed a mutant rat α-MyHC with expression driven by the α-MyHC promoter (29). The transgene coding region contained 2 mutations, a point mutation, R403Q, and a deletion of 59 amino acids in the actin binding site bridged by the addition of 9 nonmyosin amino acids. Male and female WT and HCM mice were fed a soy diet, a casein (phytoestrogen-free) diet, or a casein diet supplemented with phytoestrogens ad libitum. Mice were genotyped by PCR from a 2-mm tail sample prior to study inclusion. Given the large number of animals included in this study, repeat genotyping was performed on a separate tissue source of protein (casein in the D10001 versus alfalfa and soybean in the 8656 diet), carbohydrate (sucrose and corn starch in the D10001 versus soybean oil in the 8656 diet). The effective caloric content was different between the diets as well (D10001, 3.9 kcal/g versus 8656, 3.25 kcal/g).

Surgical gonadectomy. At 1 month of age, mice were anesthetized with 2.5% tribromoethanol (Avertin) by intraperitoneal injection. Females underwent bilateral oophorectomy via a transverse scrotal incision. The incisions were repaired with suture and skin staples. While still under anesthesia, sustained release (90 day) hormone pellets (Innovative Research of America) of 17β-estradiol, testosterone, or vehicle placebo were implanted subcutaneously in the nape of the neck. Sham-operated animals were handled identically except the gonads were not removed.

Growth measurements. At 2 or 8 months of age, mice were euthanized using approved methods. Each mouse was weighed, and the length of the right tibia was recorded. Hearts were rapidly excised and washed with ice-cold normal saline (9% NaCl w/v). The great vessels and all atrial tissue were removed under a dissecting scope (Carl Zeiss), and the ventricles were blotted dry and weighed. The RVs and LVs were rapidly separated and individually flash frozen in liquid nitrogen. The samples were stored at −80°C until further analysis was performed.

Echocardiography. Echocardiography was performed on animals at 8 months of age as previously described (66). A VingMed System Five echocardiography machine (GE Medical Systems) with a 10-MHz–phased array transducer was used for digital image acquisition. The mice were positioned prone, and M-mode recordings were obtained and saved on magnetic optical media for offline analysis, which was performed using EchoPAC software (version 6; GE Medical Systems). Fractional shortening was calculated using the average over 3 cardiac cycles.

Blood pressure measurement. In vivo systemic blood pressure measurement was performed on animals at 8 months of age. Each mouse was injected intraperitoneally immediately before evaluation with 2.5% tribromoethanol (Avertin). A longitudinal skin incision was performed over the trachea, and the right carotid artery was isolated using blunt dissection under sterile conditions (Carl Zeiss). The cephalad portion of the carotid artery was ligated at the base of the skull. A small nick was made in the midportion of the artery, and a 1.4-Fr Millar Mikro-tip pressure transducer (Millar Instruments) was advanced through the nick and into the aorta. After a period of equilibration, aortic pressures were recorded. Following blood pressure measurements, the animals were euthanized.

Histology. Following euthanasia at 8 months of age, hearts were rapidly excised and washed, and whole-heart weight was recorded. The entire heart was placed in 10% neutral-buffered formalin for 24 hours for fixation prior to histological evaluation. The fixed hearts were processed, embedded in paraffin, sectioned, and stained with picrosirius red according to standard protocols. Thin collagen fibers showed green birefringence, and thick collagen fibers showed bright yellow/orange birefringence under polarization light microscopy. Myofibrils in series exhibited slight birefringence.

Western blot analysis. Frozen LV tissue was homogenized on ice in a protein extraction buffer: NaCl (137 mM); Tris(hydroxymethyl)-aminomethane (20 mM); 10% vol/vol glycerol; 1% vol/vol NP-40; pH 7.4. The homogenized tissue was centrifuged at 12,000 g for 10 minutes at 4°C and the supernatant removed. Protein concentration was determined using the Bradford method. SDS-PAGE separation was performed under denaturing conditions. The proteins were transferred to a PVDF membrane (Amersham Biosciences) using standard techniques. The membranes were probed with antibodies (Santa Cruz Biotechnology) specific for either total GSK3β or the phosphorylated isoform. Immunoreactivity was visualized using a Western Lightning chemiluminescence detection system (PerkinElmer) and quantified using densitometry.

MyHC expression. LV tissue was homogenized in a myosin sample buffer: urea (8 mol/l); thiourea (2 mol/l); Tris, pH 6.8 (0.05 mol/l); dithiothreitol (DTT) (0.075 mol/l); 3% SDS. Each sample was heated at 100°C for 3 minutes and then loaded in 6 wells containing escalating amounts of total
protein (0.5 to 50 µg/well). The samples were separated by 6% SDS-PAGE at 16 mA for 4.5 hours at 8°C. Gels were silver stained, and the percentages of α- and β-MHC protein were determined by densitometry (67).

Caspase-3 activity assay. Caspase-3 activity was determined by monitoring the rate of cleavage of a fluorogenic caspase-3 specific substrate (Acetyl-AspGluValAsp-AMC; Calbiochem). To do this, frozen hearts were mechanically disrupted in an ice-cold lysis buffer (0.02 ml/g tissue): Tris(hydroxymethyl)-aminomethane (20 mM); NaCl (137 mM); EDTA (0.2 mM); EGTA (0.5 mM); Triton X-100 (1%); glycerol (10%), pH 7.4. In a 96-well plate, 0.5 mg of protein was added to each well with an equal volume (50 µl) of caspase-3 activity assay buffer containing: Tris(hydroxymethyl)-aminomethane (50 mM); EDTA (0.5 mM); glycerol (20%); caspase-3 substrate (0.02 mM); DTT (0.004 mM), pH 7.0. Cleavage of the substrate was monitored by excitation at 380 nm and emission at 460 nm with a Fluoroscan Ascent Microplate fluorometer (Thermo Electron Corp.). Caspase-3 activity was determined by calculating the slope of the linear portion of the cleaved substrate and then normalized to protein content (fluorescent units/min/mg protein).

Data and statistical analysis. Results are presented as mean ± SEM. Between group differences of morphometric, protein, and blood pressure, data were determined using a 2 between (male, female) by 2 between (soy, casein) by 2 between (WT, HCM) ANOVA at 8 months of age. Between group differences were determined using a 2 between (male, female) by 3 between (soy, casein, supplemented) ANOVA. Interactions and/or specific main effects differences were examined by Tukey post hoc analyses. P < 0.05 was considered significant a priori.

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