

# Exercise Can Prevent and Reverse the Severity of Hypertrophic Cardiomyopathy

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**Abstract**—Hypertrophic cardiomyopathy (HCM) is the most common form of sudden death in young competitive athletes. However, exercise has also been shown to be beneficial in the setting of other cardiac diseases. We examined the ability of voluntary exercise to prevent or reverse the phenotypes of a murine model of HCM harboring a mutant myosin heavy chain (MyHC). No differences in voluntary cage wheel performance between nontransgenic (NTG) and HCM male mice were seen. Exercise prevented fibrosis, myocyte disarray, and induction of “hypertrophic” markers including NFAT activity when initiated before established HCM pathology. If initiated in older HCM animals with documented disease, exercise reversed myocyte disarray (but not fibrosis) and “hypertrophic” marker induction. In addition, exercise returned the increased levels of phosphorylated GSK-3 $\beta$  to those of NTG and decreased levels of phosphorylated CREB in HCM mice to normal levels. Exercise in HCM mice also favorably impacted components of the apoptotic signaling pathway, including Bcl-2 (an inhibitor of apoptosis) and procaspase-9 (an effector of apoptosis) expression, and caspase-3 activity. Remarkably, there were no differences in mortality between exercised NTG and HCM mice. Thus, not only was exercise not harmful but also it was able to prevent and even reverse established cardiac disease phenotypes in this HCM model. (*Circ Res.* 2006;98:540-548.)

**Key Words:** apoptosis ■ exercise ■ hypertrophic cardiomyopathy ■ remodeling

Treatment strategies for hypertrophic cardiomyopathy (HCM) depend on clinical symptoms and stratification of sudden death risk with little evidence supporting prophylactic treatment of asymptomatic HCM.<sup>1</sup> It is becoming increasingly evident that cardiac rehabilitation for patients with other forms of cardiac disease such as ischemic heart disease, hypertension, and congestive heart failure (CHF) includes regular exercise, particularly aerobic exercise, and that exercise reduces cardiovascular morbidity and mortality in these subjects.<sup>2,3</sup> Yet there are no reports in the literature that discuss the impact of mild, monitored exercise as a therapeutic measure for HCM patients. Exercise rehabilitation in HCM patients, however, is somewhat counterintuitive considering the high incidence of sudden cardiac death in young athletes and that both the American Heart Association and the European Society of Cardiology strongly encourage preparticipation cardiovascular screening of HCM for young competitive athletes.<sup>4,5</sup> However, we hypothesized that voluntary cage wheel running as a form of mild exercise would improve and/or prevent the pathologic cardiac phenotype in a murine model of HCM. Voluntary cage wheel was chosen as the exercise intervention because it eliminates physical and

psychological stressors associated with forced exercise paradigms that may exacerbate the HCM syndrome, and we have previously demonstrated cardiac adaptation to voluntary cage wheel exercise.<sup>6</sup> The particular HCM mouse model developed by our group<sup>7</sup> is ideal to address this hypothesis because it displays typical histologic features of HCM such as myocyte disarray and interstitial fibrosis and significant left ventricular and right ventricular hypertrophy by 4 months of age.<sup>7</sup> Much like their human counterparts, these animals are also characterized by a pathological spectrum of disease phenotypes that can include sudden cardiac death and progressive deteriorating left ventricular function that leads to CHF.<sup>7,8</sup> More importantly, the HCM mice show impaired exercise tolerance by treadmill running.<sup>8</sup> In a similar HCM model, one of five mice died during swimming.<sup>9</sup>

To assess the impact of the exercise intervention, we measured an array of pathologic markers that are characteristic of HCM including transcriptional regulators, expression of hypertrophic genes and associated proteins, myocyte ultrastructure and fibrosis, cardiac function, and apoptosis.<sup>7,8,10</sup> We demonstrate that voluntary cage wheel exercise reversed and/or prevented the pathologic course associated

Original received October 24, 2005; revision received December 15, 2005; accepted January 17, 2006.

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DOI: 10.1161/01.RES.0000205766.97556.00

with HCM and identify signaling pathways that are important for this beneficial outcome.

## Materials and Methods

### Experimental Animals

The experimental murine HCM model has been detailed previously.<sup>7</sup> Previously described mice containing myocyte enhancer factor 2 (MEF2)<sup>11</sup> or nuclear factor of activated T cells (NFAT)<sup>12</sup> controlling a lacZ reporter gene were also generated for these studies (see supplementary methods, available online at <http://circres.ahajournals.org>). Animals were euthanized and hearts were equally sectioned for the assays described below. All of the animals were handled according to approved protocols of the University of Colorado.

### Voluntary Cage-Wheel Exercise

Individual HCM or NTG male animals were subjected to voluntary cage-wheel exercise as previously described.<sup>6</sup> Animals were subjected to cage wheel exposure starting at either 2 months or at 6 months of age. Each group continued voluntary exercise until 8 months of age. All animals (sedentary and exercised) were euthanized at 8 months of age.

### NFAT/lacZ and MEF2/lacZ Activity Assays

Using a portion of the excised hearts, MEF2/lacZ assays were performed as previously described.<sup>6</sup> NFAT/lacZ assays were performed in an identical manner.

### RNase Protection Assay

RNase protection assays were performed as previously described.<sup>13</sup> Total RNA was extracted and hybridized overnight with radiolabeled RNA fragments for hypertrophic genes (see supplemental methods).

### Western Blot Analysis

Preparation of heart samples for Western immunoblotting were performed as previously described.<sup>6</sup> All antibodies were obtained commercially from Cell Signaling Technology (Beverly, Mass), except Bcl-2, which was obtained from BD Transduction Laboratories (San Diego, Calif).

### Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis

Hearts extracts were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) for cardiac MyHC content as previously described<sup>10,14</sup> (see supplemental methods).

### Histology and Picrosirius Red Staining

Hearts were rapidly excised after euthanasia, washed, and whole heart weight was recorded. The entire heart was fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned, and stained with picrosirius red according to standard protocols. Fibrosis was quantified as described in the supplementary data.

### Caspase-3 Activity Assay

Frozen hearts were mechanically disrupted in an ice-cold lysis buffer for caspase-3 activity analysis. Caspase-3 activity was determined by monitoring the rate of cleavage of a fluorogenic caspase-3 specific substrate (Acetyl-AspGluValAsp-AMC; Calbiochem) as described in greater detail in the supplementary data.

### Data and Statistical Analysis

Results are presented as mean±SEM. The differences between groups were analyzed with a one-way ANOVA followed by a Student *t* test with a post hoc Bonferroni correction to assess difference among mean values.

## Results

### HCM Mice Exercise to the Same Extent as NTG Mice

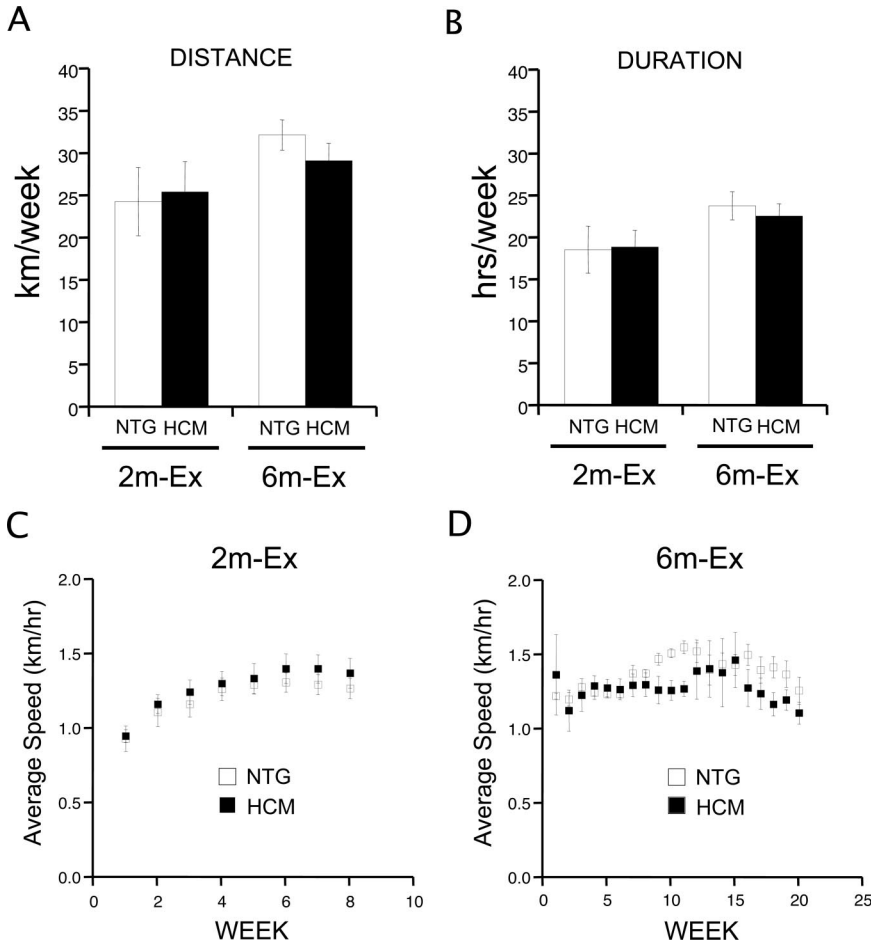
NTG and HCM male mice were exposed to a voluntary cage wheel at two different time points to assess prevention or reversal of disease phenotypes as described in the Methods section and all animals were euthanized at 8 months of age. The 8-month time point was chosen based on previous data indicating significant functional, biochemical, and histological pathology in HCM hearts.<sup>7,8,10,15</sup> Despite the previous observation of impaired treadmill running in HCM animals,<sup>8</sup> voluntary cage wheel performance was not different between NTG and HCM in either paradigm (Figure 1). Although the cause of death was unknown, one HCM animal from each running group died (of 20 exercised mice; supplemental Table I, available online at <http://circres.ahajournals.org>), and one NTG animal (of 18 exercised mice; supplemental Table I) from the long-term exercised group died. All sedentary animals survived the duration of the experimental protocol. Therefore, there was no difference in mortality between HCM and NTG exercised mice.

Wheel running speeds (weekly) were calculated and both NTG and HCM mice showed a gradual increase in wheel running speeds over the exercise protocol in both the 2-month (Figure 1C) and 6-month (Figure 1D) exercise groups. The speed for the NTG mice exercised for 6 months (NTG-6m-Ex) group was significantly ( $P<0.01$ ) higher than all other groups.

### Cardiac Mass/Adaptation

Each animal was analyzed for body and cardiac mass after exercise. Sedentary HCM mouse hearts are enlarged at 8 months of age (supplemental Table I).<sup>7,8</sup> Cardiac hypertrophy has also been demonstrated in the wild-type mouse in response to 3 to 4 weeks of voluntary cage wheel exercise.<sup>6</sup> In both NTG mice and HCM, 2 months of cage wheel exposure starting at 6 months of age significantly increased heart weight:body weight (HW/BW) ratio and heart weight:tibial length (HW/TL) compared with sedentary mice of each respective genotype. However, the hearts of NTG and HCM mice exposed to a cage wheel for 6 months (starting at 2 months of age) were not different in size from the hearts of NTG and HCM sedentary mice, respectively.

To more clearly represent the impact of exercise on cardiac mass in NTG and HCM animals, the percent difference from each respective sedentary control was calculated for each exercise group (Figure 2). As indicated in Figure 2, cardiac mass relative to sedentary counterparts was the same for both NTG and HCM mice in each exercise group. HCM animals exercised for 2 months (2m-Ex) exhibited the most pronounced, significant effect of exercise, with a  $11.3\pm 3.2\%$  increase in heart size relative to HCM sedentary animals (Figure 2), an increase that was 2-fold greater than NTG animals ( $4.6\pm 3.3\%$ ) exercised for the same duration (Figure 2). Interestingly, the hearts of both NTG and HCM animals from the long-term exercise group (6m-Ex) were smaller than their sedentary counterparts by  $5.9\pm 3.5\%$  and  $3.7\pm 2.3\%$ ,



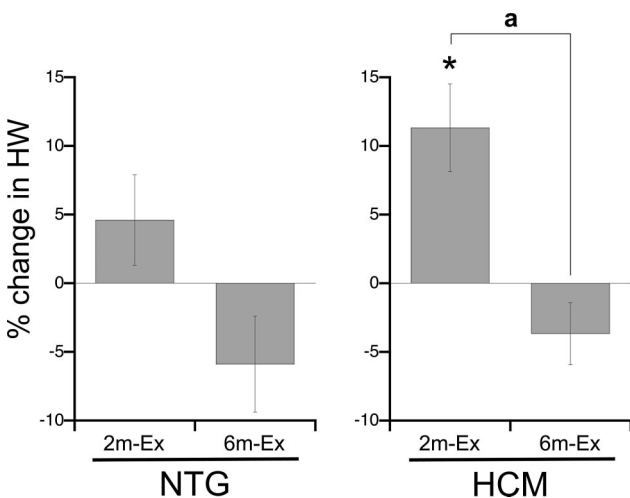
**Figure 1.** Voluntary cage wheel performance in NTG and HCM male mice exercised for either 2 months (2m-Ex) or 5 to 6 months (6m-Ex). A, Average running distance (km/week) determined for every 7 days over the 2- or 6-month study period. B, Average running duration (hour/week) determined for every 7 days over the 2- or 6-month study period. C, Calculated running speed (km/hour) averaged for every 7 days over the 2-month study period for NTG (open symbols) and HCM (closed symbols) animals. D, Calculated running speed (km/hour) averaged for every 7 days over the 6-month study period for NTG (white symbols) and HCM (black symbols) animals. There were no significant differences among the experimental groups studied.

respectively. Whereas the difference between NTG exercise groups was not significant, the difference in percent change achieved statistical significance between HCM animals. Therefore, despite having a directional impact that was

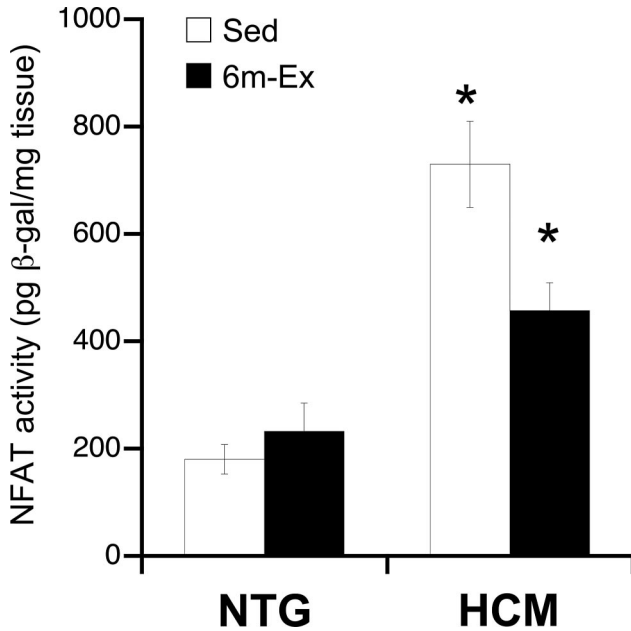
similar, exercise appears to have a greater effect on cardiac adaptation in the context of cardiac disease.

**NFAT but not MEF2 Is Decreased by Exercise in HCM Mice**

Calcineurin, a potent effector of cardiac hypertrophy, dephosphorylates NFAT, which then translocates to the nucleus.<sup>16</sup> We wished to determine whether NFAT activity is increased in HCM mice and, if so, how NFAT activity might change with exercise. NFAT activity was significantly elevated in HCM versus NTG sedentary animals (729.7±80.2 versus 180.3±27.3 pg β-galactosidase/mg tissue; Figure 3). Long-term exercise in HCM mice significantly reduced NFAT activity (457.1±49.6 pg β-galactosidase/mg tissue). Long-term (6m-Ex) exercise in NTG animals did not affect NFAT activity (232.1±52.8 pg β-galactosidase/mg tissue). Of the three 2m-Ex HCM animals that were studied, two had reduced NFAT activity levels (68.0 and 117.9 pg β-galactosidase/mg tissue), whereas one had an elevated level (6919 pg β-galactosidase/mg tissue). However, the heart with the elevated NFAT activity levels had a HW/BW of 7.2 mg/g, indicative of a transition to CHF. We conclude that these results are consistent with a role for NFAT activity in pathologic but not physiologic cardiac hypertrophy<sup>17</sup> and support the hypothesis that decreased NFAT activity may contribute to the beneficial effect of exercise on the HCM phenotypes.



**Figure 2.** Relative cardiac mass for each experimental group. The absolute cardiac mass of each animal in each experimental group was compared with the mean cardiac mass of each respective sedentary control and expressed as a percent difference (see Methods). Sed, NTG (n=30) or HCM (n=36) sedentary male mice; 2m-Ex, NTG (n=14) or HCM (n=14) male mice exercised for 2 months; 6m-Ex, NTG (n=4) or HCM (n=6) male mice exercised for 5 to 6 months. (\*P<0.05 from sedentary counterparts; a, P<0.05.)



**Figure 3.** NFAT transcriptional activity in sedentary and exercised NTG and HCM animals. NFAT transcriptional activity was determined by the NFAT-dependent expression of β-galactosidase. Sed, NTG (n=5) or HCM (n=17) sedentary male mice; 6m-Ex, NTG (n=5) or HCM (n=11) male mice exercised for 5 to 6 months. (\**P*<0.05 from measured values in NTG sedentary controls; †*P*<0.05 from measured activity in HCM sedentary animals.)

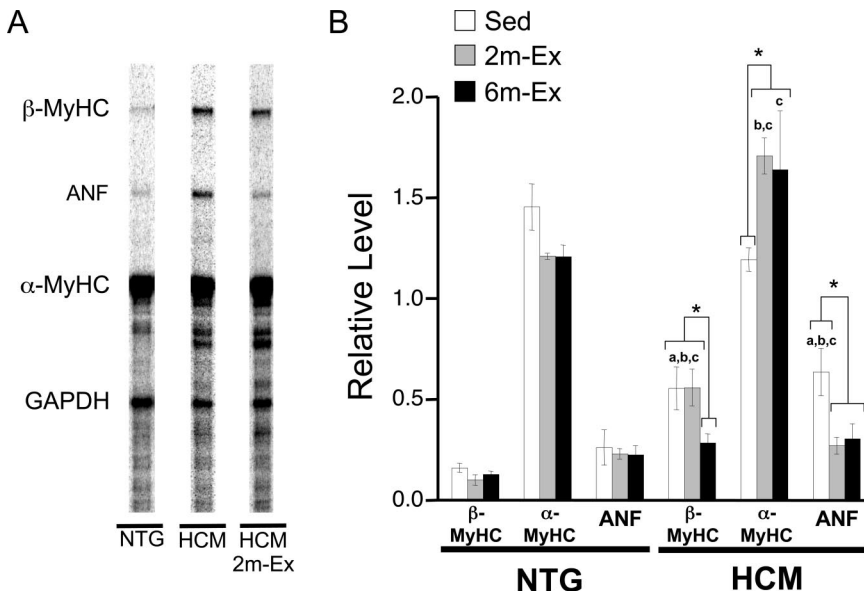
The MEF2 family of transcription factors has also been implicated as a mediator of pathological hypertrophy in mice,<sup>18,19</sup> yet has not been evaluated in HCM. MEF2 activity appears to play a minimal role in the response of cardiac muscle to exercise.<sup>6</sup> This is consistent with a specific role for MEF2 in pathologic cardiac hypertrophy. Using a similar MEF2-dependent lacZ reporter transgene,<sup>11</sup> sedentary HCM animals demonstrated a 15-fold elevation in β-galactosidase levels (1489.7±520.6 pg β-galactosidase/mg tissue) compared with NTG controls (104.0±17.4 pg β-galactosidase/mg tissue)

that was unchanged by 2 months of exercise (1991.5±640.3 pg β-galactosidase/mg tissue). Therefore, unlike NFAT, changes in MEF2 activity do not appear to contribute to the beneficial effect of exercise on HCM phenotypes.

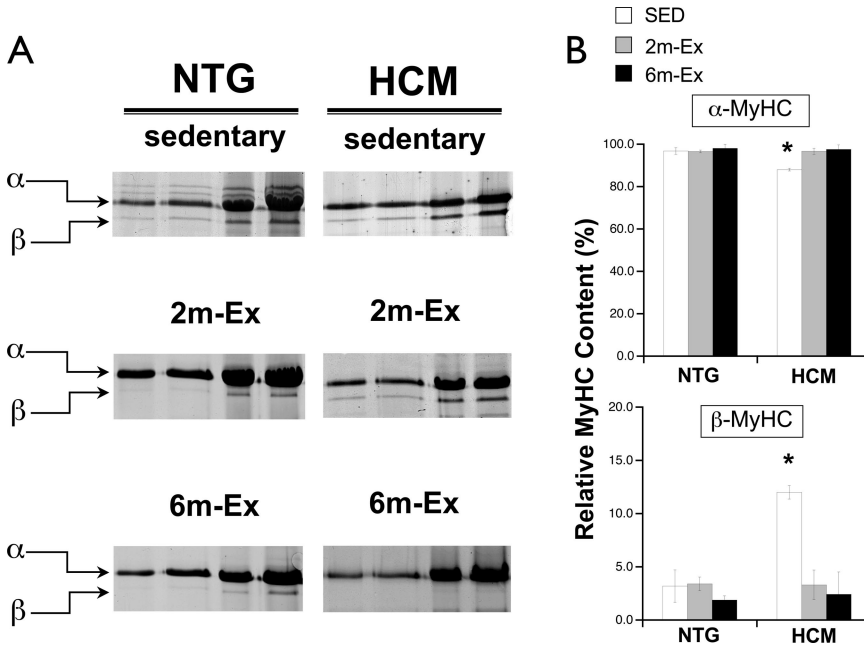
**Induction of Hypertrophic Markers Is Reduced by Exercise**

Pathologic cardiac hypertrophy and this model of HCM are characterized by the induction of genes normally expressed during fetal development such as β-MyHC and ANF.<sup>7,20</sup> In contrast, exercise induces expression of α-MyHC.<sup>21</sup> We aimed to determine whether exercise training prevented or reversed the induction of β-MyHC and ANF in HCM mice. An RPA was used for this purpose; an example of the raw data is displayed in Figure 4A. Consistent with previous data,<sup>7,8</sup> there was increased expression of ANF and β-MyHC mRNA in sedentary HCM mice (Figure 4B). In NTG mice, exercise did not affect the expression of hypertrophic mRNA markers. Likewise, 2m-Ex HCM mice were indistinguishable from HCM sedentary counterparts with respect to β-MyHC mRNA. However, 6m-Ex HCM animals expressed significantly less β-MyHC mRNA than sedentary and 2m-Ex HCM mice, an amount that was similar to NTG control levels. In both HCM exercise groups, voluntary cage wheel exposure lowered ANF expression to that of NTG sedentary controls.

To determine whether MyHC protein reflected RNA levels, MyHC isoform separating gels were performed. Figure 5A illustrates four of the mid-range dilutions demonstrating the ability to clearly separate α-MyHC and β-MyHC. Consistent with previous data from our laboratory,<sup>10</sup> β-MyHC represented 12.0±0.6% of total MyHC in sedentary HCM mice (Figure 5A). This value was significantly greater than NTG sedentary counterparts that expressed 3.2±1.5% of total MyHC as β-MyHC. Two or 6 months of exercise in NTG animals did not alter relative β-MyHC content (3.4±0.6% and 1.9±0.4%, respectively). In contrast, both exercise protocols significantly reduced the amount of β-MyHC protein expressed in HCM animals (3.3±1.4% and 2.4±2.1% for



**Figure 4.** Expression of hypertrophic mRNA markers in sedentary and exercised NTG and HCM animals by RPA. A, Representative RPA displaying expression of hypertrophic markers: ANF, α-MyHC and β-MyHC; GAPDH served as a loading control. B, Bar graph representation of each hypertrophic marker. Sed, NTG (n=3) or HCM (n=5) sedentary male mice; 2m-Ex, NTG (n=3) or HCM (n=4) male mice exercised for 2 months; 6m-Ex, NTG (n=4) or HCM (n=4) male mice exercised for 5 to 6 months. (\**P*<0.05 from measured values in HCM sedentary controls; a, *P*<0.05 from NTG sedentary mice; b, *P*<0.05 from NTG 2m-Ex; c, *P*<0.05 from NTG 6m-Ex).



**Figure 5.** Cardiac MyHC isoform expression in sedentary and exercised NTG and HCM animals. The relative amount of  $\alpha$ -MyHC and  $\beta$ -MyHC isoform expression was determined by SDS-PAGE. A, Representative silver-stained gels illustrating 4 dilutions of an individual sample in each of the experimental groups studied. B, (top panel) Bar graph representation of the relative  $\alpha$ -MyHC expression; (bottom panel) bar graph representation of the relative  $\beta$ -MyHC expression. Sed, NTG (n=3) or HCM (n=4) sedentary male mice; 2m-Ex, NTG (n=4) or HCM (n=6) male mice exercised for 2 months; 6m-Ex, NTG (n=3) or HCM (n=4) male mice exercised for 5 to 6 months. (\* $P < 0.05$  from measured values in NTG sedentary controls.)

2m-Ex and 6m-Ex, respectively). Figure 5B summarizes the relative percent of  $\alpha$ -MyHC (top panel) and  $\beta$ -MyHC (bottom panel) for each experimental group. Thus, based on these data, exercise in HCM animals prevented or reversed the induction of  $\beta$ -MyHC.

**Histopathology and Fibrosis Are Reduced by Exercise**

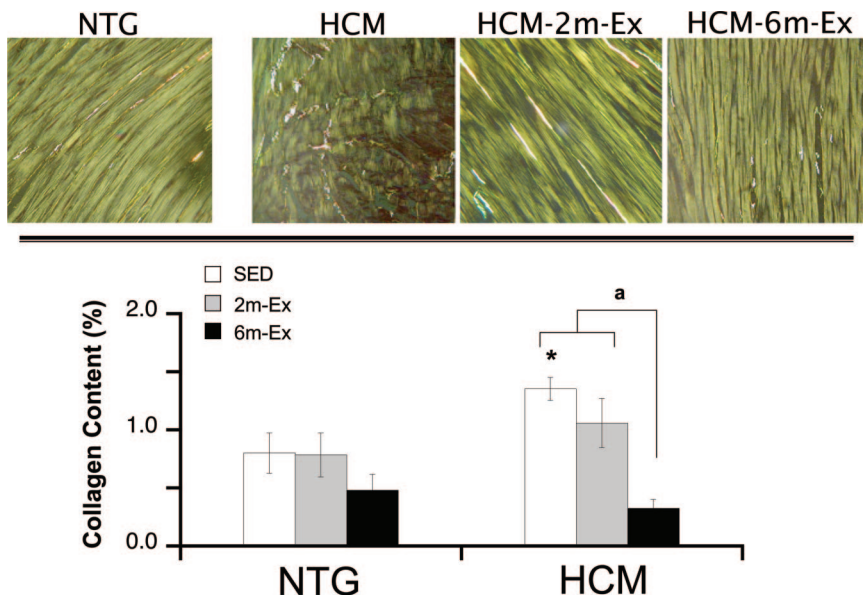
Fibrosis and myocellular disarray were evaluated in sedentary and exercised hearts by picrosirius red staining. Quantitative analysis for picrosirius red polarization (Figure 6) indicated significantly larger fractional area of collagen in HCM sedentary hearts ( $1.35 \pm 0.10\%$ ) compared with NTG sedentary hearts ( $0.80 \pm 0.17\%$ ). Collagen content in the hearts from HCM animals that began exercise at 2 months of age (HCM-6m-Ex) was significantly less ( $0.32 \pm 0.08\%$ ) than HCM sedentary coun-

terparts. There were no significant differences in collagen content in NTG-2m-Ex ( $0.78 \pm 0.19\%$ ), NTG-6m-Ex ( $0.48 \pm 0.14\%$ ), or HCM-2m-Ex ( $1.06 \pm 0.21\%$ ).

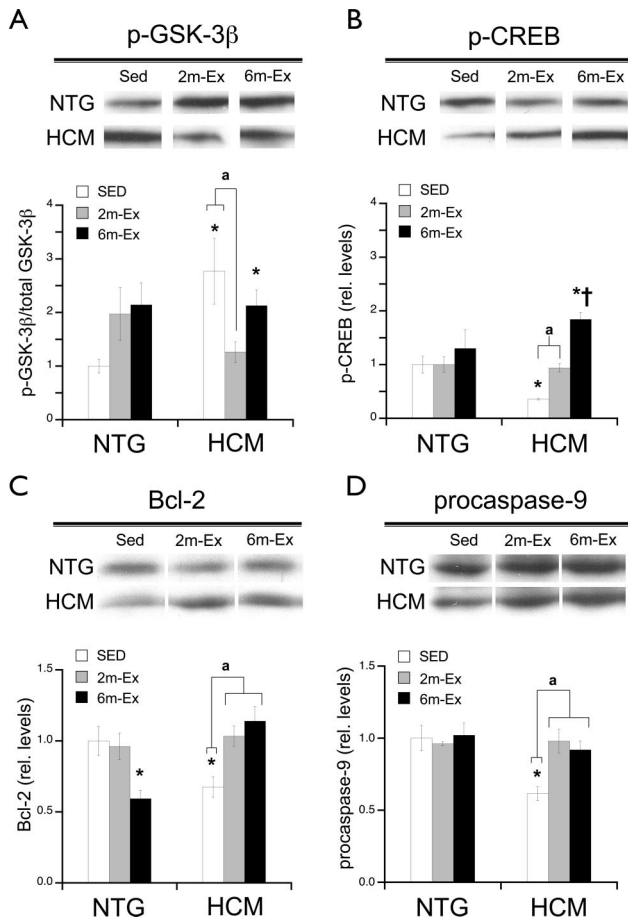
HCM sedentary hearts exhibited more pronounced myocellular disarray than NTG animals. Despite the continued presence of thick collagen fibers in the HCM 2m-Ex group, the disarray was considerably improved as indicated by the uniform myofilament birefringence. HCM mice in the 6m-Ex group were indistinguishable from NTG counterparts suggesting that exercise was able to prevent the typical myocellular disarray and fibrotic phenotype in HCM mice.

**Inactivation of GSK-3 $\beta$  and CREB in HCM Mice Is Reversed and/or Prevented by Exercise**

GSK-3 $\beta$  is a ubiquitous serine/threonine protein kinase that in the active, unphosphorylated state acts as a negative



**Figure 6.** Cardiac fibrosis in sedentary and exercised NTG and HCM animals. Collagen content was quantified in each of the experimental groups (top panel) and summarized in the bar graph (bottom panel). Collagen deposition is visualized by yellow/orange birefringence and myocellular uniformity is evident by green birefringence. (\* $P < 0.05$  from measured values in NTG sedentary controls; a,  $P < 0.05$  from HCM sedentary and HCM-2m-Ex mice).



**Figure 7.** Signaling intermediates in sedentary and exercised NTG and HCM animals. A, Ratio of phosphorylated GSK-3 $\beta$  to total GSK-3 $\beta$  protein. B, Relative levels of phosphorylated CREB protein (p-CREB). C, Relative levels of Bcl-2 protein. D, Relative levels of procaspase-9 protein. Sed, NTG (n=5) or HCM (n=5) sedentary male mice; 2m-Ex, NTG (n=3) or HCM (n=5) male mice exercised for 2 months; 6m-Ex, NTG (n=3) or HCM (n=4) male mice exercised for 5 to 6 months. (\* $P$ <0.05 from measured activity in NTG sedentary controls; † $P$ <0.05 from measured activity in HCM sedentary animals; a,  $P$ <0.05)

regulator of cardiac hypertrophy whereas phosphorylation leads to inactivation promoting cardiac hypertrophy.<sup>22,23</sup> We have previously shown that exercise inactivates GSK-3 $\beta$ .<sup>6</sup> The 3-fold elevated phosphorylation state of GSK-3 $\beta$  as a fraction of total GSK-3 $\beta$  in sedentary HCM mice was significantly reduced with 2 months of exercise (Figure 7A), a level that was equivalent to that of NTG-sedentary controls. The p-GSK-3 $\beta$ /total GSK-3 $\beta$  ratio in the HCM-6m-Ex group was similar to NTG-2m-Ex and NTG-6m-Ex animals.

Previous studies have provided evidence that the transcriptional activity of CREB is important for cell proliferation<sup>24</sup> and cardiac myocyte gene regulation and function.<sup>25</sup> In the heart, transgenic expression of a dominant-negative CREB induced a severe dilated cardiomyopathy characterized by both anatomical and hemodynamic disease consistent with CHF.<sup>25</sup> More importantly, the progressive deterioration in cardiac function of these mice could not be prevented by treadmill exercise, suggesting an integral role for CREB in exercise-mediated recovery.<sup>26</sup> Considering the potential role of CREB regulation in cardiac disease and exercise,<sup>25,26</sup> we measured the level of CREB phosphoryla-

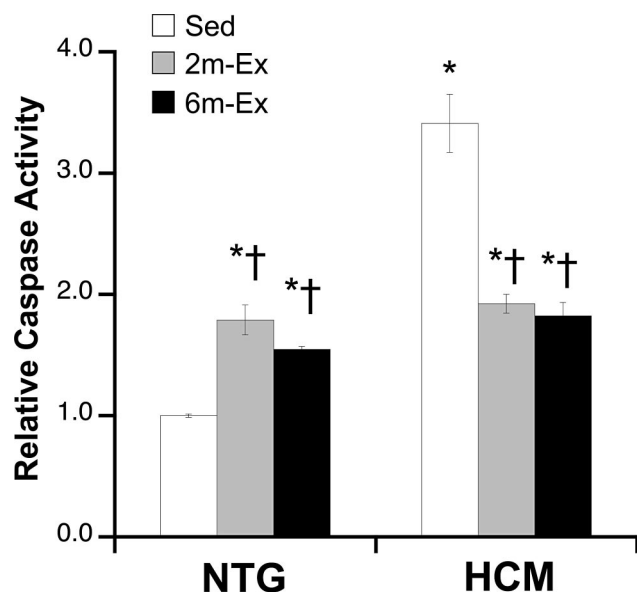
tion at the critical Ser133 residue by Western blot in HCM hearts before and after exercise. In HCM sedentary hearts, there was a 3-fold decrease in the phosphorylated, active form of CREB (p-CREB) compared with NTG counterparts (Figure 7B). Exercise returned the level of p-CREB to that of NTG-sedentary controls in the HCM-8 weeks-Ex group. Interestingly, the level of p-CREB in the HCM-6m-Ex group was  $\approx$ 2-fold higher than NTG-sedentary controls, an increase that was significantly different from all other groups.

### Apoptosis Is Inhibited by Exercise

CREB can also control Bcl-2 gene expression through CRE-responsive elements in the Bcl-2 promoter region.<sup>27</sup> Bcl-2 acts as an anti-apoptotic intermediate by preventing the release of mitochondrial membrane proteins, such as cytochrome *c*, that perpetuates the death signal.<sup>28</sup> Cytochrome *c* forms an activation complex with apoptotic protein-activating factor-1 and caspase-9 that activates downstream effector caspases including caspase-3. Therefore, we examined Bcl-2 and procaspase-9 (the zymogen of caspase-9) expression by Western blot analysis in sedentary and exercised NTG and HCM mice (Figure 7C and 7D). In HCM sedentary hearts, Bcl-2 expression was reduced by  $\approx$ 33%, consistent with the positive regulation of Bcl-2 expression by CREB activity (Figure 7C). Exercise in HCM animals (2m-Ex and 6m-Ex) returned Bcl-2 levels to those of NTG sedentary controls. The amount of procaspase-9 was significantly decreased in HCM sedentary mice compared with NTG sedentary mice suggesting an increased rate of procaspase-9 cleavage into the active form (Figure 7D). Similarly, the amount of procaspase-9 in exercised HCM animals (2m-Ex and 6m-Ex) was restored to levels not different from NTG sedentary controls. BAX expression, a pro-apoptotic protein, was not affected by cardiac disease or exercise (data not shown). These data suggest that there is less endogenous apoptotic inhibition in sedentary HCM animals and, thus, there is a propensity toward increased apoptotic activity in these animals. Exercise in HCM mice returned each signaling component to control levels suggesting a reversal of this pro-apoptotic process.

### Caspase-3 Activity Is Reduced by Exercise

To determine whether the Western blot data were consistent with apoptotic activity, whole heart homogenates from each experimental group were examined for apoptotic activity by monitoring the rate of cleavage of a fluorogenic caspase-3 specific substrate, and thus apoptotic activity.<sup>28</sup> In this study, caspase-3 activity was normalized to NTG sedentary animals and was used as a measure of relative apoptosis for each group. As illustrated in Figure 8, HCM sedentary animals exhibited 3.5-fold higher caspase-3 activity when compared with NTG sedentary controls. In NTG animals, each exercise protocol significantly increased caspase-3 activity over sedentary counterparts by 2- and 1.5-fold in 2- and 6-month exercised animals, respectively. It should be noted that the increase in caspase-3 activity with exercise in NTG animals was still lower than that of the HCM sedentary group. Caspase-3 activity measured in exercised HCM animals under either protocol was significantly reduced and/or attenuated (2-fold in 2m-Ex and 6m-Ex animals) when compared



**Figure 8.** Caspase-3 activity in sedentary and exercised NTG and HCM animals. Caspase-3 activity as an indicator of apoptosis was determined by monitoring the rate of cleavage of a fluorogenic caspase-3 specific substrate in each experimental group studied. Sed, NTG (n=13) or HCM (n=7) sedentary male mice; 2m-Ex, NTG (n=4) or HCM (n=9) male mice exercised for 2 months; 6m-Ex, NTG (n=4) or HCM (n=5) male mice exercised for 5 to 6 months. (\* $P < 0.05$  from measured activity in NTG sedentary controls; † $P < 0.05$  from measured activity in HCM sedentary animals).

with HCM sedentary mice but remained significantly elevated over NTG sedentary controls. There were no measurable differences in caspase-3 activity when a caspase-3 specific inhibitor in addition to a cocktail of protease inhibitors to the reaction was included in the reaction buffer for NTG and HCM sedentary animals (data not shown). These data demonstrate that (1) pro-apoptotic signaling was elevated in HCM sedentary animals as determined by a decrease in anti-apoptotic components and an increase in apoptotic activity markers, and (2) exercise was able to return apoptotic signaling to control levels.

### Discussion

In this study, we chose to measure parameters that are generalized characteristics of HCM and, therefore, these studies may be applicable to their human counterparts that share these common phenotypes. Furthermore, although we chose to examine cellular components hypothesized as integral to the HCM pathology/exercise recovery signaling axis, more comprehensive studies are needed to attribute causality to these potential mediators. Nevertheless, in our study, not only did moderate exercise in HCM mice prove to be beneficial but also it did not induce any sudden death events. The first observation of this study was that voluntary cage wheel performance was similar between NTG and HCM animals, despite a significant impairment in exercise tolerance by treadmill running.<sup>8</sup> This may not be surprising considering that treadmill exercise has a high stress component to it. Another important observation was that the hearts of HCM mice responded to each exercise protocol in a similar

manner as NTG animals (Figure 2), suggesting that exercise selectively activated a hypertrophic signaling pathway separate from that induced by the  $\alpha$ -MyHC mutation. One important distinction was that HCM animals with established hypertrophy (2m-Ex) responded to voluntary cage wheel running with the greatest relative increase in cardiac mass. This may indicate that the hearts of these HCM animals are at a basal state that is more *permissive* to cardiac hypertrophy as a result of the established pathology at this time point<sup>7,8,10,15</sup> and, thus, may initially include a greater compensatory increase in cardiac mass with exercise followed by a period of beneficial remodeling as demonstrated by the improved phenotype. This contention is supported by the increased phosphorylation status (inactivation) of GSK-3 $\beta$ , and thus removal of an inhibitory component of cardiac hypertrophy in HCM sedentary mice. Moreover, the HCM animals that began cage wheel exposure at 2 months of age, before the appearance of a pathologic phenotype, never reached the cardiac mass of HCM sedentary animals. This suggests that the exercise and disease pathways in these animals can be antithetical with respect to cardiac mass.

Along with an array of genes such as ANF, brain natriuretic peptide and  $\alpha$ -skeletal actin, the heart expresses  $\beta$ -MyHC in response to pathologic stressors.<sup>20</sup> In HCM sedentary animals, the significant elevation in  $\beta$ -MyHC mRNA and protein was decreased by exercise and accompanied by increased  $\alpha$ -MyHC protein. Interestingly, the RNA data suggest that the  $\alpha$ -MyHC mRNA is driving the stoichiometry of MyHC protein expression. In addition, exercise in HCM animals reduced ANF mRNA expression to that of NTG controls. Although the functional consequences of MyHC isoform switches in exercised HCM animals have not been examined, similar shifts in myosin isozyme content in hypothyroid or other transgenic-diseased mice result in a decrease in the time derivative of both pressure development (dP/dt<sub>max</sub>) and relaxation (dP/dt<sub>min</sub>) in isolated work-performing hearts<sup>29</sup> and intact closed-chest preparations.<sup>30</sup>

A hallmark of HCM regardless of etiology is interstitial fibrosis and myocellular disarray.<sup>1</sup> In this study, we show that fibrosis per se was not reduced by exercise in HCM mice with established histopathology (2m-Ex group) but that exercise improved myocellular organization. Previous studies showed that treadmill exercise training was not able to alter collagen content but was able to increase collagen turnover and alter collagen cross-linking and subtype in the aging rat.<sup>31</sup> It has been shown that improvements in the characteristics in collagen cross-linking lead to enhanced diastolic properties.<sup>32</sup> Perhaps more importantly, exercise before phenotypic presentation (6m-Ex group) prevented the characteristic HCM histopathology such that 6m-Ex HCM hearts were indistinguishable from their NTG-counterparts.

It has been previously demonstrated that pathologic cardiac hypertrophy is associated with increased transcriptional activity of NFAT and MEF2.<sup>16</sup> The reduction of NFAT activity, but not MEF2, strongly implicates NFAT as a mediator of pathologic hypertrophy.<sup>16,19</sup> Given the lack of an effect of exercise on NFAT activity in NTG animals, this reduction in HCM animals attributable to exercise may be an indication that these hearts are returning to a nonpathologic state.

The activation of NFAT in HCM sedentary hearts is consistent with the inactivation (increase in relative phosphorylation) of GSK-3 $\beta$ .<sup>22,23</sup> Because nuclear translocation of NFAT is dependent on its phosphorylation status, a decrease in GSK-3 $\beta$ -dependent phosphorylation could make the non-phosphorylated NFAT more readily available for entrance into the nucleus. Interestingly, in short-term (2 month) HCM runners, the amount of phosphorylated (inactive) GSK-3 $\beta$  was significantly reduced to NTG sedentary levels suggesting perhaps an inhibition or reversal of the HCM phenotype. However, the GSK-3 $\beta$  phosphorylation pattern in long-term (5 to 6 months) HCM runners mimicked that of NTG runners, suggesting that the inactivation (phosphorylation) of GSK-3 $\beta$  in these hearts is more related to exercise than HCM pathology. One potential explanation is that both pathologic and physiologic stimuli can result in GSK-3 $\beta$  phosphorylation by different mechanisms. In addition, NFAT activity levels were lower in HCM exercised animals, despite this increase in GSK-3 $\beta$  phosphorylation. These data indicate that the regulation of GSK-3 $\beta$  is dependent on multiple factors. One of these factors may be related to the phosphorylation status of Akt (protein kinase B),<sup>22</sup> which we have previously shown to be regulated by exercise.<sup>6</sup> Consistent with GSK-3 $\beta$  phosphorylation, the ratio of phosphorylated Akt to total Akt was reduced in HCM-2m-Ex hearts compared with NTG and HCM sedentary controls and HCM-6m-Ex (see supplemental data). Moreover, as evidenced in the NTG exercised mice, GSK-3 $\beta$  and NFAT activity can be uncoupled during a physiologic stimulus.

It is also becoming evident that cardiac disease progression, with decreasing left ventricular pump function and wall thinning/dilation, can be hastened and/or caused by an increase in the rate of myocardial cell loss, or apoptosis. In fact, high levels of apoptosis have been demonstrated in the failing human heart in some studies<sup>33</sup> and more moderate amounts have been published in other studies.<sup>34,35</sup> It has been shown that levels of apoptosis that are 4- to 10-fold lower than those observed in failing human hearts can lead to profoundly detrimental morphological and physiologic cardiac defects in a mouse model.<sup>36</sup> In this study, we asked whether mediators of apoptosis were activated in HCM hearts and, if so, whether exercise would reduce them.

As mentioned, CREB possesses a dual role as negative regulator of cardiac remodeling and apoptosis.<sup>25,27</sup> Reduced CREB activity indicated by lower levels of p-CREB in HCM sedentary hearts supports this contention. This pattern of reduced p-CREB in pathologic hypertrophy has been observed in multiple rodent models of cardiac hypertrophic disease (Watson, in review). Moreover, this reduction in p-CREB is consistent with the lower expression of Bcl-2, an anti-apoptotic factor, in HCM sedentary animals compared with NTG controls. We also showed increased caspase-9 cleavage/activity (further downstream in apoptotic signaling) indicated by lower levels of procaspase-9, the pro-peptide of caspase-9. In turn, caspase-9 can propagate apoptotic signaling by activating downstream effector caspases, including caspase-2, -3, -6, -7, -8, and -10.<sup>28</sup> The functional consequence of elevated caspase-9 activity was demonstrated by an increased rate of cleavage of a caspase-3-specific fluorescent

peptide that was 3-fold higher in HCM sedentary animals compared with NTG sedentary controls. Taken together, these results are consistent with apoptosis being a central mediator in the pathogenesis of HCM in this model. More importantly, exercise was able to reverse the apoptotic phenotype in HCM animals to that of NTG controls.

### Acknowledgments

This work was supported by NIH grant to L.A. Leinwand (HL 56510) and by a National Research Service Awards from the NIH awarded to J.P. Konhilas (F32 HL 70509), S.W. Luckey (F32 HL 72565), and B.L. Stauffer (F32 HL 67543). We are grateful to Genevieve Sparagna, PhD for aid with the caspase-3 activity assay and to Karin Nunley for aid with the RNase protection assays. We also thank Premier Histology (Boulder, Col) for their help with the picrosirius red staining.

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