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Sex modifies exercise and cardiac adaptation in mice

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Abstract

How an individual's sex and genetic background modify cardiac adaptation to increased workload is a topic of great interest. We systematically evaluated morphological and physiological cardiac adaptation in response to voluntary and forced exercise. We found that sex/gender is a dominant factor in exercise performance (in two exercise paradigms and two mouse strains) and that females of one of these strains have greater capacity to increase their cardiac mass in response to similar amounts of exercise. To explore the biochemical mechanisms for these differences, we examined signaling pathways previously implicated in cardiac hypertrophy. Ca^{2+}/calmodulin-dependent protein kinase (CaMK) activity was significantly greater in males compared with females and increased after voluntary cage-wheel exposure in both sexes, but the proportional increase in CaMK activity was twofold higher in females compared with males. Phosphorylation of glycogen synthase kinase-3β (GSK-3β) was evident after 7 days of cage-wheel exposure in both sexes and remained elevated in females only by 21 days of exercise. Despite moderate increases in myocyte enhancer factor-2 (a downstream effector of CaMK) transcriptional activity and phosphorylation of Akt with exercise, there were no sex differences. Mitogen-activated protein kinase signaling components (p38 mitogen-activated protein kinase and extracellular regulated kinase 1/2) were not different between male and female mice and were not affected by exercise. We conclude that females have increased exercise capacity and increased hypertrophic response to exercise. We have also identified sex-specific differences in hypertrophic signaling within the cardiac myocyte that may contribute to sexual dimorphism in exercise and cardiac adaptation to exercise.

Keywords

hypertrophy; myocyte signaling; workload; glycogen; calmodulin

Despite increasing knowledge of the mechanisms of cardiac adaptation to increased workload, there are significant sex/gender differences that remain poorly understood. For example, in response to pathological stimuli such as systemic hypertension or aortic stenosis, women respond with more left ventricular hypertrophy and augmented contractility than men (51, 54), whereas men progress to poor contractility, chamber dilation, and wall thinning (7,11, 24,39,50). Yet in the face of ischemic heart disease, females fare worse than their male counterparts (12,14,21).

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Physiological stimuli such as exercise can also elicit a sexually dimorphic cardiac response. Despite conflicting reports on the functional impact of exercise on the heart, it is apparent that there is differential remodeling between the sexes in response to aerobic exercise (16,49). Moreover, the sexually dimorphic cardiac response to exercise likely depends on the type of activity performed (aerobic vs. anaerobic, chronic vs. acute) as demonstrated in exercise-trained male and female rats. Male and female animals exercised by treadmill running have similar heart mass compared with sedentary controls (42,44). In both sexes, run training causes hypertrophy when heart weight is normalized for body weight. However, the hearts of male rats adapt to treadmill training with improved intrinsic performance, whereas the hearts of female rats do not (42). Compared with males, swim-trained female rats exhibit a substantial increase in absolute heart mass that is associated with an increase in tension development in both an isolated papillary preparation and a whole heart model (31).

Although the mechanisms that underlie these phenotypical variations between the sexes are presently unresolved, it is clear that there are strong genetic modifiers to cardiac adaptation and exercise capacity (17,25,46). Candidates for these modifiers are likely to lie in known hypertrophic signaling pathways within the cardiac myocyte. For example, the serinethreonine protein kinase Akt [or protein kinase B (PKB)] when expressed as a transgene in constitutively active form in the heart can induce hypertrophy that is greater in females than males when normalized to body weight (27). The active, phosphorylated form of Akt (phospho-Akt) is increased in hearts of young women compared with age-matched men (5-fold) and postmenopausal women (16-fold). A twofold increase in nuclear myocardial phospho-Akt (but not total Akt) is seen in female mice compared with age-matched males and is associated with a concomitant increase in Akt kinase activity (6). Moreover, there is interplay between sex-specific factors such as estrogen and signaling pathways. This is illustrated by a study in which nuclear localization of Akt was induced by an estrogen receptor agonist in vitro (6). Recently, it was shown that the α_1-adrenergic system is critical in determining heart size and in the ability of the heart to respond to both pathological and physiological stimuli, but only in male mice. Intriguingly, this sex difference does not disappear after ovariectomy of females (38).

A coherent analysis of the potential mediators of sex differences in the heart is strongly justified. We wished to determine whether sex was the dominant factor over type of exercise, age, and strain. Sex- and strain-specific differences in exercise performance and cardiac adaptation are demonstrated here under conditions of increased physiological workload using both voluntary and involuntary running paradigms. We employed these different paradigms to decrease the impact of behavior on exercise performance. We show that sex is a dominant factor (over exercise paradigm, age, and genetic background) in exercise performance. We explored the basis for these differences by examining cardiac hypertrophic signaling molecules that have been implicated in physiological, pathological, or sex-specific regulation in sedentary and exercised wild-type male and female mice (3,6,27,38). These include mitogen-activated protein kinases (MAPKs), glycogen synthase kinase-3β, and Akt. Because Ca^{2+} handling plays a central role in normal myocyte function, we also measured Ca^{2+}/calmodulin-dependent protein kinase (CaMK) activity and one of the many targets of CaMK, myocyte enhancer factor-2 transcription factor (MEF-2). Accordingly, we have identified signaling pathways that may contribute to these observed differences.

METHODS

All experiments were performed according to institutional guidelines concerning the care and use of experimental animals.

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Voluntary cage-wheel exercise

Voluntary running was performed by inbred male and female C57BL/6J and FVB/NJ mice that were 12 wk old at the start of cage-wheel exposure. Individual animals were housed in a cage (47 × 26 × 14.5 cm) that contained a free wheel for 7, 10, or 21 days. The exercise wheels used have been previously described (1). Briefly, this system consists of an 11.5-cm-diameter wheel with a 5.0-cm-wide running surface (model 6208; PetsMart; Phoenix, AZ) equipped with a digital magnetic counter (model BC 600; Sigma Sport; Olney, IL) that is activated by wheel rotation. For a given litter, mice were randomly assigned to the particular exercise duration or sedentary littermate control. All animals were given water and standard pelleted rodent feed ad libitum. Daily exercise values for time and distance run were recorded for each exercised animal throughout the duration of the exercise period. At the end of the specific exercise period, exercised and sedentary control mice were euthanized by cervical dislocation under inhaled anesthesia. Body mass was measured and hearts were rapidly excised and washed with a modified ice-cold PBS solution that contained (in mmol/l) 136.9 NaCl, 3.35 KCl, 12 NaH_2PO_4, and 1.84 KH_2PO_4 (pH 7.4). Hearts were frozen in isopentane cooled in liquid nitrogen or used fresh for biochemical assays.

Treadmill exercise tests

Mice were exercised in the absence of light on an eight-lane treadmill with adjustable belt speed (0–50 m/min) as previously described (25). The treadmill apparatus was equipped with adjustable amperage (0–2 mA) shock bars at the rear of the belt to stimulate each mouse to run, and an air gun was used as an additional stimulus when mice attempted to rest on the shock grid. A double-beam infrared photon detector located above the shock grid allowed for quantification of the number of shock stimuli received by each mouse. Over a 2-wk period, mice were acclimated to the treadmill via three 15-min running sessions at a 7° incline as follows: *1*) no shock activation and 2 m/min belt speed; *2*) mild shock stimulation and 5 m/min belt speed; *3*) high shock stimulation and 15 m/min belt speed. After acclimation, two exercise tests were performed: an endurance exercise tolerance test and an exercise stress test. The endurance exercise test consisted of a 30-min treadmill run at 20 m/min with a 7° incline. During the test, the number of beam breaks per minute was recorded and used as an indication of a mouse's ability to sustain the required workload. Thus for this test, a high number of beam breaks per minute is indicative of poor performance. This test was used as an indicator of muscle endurance and the ability of the physiological systems to contend with a prolonged, constant exercise stimulus. Each mouse was tested three times (2–3 days between each test), and the average values across all exercise sessions for each mouse were calculated. After completion of the exercise tolerance tests, mice were subjected to a graded exercise stress test. Such tests consist of an incremental protocol with increasing workloads and are commonly used to screen for cardiovascular disease and evaluate cardiovascular fitness. This test began with a 7° incline and a 20-m/min belt speed. Belt speed was then increased linearly by 1.5 m/min every 2 min until 45 m/min were reached. "Failure" was defined as the mouse's inability to continue regular treadmill running despite the extra stimulus of pressurized nitrogen from an air gun. Upon completion of the treadmill tests, the mice were killed as described above.

CaMK II and LacZ activity assays

Freshly excised or frozen hearts were mechanically disrupted in ice-cold buffer solutions. For the CaMK assays, buffer contained (in mmol/l) 50 Tris(hydroxymethyl)-aminomethane, 0.5 EGTA, 1 EDTA, and 0.5 dithiothreitol (pH 7.0); for the β-galactosidase (β-gal) assays, 40 Tris (hydroxymethyl)-aminomethane, 150 NaCl, and 1 EDTA. Each of the buffers also contained (in mmol/l) 0.1 leupeptin, 0.1 pepstatin, and 0.1 phenylmethylsulfonyl fluoride to prevent nonspecific proteolysis. The homogenized tissue was then centrifuged at 10,000 *g* (Beckman J2-HS centrifuge) for 5 min at 4°C. CaMK II activity was performed by means of SignaTECT

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CaMK II assay system (Promega; Madison, WI). β-Galactosidase assays (β-gal; Galacto-*Star*; Tropix; Bedford, MA) were performed on cardiac extracts from MEF-2-indicator mice as described previously (30) under conditions of linearity with respect to time and protein concentration.

Western blot analysis and gel electrophoresis

Preparation of heart samples for SDS-PAGE and subsequent Western immunoblotting for detection of proteins and particular sites of phosphorylation on each protein began by homogenization of whole hearts (minus the atria) in a protein extraction buffer that contained 137 mmol/l NaCl, 20 mmol/l Tris(hydroxymethyl)-aminomethane, 10% (vol/vol) glycerol, and 1% (vol/vol) Nonidet P-40 (pH 7.4) (48). The buffer also contained (in mmol/l) 0.1 leupeptin, 0.1 pepstatin, and 0.1 phenylmethylsulfonyl fluoride (to prevent nonspecific proteolysis) and 1 sodium pyrophosphate and 1 sodium vanadate (to prevent nonspecific phosphorylation or dephosphorylation, respectively). The homogenized tissue was then centrifuged at 12,000–14,000 *g* (Beckman J2-HS centrifuge) for 10 min at 4°C. The supernatant was removed, and protein concentration was determined using the Bradford method. SDS-PAGE was performed on the heart extracts followed by the transfer to a membrane support (polyvinylidene difluoride). The membranes were probed according to specifications with antibodies specific for Akt, GSK-3β, and MAPK components [extracellular regulated kinase 1/2 (ERK1/2), c-Jun NH_2-terminal kinase, and p38 MAPK] including the phosphorylated form of each respective protein. All antibodies were obtained commercially from Cell Signaling Technology (Beverly, MA) except the antibodies specific for GSK-3β and phosphorylated GSK-3β (phospho-GSK-3β), which were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Data and statistical analysis

Results are presented as means ± SE. The percent change in cardiac mass with exercise was determined by comparing the mass of each exercised animal to the mean cardiac mass of the sedentary group. The difference in cardiac mass was then expressed as a percent change from sedentary animals for each respective animal. Similarly, cardiac mass normalized to cage-wheel activity was determined by subtracting the cardiac mass of each exercised animal from the mean cardiac mass of the sedentary group; this value was then normalized to the cage-wheel activity of each corresponding animal. The differences between male and female groups were analyzed with one-way ANOVA; to determine age-specific differences between male and female mice, two-way ANOVA was performed with sex and age as independent factors. $P < 0.05$ was considered as significant.

RESULTS

Exercise performance

Female C57BL/6J (4 mo old) mice exhibited enhanced wheel-running performance over their male counterparts as measured by both distance and duration. As illustrated in Fig. 1*A*, female C57BL/6J mice (4 mo old) ran an average of 5.26 ± 0.4 km, whereas male mice ran 3.43 ± 0.4 km during each 24-h period. To complete these distances, females spent 4.11 ± 0.2 h on the wheel, which is a significantly longer duration than the 2.99 ± 0.3 h spent on the wheels by the males during each 24-h period (data not shown). This resulted in significantly elevated wheel-running speeds (1.22 ± 0.04 km/h) in female mice over male mice (1.10 ± 0.03 km/h; Fig. 1*B*) when averaged over the 3-wk time period. The enhanced performance of females compared with males remained evident in older but still premenopausal animals (12–15 mo old), which suggests that the sex/gender difference in exercise performance is not an age-specific phenomenon (Fig. 1*A*). It should be noted that performance in 12- to 15-mo-old animals was less than that of 4-mo-old animals of both sexes, yet the proportional differences in both time

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and distance run between sexes increased from ~1.4-fold in the younger animals to ~2.0-fold in the older animals.

A previous study by our group demonstrated a lack of correlation between voluntary wheel performance and treadmill performance in seven strains of male mice including C57BL/6J mice (25). Therefore, to examine whether the enhanced exercise of females persisted across exercise paradigms, we examined both endurance and stress treadmill performance as described previously (25). In the treadmill endurance test, female mice performed significantly better than their male counterparts as assessed by the number of times per minute the mice broke a beam of light at the rear of the treadmill (10.3 ± 1.4 vs. 19.8 ± 3.0 beam breaks/min). For the treadmill-based exercise stress test, mean female maximum speed was also significantly greater than male maximum speed (33.9 ± 1.33 vs. 24.7 ± 0.4 m/min; Fig. 1*C*).

We also wished to determine whether the sex difference in exercise transcended mouse strains (8,17,22,25). We therefore exposed 4-mo-old animals of a second mouse strain, FVB/NJ, to the identical free wheel exercise paradigm. As illustrated in Fig. 1*D*, despite quantitative increases in duration and distance in both sexes of FVB/NJ compared with C57BL/6J mice, there was a similar sex/gender disparity as demonstrated by elevated exercise performance in female FVB/NJ compared with male mice (Fig. 1*D*). Similarly, wheel-running speeds were greater in female than male mice (1.58 ± 0.2 vs. 1.06 ± 0.05 km/h).

Cardiac mass and adaptation

Given the enhanced running performance in females, we wished to determine whether there was also a sexually dimorphic adaptive response to voluntary cage-wheel exercise in the myocardium. The adaptation of the mouse heart to voluntary cage-wheel exercise is noted by an increase in cardiac mass (1). The results of these investigations are summarized in Table 1 and are graphically depicted in Fig. 2. The absolute heart mass of male C57BL/J6 and FVB/NJ sedentary mice was significantly greater than that of female mice. Cardiac mass values after cage-wheel activity significantly increased in both male and female C57BL/J6 and FVB/NJ mice compared with each respective sedentary control group (Table 1; Fig. 2*A*). Because body weights were significantly greater in male mice compared with female mice, heart weight was normalized to body weight (HW/BW, in mg/g). Exercise significantly increased the HW/BW ratio in both sexes and in strains from each respective sedentary control group (Fig. 2*B*; Table 1). In a subset of C57BL/J6 mice, heart weight was normalized to both body weight and tibial length. In this group of animals, there were identical changes in cardiac mass and HW/BW ratio. Similarly, when heart weight was normalized to tibial length (in mg/mm), there was a significant increase after exercise in both males (7.2 ± 0.2 vs. 8.1 ± 0.2 mg/mm) and females (6.0 ± 0.2 vs. 7.3 ± 0.4 mg/mm).

To better compare the magnitude of hypertrophy, we calculated the percent increase in cardiac mass. As shown in Fig. 3*A*, the percent increase in cardiac mass was significantly greater in female C57BL/J6 mice compared with their male counterparts (15.9 ± 2.0 vs. 5.0 ± 1.6%). In addition, as in C57BL/J6 mice, cardiac hypertrophy after exercise in FVB/NJ mice was even more pronounced in females (24.2 ± 5.4%) than in males (9.0 ± 2.2%; Fig. 3*A*). Given the enhanced exercise performance of female mice, the increase in cardiac mass was normalized to cage-wheel activity. Adjusting for time on the wheel, female C57BL/6J mice showed a significantly greater increase in heart mass for every hour of activity than males (4.0 ± 0.6 vs. 1.9 ± 0.6 mg of HW/h of activity) and for every kilometer of distance run (3.3 ± 0.5 vs. 1.6 ± 0.6 mg of HW/km) during each 24-h period (Fig. 3*B*; *top*). When the gain in cardiac mass was normalized to cage-wheel activity in FVB/NJ mice, females demonstrated no significant differences when normalized to time on the wheel (3.8 ± 1.0 vs. 2.8 ± 0.6 mg of HW/h of activity in a 24-h period) and/or distance run (2.3 ± 0.4 vs. 2.6 ± 0.6 mg of HW/km in a 24-h period; Fig. 3*B*, *bottom*).

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Ca^{2+}-dependent signaling

Little is known about either the mediators of cardiac hypertrophy in response to a physiological stimulus or the molecular players responsible for cardiovascular phenotypical differences between the sexes. To address both of these issues, signaling molecules previously shown to be mediators of the hypertrophic response were examined. Because CaMK II activity has been associated with cardiac hypertrophy (55), we tested the hypothesis that CaMK II activity would change with exercise and differ between the sexes. CaMK II activity in female sedentary mice (4.48 ± 0.77 pmol·min^{-1} ·μg protein^{-1}) was significantly lower than in sedentary male mice (8.34 ± 0.37 pmol·min^{-1} ·protein μg^{-1};Fig. 4*A*). Although mice exposed to a cage wheel for 7 days do not have hypertrophied hearts (1), hypertrophic signals important for the induction of cardiac hypertrophy are evident at very early time points in myocardial cells exposed to a hypertrophic stimulus (41). Consequently, CaMK II activity was measured after 7 days of cage-wheel exposure. Exercise significantly increased CaMK II activity to 10.40 ± 1.06 and 6.67 ± 0.69 pmol·min^{-1} ·μg protein^{-1} in male and female mice, respectively, while maintaining the significant difference between the sexes. Although the absolute increase in CaMK II activity was not different between the sexes, the relative increase was twofold greater in female mice (48.9 ± 15.3%) compared with male mice (24.7 ± 12.7%). CaMK II activity measured after 21 days of cage-wheel exposure was significantly reduced below sedentary levels in both males and females (3.2 ± 0.2 and 3.3 ± 0.2 pmol·min^{-1} ·μg protein^{-1}, respectively). Moreover, there was no longer a difference between the sexes.

Although multiple downstream targets exist for CaMK, CaMK signaling activates transcription factors of the MEF-2 family (40). MEF-2 has been associated with pathological hypertrophy (33,40). Although it has been demonstrated that MEF-2 activity increases in skeletal muscle after a physiological stimulus (52), MEF-2 activity has never been reported in heart after a physiological stimulus such as exercise. Because MEF-2 activity is difficult to measure directly, we utilized a previously described transgenic mouse line that harbors a LacZ transgene under the transcriptional control of three MEF-2 consensus DNA-binding sites (5). In this mouse line, LacZ is only expressed in tissues with MEF-2 activity (brain andskeletal and cardiac muscle), and MEF-2 is virtually inactive in the normal adult heart. We could then measure MEF-2 activity by measuring β-gal after exercise in mouse hearts in vivo (5,23,36). These data are displayed in Fig. 4*B*. MEF-2 activity as measured by the level of β-gal in sedentary and exercised male hearts was not different from that of female hearts at any time point studied. In males, exercise increased MEF-2 activity modestly after 7 and 10 days of cage-wheel exposure, and this elevation achieved statistical significance at 10 days only (133.5 ± 46.2 and 215.1 ± 47.1 pg of β-gal/mg of tissue, 7 and 10 days, respectively) over sedentary controls (98.3 ± 10.8 pg of β-gal/mg of tissue). In female mice, exercise significantly increased MEF-2 activity measured after 7 and 10 days of cage-wheel exposure (187.1 ± 43.5 and 196.2 ± 47.5 pg of β-gal/mg of tissue; 7 and 10 days, respectively) over sedentary controls (85.8 ± 9.2 pg of β-gal/mg of tissue). Interestingly, after 21 days of cage-wheel exposure, MEF-2 activity was attenuated to that of sedentary control animals in both male (98.1 ± 11.1 pg of β-gal/mg of tissue) and female (104.3 ± 9.9 pg of β-gal/mg of tissue) mice.

Akt, GSK-3β, and MAPK

To examine differences in intracellular signaling molecules and posttranslational modifications of these molecules in sex-specific and exercise-induced hypertrophy, we performed Western blots from whole cardiac extracts of Akt (PKB), phospho-Akt, GSK-3β, and phospho-GSK-3β and aspects of the MAPK pathway. Akt protein levels in C57BL/6J mice were not affected by exercise or sex (Fig. 5*A*). However, after 7 days of cage-wheel exposure, the levels of phosphorylated or activated Akt (phospho-Akt) were elevated (2.3- and 2.0-fold higher in female and male runners, respectively) over their sedentary counterparts (Fig. 5*B*). The amount of phospho-Akt was not different between the sexes. Twenty-one days of cage-wheel exposure

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returned the levels of phospho-Akt to those of sedentary mice in both females and males (Fig. 5*B*). Because Akt is a principal kinase that phosphorylates the inhibitory site of GSK-3β (10), GSK-3β and the phosphorylation state of GSK-3β were also analyzed. Similar to Akt, the level of GSK-3β protein was not affected by cage-wheel exposure or sex at any time point compared with sedentary control animals (Fig. 5*A*). Cage-wheel exposure for 7 days increased the amount of phospho-GSK-3β by 2.6-fold in female mice and 2.1-fold in male mice relative to each respective sedentary control group (Fig. 5*B*). After 21 days of cage-wheel exposure, the levels of phospho-GSK-3β were attenuated in male mice to that of sedentary males, whereas the level of phospho-GSK-3β remained elevated in female mice (2.1-fold increase). Neither cage-wheel exposure nor sex altered levels of p38 MAPK or ERK1/2 from those of sedentary control mice as determined by immunoblotting. Furthermore, the phosphorylation states of p38 MAPK and ERK1/2 were not different between the sexes and were unaltered by cage-wheel exposure (Fig. 5).

DISCUSSION

Multiple elements contribute to aerobic exercise capacity including the physiological influence of the cardiovascular, musculoskeletal, and autonomic nervous systems. Perhaps because of this complexity, studies of exercise capacity have led to conflicting results. For example, early reports on rats demonstrate that male and female rats trained by either swimming or running regimens have no increase in heart mass over sedentary control animals, whereas other studies show a significant increase in cardiac mass in females (9,31,42–44). C57BL/6J female mice respond to swim training with an increase in cardiac mass and HW/BW ratio (20). These studies are undoubtedly influenced by differences in age and strain of animals used and differences in exercise paradigms. Thus far, only males have been examined for cardiac adaptation in response to voluntary cage-wheel exposure (1). In that study, males significantly increased heart mass when exposed to a cage wheel (1). A previous study performed with a strain of ICR mice selected for increased wheel-running behavior demonstrates enhanced free wheel exercise performance for both duration and distance in female mice compared with male mice, but that study did not examine cardiac adaptation (18). Therefore, this is the first study to examine cardiac adaptation between two sexes and the pathways underlying it. Furthermore, this study demonstrates that sex/gender has a profound and significant influence on exercise capacity in two distinct exercise paradigms, in two strains of mice, and at two disparate ages.

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Involuntary and voluntary running paradigms both have strong behavioral components, but they are controlled by separate sets of genes as demonstrated by the lack of correlation between running paradigms in seven different mouse strains (25). Our data show that the sex difference in exercise performance clearly transcends the type of exercise. Furthermore, the fact that the findings were consistent between two strains of mice minimizes the contribution of genetic background as a modifier of the sex difference. Thus it is likely that the enhanced aerobic performance in females is largely due to differences in physiological capacity. Because exercise capacity is higher in females at onset of exercise, there is a difference in basal exercise capacity.

Although male and female mice enlarge cardiac mass in response to cage-wheel exposure, female mice of both strains exhibit a greater percent increase in cardiac mass than their male counterparts (see Fig. 3*A*). When the gain in cardiac mass was normalized for cage-wheel activity, C57BL/6J females have an augmented hypertrophic response for a given physiological stimulus compared with males (see Fig. 3*B*). This analysis also suggests that the female cardiac response of the FVB/NJ strain is proportionately less per unit of exercise/activity than that of C57BL/6J mice. However, it appears that exercise performance is not dependent on the extent of cardiac hypertrophy when animals were analyzed individually. Exercise performance may, however, be dependent on the presence of estrogen in females as demonstrated by reduced wheel running in ovariectomized mice (A. Maass, unpublished observations) and, similarly,

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in rats (19). Furthermore, female mice have enhanced exercise performance at the outset, as demonstrated by enhanced running speeds (see Fig. 1*B*) compared with males. Taken together, these data suggest that it is unlikely that the more profound cardiac adaptation in females is the basis for their enhanced aerobic capacity or performance over males and is most likely due to intrinsic differences in both heart and skeletal muscle. Moreover, both strains of female mice exhibited a hypertrophic response that was 2.5- to 3-fold greater than the respective males, which suggests that the sex-specific difference in hypertrophic capacity is dependent more on sex than strain and that this is indicative of sexually dimorphic hypertrophic mechanisms.

To probe for potential mechanisms underlying these observations, we examined signaling factors that have been implicated in cardiac hypertrophy within the cardiac myocyte. It has been demonstrated that one of the primary signaling pathways whereby cardiac muscle increases myocyte size is the Ca^{2+}/calmodulin-dependent system. Activated CaMK targets cellular proteins involved in Ca^{2+} handling in the cardiac myocyte and has been implicated as an integral component in hypertrophic signaling. Constitutive activation of CaMK IV (40) or overexpression of CaMK II (55) in heart results in significant cardiac hypertrophy. Although implicated in skeletal muscle hypertrophy (2,13) and pathological cardiac hypertrophy, the role of CaMK in physiological hypertrophy has not been elucidated.

Here, we demonstrated that the hearts of male sedentary mice have elevated CaMK activity compared with sedentary females. In both males and females, 7 days of exercise increased CaMK activity. Although the absolute increase in CaMK activity after exercise was similar among the sexes, the proportional increase in CaMK activity in females was twofold greater than that of males. This corresponds with the augmented hypertrophic response in female mice compared with male mice after an exercise stimulus. Accordingly, we examined the relationship between heart size and CaMK activity (Fig. 6). Absolute heart weight and CaMK activity correlate tightly (r= 0.994), which suggests that CaMK is an important agent of exercise-induced cardiac hypertrophy, and it may be important in determining basal cardiac size. In addition, data in Fig. 6 are consistent with the idea that male mice may be limited in their extent of cardiac hypertrophy compared with female mice, and that this may be dependent on CaMK activity. Interestingly, when CaMK activity was measured after 3 wk of cage-wheel exposure, the levels decreased in male and female mice to below those of sedentary mice, which suggests that CaMK may be important for the induction but not the maintenance of cardiac hypertrophy.

The mechanism by which CaMK induces hypertrophy is thought to operate at least partially through activation of MEF-2 (26,28,40). To test whether the phenotypical differences and the exercise-induced cardiac hypertrophy in males and females is mediated through the MEF-2 family of transcription factors, MEF-2 activity was measured by MEF-2- dependent expression of β-gal. MEF-2 activity was not different between sedentary males and females. However, there was a modest although significant elevation in MEF-2 activity after 7 and 10 days of cage-wheel exercise concomitant with an increase in CaMK activity after 7 days of exercise in both males and females. Unlike CaMK activity, there remained a lack of effect of sex on MEF-2 activity. Upon completion of the 21-day exercise protocol, MEF-2 activity levels returned to those of sedentary control animals and correlated with the decrease in CaMK activity. Nevertheless, the augmentation in MEF-2 activity was 2-fold at best, which is far below the 100-fold elevations reported in the CaMK IV overexpression model of cardiac hypertrophy (40). It appears, then, that CaMK activity may be partially uncoupled from MEF-2 activation during physiological hypertrophy, which suggests that the induction of cardiac hypertrophy by exercise is potentially mediated through alternative transcriptional targets, although a role for MEF-2 cannot be completely eliminated based on these data.

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It is likely, however, that signaling pathways in addition to CaMK are contributing to physiological cardiac adaptation. Previous studies have highlighted the importance of the MAPKs as positive regulators of cardiac hypertrophy (for reviews, see Refs. 15 and 32). Several studies have examined the MAPK pathways in exercised skeletal muscles. Marathon running in men was shown to significantly increase phosphorylated ERK1/2 and p38 MAPK levels (downstream effectors of MAPK signaling) in skeletal muscle (53). Studies that examined these pathways in rodent heart have shown that treadmill exercise induced activation of p38 MAPK and ERK1/2 (35), whereas swimming induced phosphorylation of Akt but not p38 MAPK or ERK1/2 (29). However, in the aforementioned studies, the mice that were exercised by treadmill did not undergo cardiac hypertrophy, whereas mice that were subjected to swim training had significant cardiac hypertrophy. Moreover, female mice were not examined in either study. In the present study, cardiac hypertrophy induced by voluntary cage-wheel exposure led to no obvious alterations in these components of the MAPK pathway.

Growth factors and their downstream targets have also been implicated in cardiac hypertrophy (15,37,45,47). Phosphatidylinositol 3-kinases (PI3-kinases) are downstream of many receptor tyrosine kinases to which growth factors bind. A downstream target of PI3-kinase is Akt (PKB). Expression of the catalytic subunit of PI3-kinase-α (P110α) in an activated form in heart results in increased phospho-Akt, significant hypertrophy, and normal contractility (48). Moreover, Akt may impart phenotypical differences between males and females (6). Here, although cage-wheel exposure increased Akt activity as measured by the increase in phospho-Akt, there was no difference between male and female mice. However, sex differences in Akt activation may be limited to the nuclear localization of phospho-Akt as demonstrated in a previous report (6); this distinction was not made in the present study. The present study, however, is consistent with a role for Akt in physiological hypertrophy as indicated in swim-trained mice (29).

GSK-3β, a negative regulator of cardiac hypertrophy, is one of the downstream effectors of Akt. It is inactivated through phosphorylation by Akt (3,4,34). As detailed (see *Akt, GSK-3β, and MAPK*), cage-wheel exposure for 7 days doubled the relative amount of the inactive (phosphorylated) form of GSK-3β in both male and female mice. Thus the antihypertrophic effects of GSK-3β are relieved, and prohypertrophic pathways are promoted. Moreover, the inactivation of GSK-3β by exercise may be mediated through the activation of Akt. After 21 days of cage-wheel exposure, phospho-GSK-3β levels remained elevated only in females despite the attenuation of phospho-Akt levels to those of sedentary control animals. The mechanism of the sustained deactivation of GSK-3β was not addressed in this study. Nevertheless, these findings suggest two important sex dimorphisms as follows: *1*)Akt activation by phosphorylation can be uncoupled from GSK-3β inactivation by phosphorylation only in females; *2*) the prohypertrophic effects of phospho-GSK-3β persist after a prolonged physiological stimulus only in females. Table 2 summarizes the data on the signaling factors examined in this study.

Here, we have identified potential targets, molecules, and pathways that may be important in imparting the phenotypical differences between male and female mice and in the sexually dimorphic cardiac adaptation to exercise. The observed differences between the sexes may be partially explained by disparities in the levels of certain hypertrophic signaling molecules. It appears that the initial pathways may involve CaMK, Akt, and GSK-3β in both sexes and that the persistence of phospho-GSK-3β in females may be permissive to further cardiac growth. Clearly, multiple systems contribute to the aerobic exercise capacity of male and female mice, and numerous signaling pathways affect cardiac adaptation.

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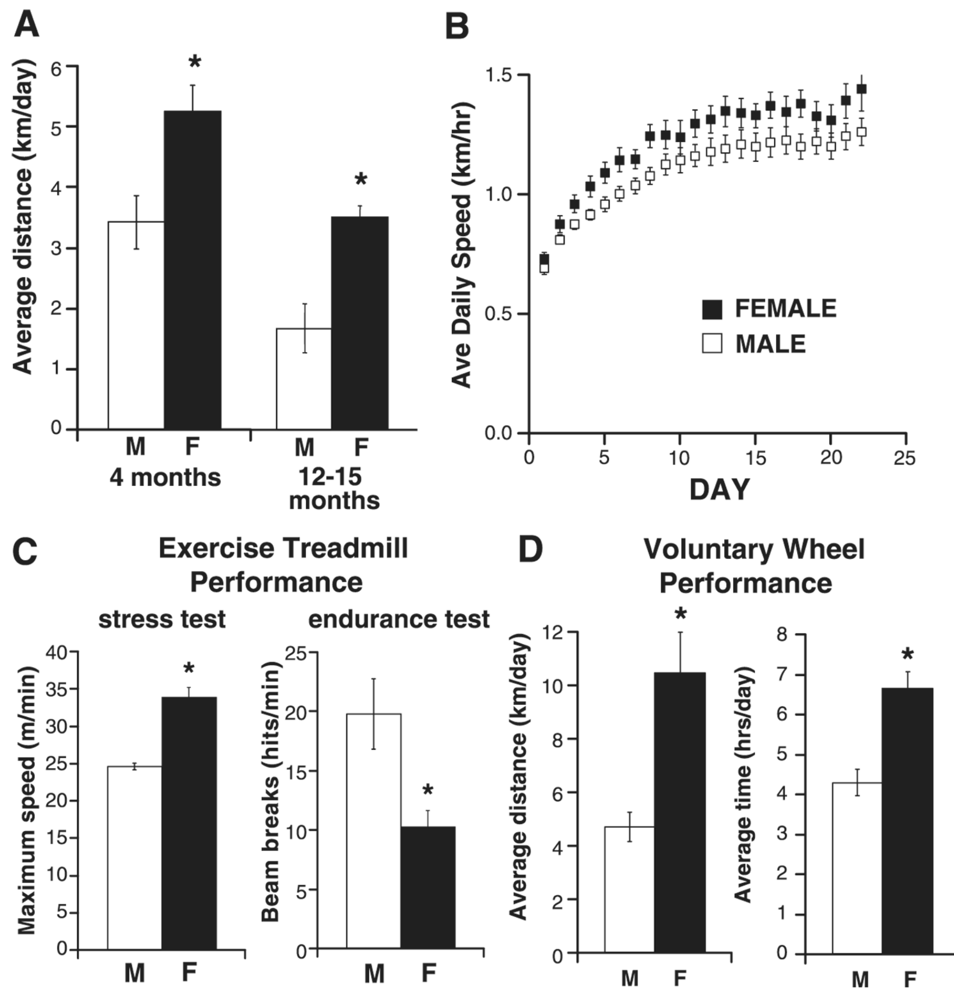

Fig. 1. Voluntary cage-wheel performance in C57BL/6J male (M) and female (F) mice

A: average running distance (in km/day) for every 24 h over the 21-day study period. *B*: average running speed (in km/h) for every 24 h over the 21-day study period. *C*: treadmill exercise performance in C57BL/6J male and female mice. Mice were subjected to treadmill stress and endurance exercise tests. Stress test illustrates that female mice reached greater maximum speeds than their male counterparts. Similarly, female mice performed significantly better than males as demonstrated by a fewer number of beam breaks per minute. *D*: voluntary cage-wheel performance in FVB/NJ male and female mice. Female FVB/NJ mice exhibited enhanced cage-wheel performance over males as measured by both average running distance (in km/day) and average running duration for every 24 h over the 21-day study period. *$P < 0.05$ from values obtained for males.

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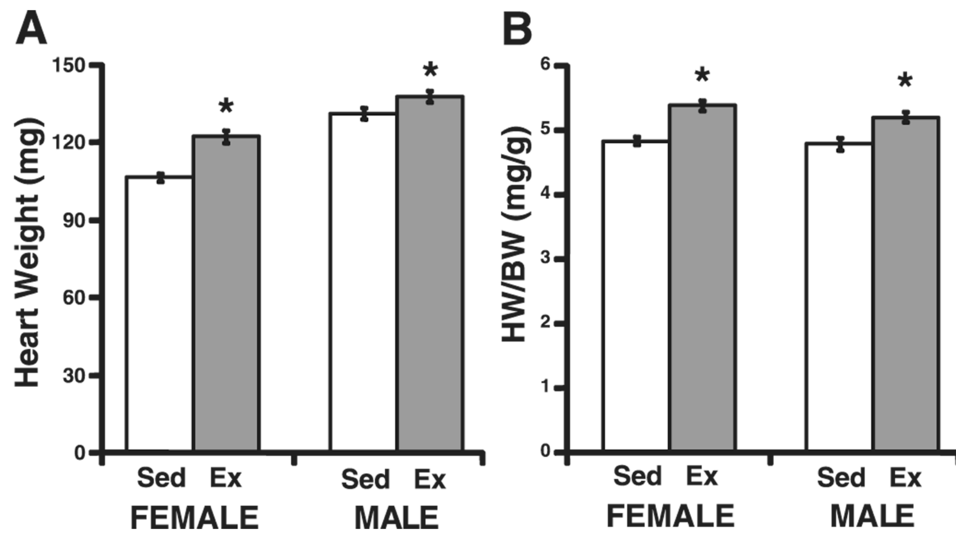

Fig. 2. Morphometric data from sedentary and exercised C57BL/6J male and female mice

A: whole heart weight (in mg) measured from sedentary (*S*ed) and exercised (Ex) male and female mice. *B*: heart weight normalized for body weight (HW/BW) determined from sedentary and exercised male and female mice. *$P < 0.05$ from values obtained for males.

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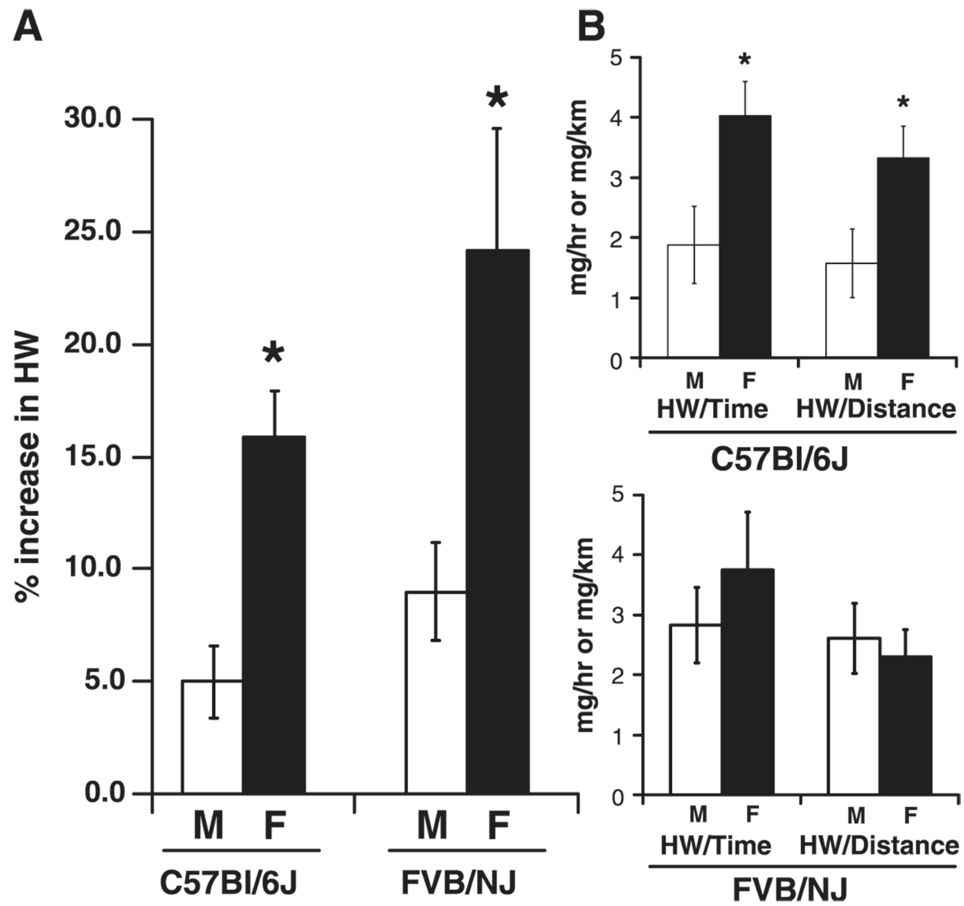

Fig. 3. Cardiac adaptation in sedentary and exercised C57BL/6J and FVB/NJ male and female mice
A: cardiac adaptation expressed as percent change from mean sedentary heart weight in the indicated study group. *B*: cardiac adaptation normalized to activity in C57BL/6J (*top*) and FVB/NJ (*bottom*) mice. Heart weight (HW) divided by mean time (HW/time) or by distance (HW/distance) run in a given 24-h period. $^*P < 0.05$ from values obtained for males.

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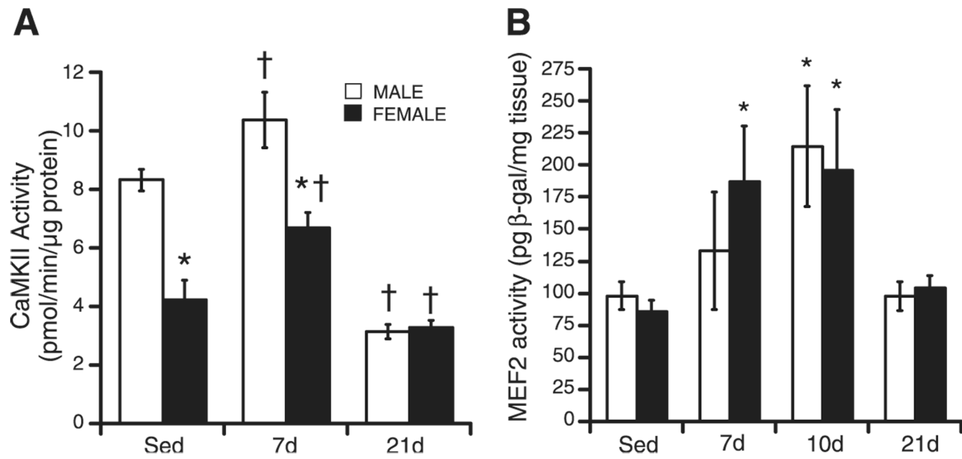

Fig. 4. Ca^{2+}-dependent signaling in sedentary and exercised C57BL/6J male and female mice

A: Hearts from mice were examined for Ca^{2+}/calmodulin-dependent protein kinase II (CaMK II) activity in sedentary (*n*= 14 males and 8 females) animals and animals exercised for 7 days (7d; *n*= 8 males and 10 females) and 21 days (21d; *n*= 4 males and 3 females). $*P < 0.05$ from values obtained for males; $†P < 0.05$ from values obtained for respective sedentary animals. *B*: myocyte enhancer factor-2 transcription factor (MEF-2) activity in sedentary and exercised C57BL/6J male and female mice. MEF-2 activity was determined by measuring MEF-2-dependent expression of β-galactosidase (β-gal) in sedentary mice (*n*= 31 males and 35 females) and in mice exercised for 7 (*n*= 8 males and 6 females), 10 (*n*= 8 males and 7 females), and 21 (21d; *n*= 8 males and 7 females) days. $*P < 0.05$ from values obtained for sedentary males.

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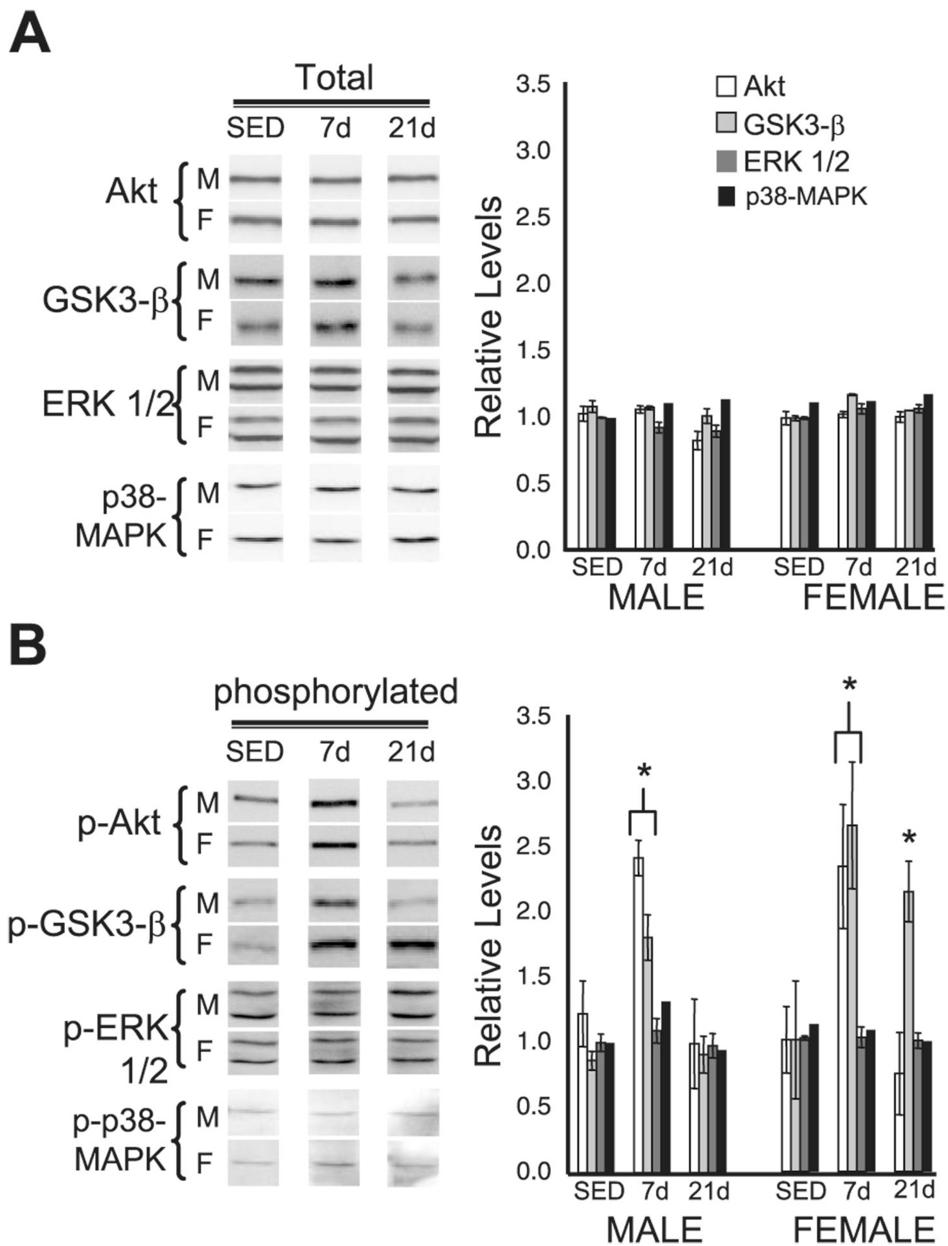

Fig. 5. Akt, glycogen synthase kinase-3β (GSK-3β), and mitogen-activated protein kinases [MAPK: extracellular regulated kinase 1/2 (ERK1/2) and p38 MAPK] protein in sedentary and exercised C57BL/6J male and female mice

A: Western blots (*left*) for Akt, GSK-3β, ERK1/2, and p38 MAPK. Mean values ± SE of immunoblot data indicate levels of total protein relative to sedentary animals (*right*). *B*: Western blots (*left*) for phosphorylated Akt (p-Akt), GSK-3β (p-GSK-3β), ERK1/2 (p-ERK1/2), and p38 MAPK (p-p38 MAPK). Mean values ± SE of immunoblot data indicate levels of each respective phosphorylated protein relative to sedentary animals (*right*). Immunoblots of ERK1/2 were quantitated by combining the densitometric values of the 42- and 44-kDa bands. *$P < 0.05$ from values obtained in sedentary animals.

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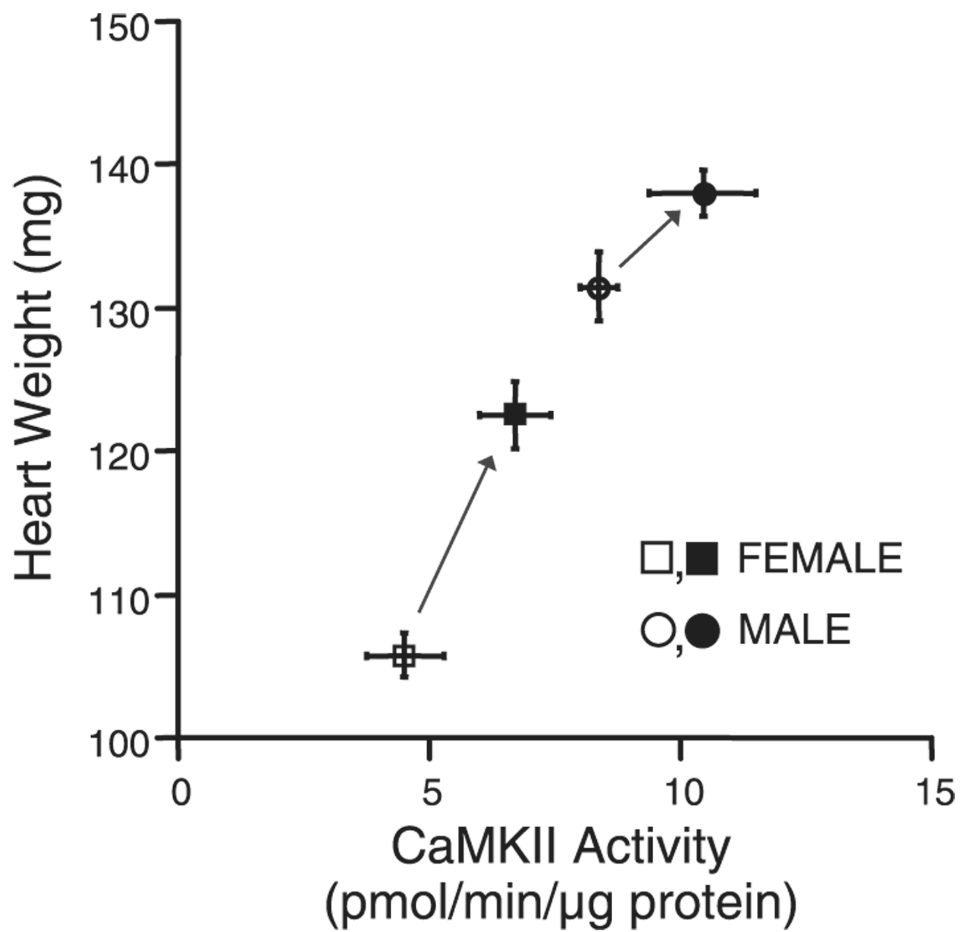

Fig. 6. Relationship between CaMK II activity and heart weight in sedentary (□, ○) and exercised (■, ●) male and female C57BL/6J mice

Heart weight was plotted against CaMK II activity measured at 7 days of cage-wheel exposure. Arrows indicate the response of the heart to an exercise stimulus as measured by an increase in cardiac mass and CaMK II activity within each sex.

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Table 1

Body and cardiac mass measurements of 4-mo-old mice

	n, No. of mice	Body Mass, g	Cardiac Mass, mg	HW/BW, mg/g
		C57BL/6J mice		
Sedentary				
Males	21	27.9±0.4	131.6±2.4	4.71±0.07
Females	20	22.2±0.3*	105.9±1.5*	4.78±0.04
Exercised				
Males	22	26.6±0.3	138.2±1.6†	5.22±0.05†
Females	23	22.8±0.4*	122.7±2.3*†	5.40±0.07†
		FVB/NJ mice		
Sedentary				
Males	4	32.8±0.9	135.5±5.0	4.13±0.08
Females	6	25.2±0.9*	99.8±3.2*	3.97±0.11
Exercised				
Males	5	32.3±0.5	147.7±2.9†	4.57±0.13†
Females	5	25.3±0.8*	123.9±5.3*†	4.89±0.12†

Values (except *n*) are means ± SE. Shown is a summary of morphometric data from sedentary and exercised C57BL/6J and FVB/NJ male and female mice. Heart rate-to-body weight ratio (HW/BW) was determined by dividing cardiac mass by body weight.

* $P < 0.05$ from values obtained for males

† $P < 0.05$ from values obtained in respective sedentary counterparts.

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Table 2

Effects of exercise on hypertrophic signaling pathways

	Males			Females		
	Sedentary	**7 days**	**21 days**	**Sedentary**	**7 days**	**21 days**
Akt						
Phospho-Akt		↑			↑	
Glycogen synthase kinase-3β						
Phospho-glycogen synthase kinase-3β		↑			↑	↑*
Mitogen-activated protein kinases						
Ca^{2+} calmodulin-dependent protein kinase		↑	↓	↓*	↑*	↓
Myocyte enhancer factor-2		↑			↑	

Hypertrophic signaling molecules were measured in sedentary mice and mice exercised for 7 and 21 days. Symbols represent changes from male sedentary animals except increase in Ca^{2+}/calmodulin-dependent protein kinase 7-day-exercise value in females, which is relative to sedentary female mice. ↑, increase; ↓, decrease; no symbol, no change

* sex-specific difference.