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Gender-specific effects of exercise on cardiac pathology in Na^+/H^+ exchanger overexpressing mice

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Abstract The Na^+/H^+ exchanger isoform 1 (NHE1) has been implicated in various cardiac pathologies including ischemia/reperfusion damage to the myocardium and cardiac hypertrophy. It is known that NHE1 levels increase in cardiac disease and we have recently demonstrated that expression of an activated NHE1 protein promotes cardiac hypertrophy in the mouse myocardium. We examined the gender-specific effects of exercise in combination with elevated cardiac expression of NHE1 on the myocardium in mice. Control mice and transgenic mice expressing elevated levels of wild type NHE1 and activated NHE1 were examined. There were gender-specific differences in the effects of NHE1 with exercise. Exercised wild type male mice showed a tendency toward increased heart weight. This was not apparent in female mice expressing elevated NHE1 levels. In some transgenic female mice, there was a significant decrease in the size of the exercised hearts, which was different from what occurred with male mice. Body weight was maintained in exercised control and transgenic male mice; however, it decreased in female mice with exercise more so in transgenic female mice expressing elevated levels of NHE1. Female mice expressing activated NHE1 had elevated HW/BW ratios compared to males, and this was exaggerated by exercise. These results suggest that gender-specific activation of NHE1 may be critical and that NHE1 plays a more critical role in promoting some types of hypertrophy in females in comparison with males.

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Introduction

The Na^+/H^+ exchanger (NHE) is a ubiquitously expressed plasma membrane protein that regulates intracellular pH. In mammalian cells, it extrudes one intracellular H^+ in exchange for one extracellular Na^+ [1, 2]. NHE1 (NHE, isoform 1) is the only plasma membrane isoform present in cardiac cells though there are several isoforms of NHE known [3–5]. NHE1 is involved in several myocardial pathologies. Increased NHE1 activity accentuates the damage that occurs during ischemia/reperfusion injury [2, 6, 7]. More recently, NHE1 activity has been shown to be an important mediator of cardiac hypertrophy [8]. Inhibitors of NHE1 [9] have been tested for treatment of these cardiac diseases. To date, however, their use has been successful in animal models, but not in clinical trials, possibly because of detrimental side effects (reviewed in [10, 11]).

Levels of the NHE1 protein are known to be elevated in the myocardium in response to cardiac disease. Protein, mRNA, and activity can increase in the disease state in the myocardium [12–16]. NHE1 is normally quiescent at physiological pHs and is activated by the reduction of intracellular pH. The "set point" of the protein is, however, modified by hormonal stimulation so that the protein is more active at more alkaline pHs [4, 17]. We have recently demonstrated that in a transgenic mouse model, elevated expression of an activated form of NHE1 promotes cardiac hypertrophy. Two transgenic mouse lines were studied; one that expresses elevated levels of a wild type NHE1 protein (N-line) and the other that expresses elevated levels of an activated NHE1 protein (K-line). Notably, mice expressing

the activated NHE1 protein were more susceptible to endocrine-induced cardiac hypertrophy and had a greater tendency toward cardiac hypertrophy [18]. While we have examined some of the basic effects of elevated NHE1 levels, the effect of expression of NHE1 in combination with many physiological phenomena have not been examined. How elevated expression interacts with physical activity and training has not been studied. In addition, we earlier examined the effects of elevated NHE1 expression in only female mice and the relative effects on genders have not been examined. In this study, we characterize the effects of elevated NHE1 expression in both male and female mice in combination with exercise. The results suggest that there is gender specificity in the effects of NHE1 expression in the myocardium.

Materials and methods

Materials

Anti-HA (12CA5) was procured from Roche Applied Science (Laval, Quebec). Secondary antibodies of specific wavelength were purchased from LI-COR Biosciences (Lincoln, Nebraska). Mouse anti-NHE1 antibody was from BD Biosciences (San Jose, CA, USA). The monoclonal antibody for alpha-myosin heavy chain (MF20) was from the Developmental Studies Hybridoma Bank of the University of Iowa, (Iowa City, IA).

NHE1 transgenic mice

All experimental procedures involving animals presented in this study were in accordance with the guidelines set out by the Canadian Council on Animal Care and were also approved by the local animal care committee. Transgenic mice expressing wild type NHE1 protein are referred to as N-line, and transgenic mice expressing an activated NHE1 protein in the myocardium are referred to as K-line. The transgenic mice expressing hemagglutinin (HA) tagged NHE1 under the control of the alpha-myosin heavy chain promoter was described earlier [19, 20]. In this study, the N-line and K-line mice expressing similar levels of NHE1 were characterized (see analysis below). N-line and K-line mice used in this study were 7 weeks of age. Littermates were used from either N-line or K-line mice as controls.

Western blotting

Hearts used for examining NHE1 protein expression were isolated from control and NHE1 transgenic mice at the age indicated. They were added to a homogenization buffer [(mM) (120 NaCl, 10 Tris (pH 7.4), 0.1 phenylmethylsulfonyl fluoride, 0.1 benzamidine), 37.5 μM ALLN (calpain I inhibitor), and a proteinase inhibitor cocktail]. Samples were homogenized at 4 °C × 30 s, incubated on ice for 30 s and were homogenized again for another 30 s using an Omni International 2000 electric homogenizer (OMNI International, Kennesaw, GA). The homogenates were subjected to a series of centrifugation steps. The initial centrifugation was for 10 min at $735\times g$. The resulting pellet was discarded and the supernatant was centrifuged at $8{,}200\times g$ for 15 min. The resulting pellet was again discarded and the supernatant was centrifuged for the last time at $40{,}000\times g$ for 1 h. The supernatant was discarded and the pellet containing the membrane fraction was resuspended in the homogenization buffer. Total protein was quantified using the Bio-Rad D_C Protein Assay kit as described by the manufacturer. 100 μg of each sample was resolved on 10 % SDS-PAGE and transferred to nitrocellulose membranes. Nitrocellulose membranes were blocked in blocking buffer solution (LI-COR Biosciences, Lincoln, Nebraska) overnight at 4 °C with gentle rocking. Membranes were incubated with anti-HA (Y11) antibody (sc-805) (Santa Cruz Biotechnology; Santa Cruz, CA) or anti-NHE1 antibody (BD Biosciences Pharmingen; San Diego, CA) overnight. Anti-NHE1 was used to examine total (endogenous and exogenous) NHE1 protein expression. Anti-HA was used to examine expression of exogenous HA-tagged NHE1. Anti-actin antibody (H-300) (Santa Cruz) was used as a loading control. The primary antibodies were diluted 1:1000 in TBS + 0.1 % Tween-20 and incubated overnight. The membrane was then washed with TBS + 0.1 % Tween-20 4× for 5 min at room temperature. Secondary antibody conjugated to HRP was used to examine expression as described earlier [21]. Total NHE1 or exogenous NHE1 expression was quantitated and corrected with the total protein expression of actin. Protein expression of the exogenous NHE1 protein was slightly larger in apparent molecular weight likely due to the HA tag.

Transgenic heart evaluation

To evaluate cardiac effects on mice, we examined heart weight to body weight (HW/BW) of transgenic mice. For HW/BW, animals were euthanized with halothane and hearts were excised. All extra cardiac structures were removed from isolated hearts and then hearts were washed in PBS, blotted, and weighed [22]. Cardiac hypertrophy was evaluated by measuring HW/BW in mg/g.

Statistical analysis

All values are expressed as mean ± SEM. Groups were compared by both Student t test and Wilcoxon signed-rank test. Differences were considered significant when P values < 0.05.

Exercise protocol

Mice, 2.5 months of age, were subjected to a regime of regular exercise for a period of 6 weeks. The exercise protocol was a medium intensity training protocol similar to that described earlier [23]. After a period of a few days exposure to acclimatize to the treadmill, mice were subject to regular exercise for 5 days a week using a six lane motorized treadmill (Columbus instruments, Treadmill Simplex II). Exercise in week 1 was 15 min at 6 m/min. Exercise in week 2 was 5 min at 6 m/min, 30 min at 10 m/min, and 5 min at 6 m/min. In week 3, it was 5 min at 6 m/min, 5 min at 10 m/min, 30 min at 14 m/min, and 5 min at 6 m/min. In weeks 4–6, it was 5 min at 6 m/min, 5 min at 10 m/min, 5 min at 14 m/min, 30 min at 18 m/min, and 5 min at 6 m/min. Mice rarely required gentle prodding and ran readily without extraneous motivation. Animals were euthanized 24 h. after the last run.

Results

Figure 1a and b illustrate the heart weights of male and female mice that were either sedentary or exercised as described in the "Materials and Methods". For the male mice (Fig. 1a), there were no significant differences between the control and the experimental groups. All the exercised mice had a tendency toward slightly increased heart weights; however, this was not statistically significant. For the female mice, the results are shown in Fig. 1b. The heart weight of control mice increased significantly with exercise. In contrast, there were no increases in the heart weight of N- or K-line mice with exercise, and in N-line mice the heart weight decreased slightly with exercise. K-line female mouse hearts were larger than that of the N-line or controls, but their weight was not further elevated exercise. We compared the relative changes that occurred with exercise, in male versus female mice. For wild type mice, exercise increased the heart weight in both sexes. In the N-line female mice, there was a decrease in heart weight with exercise. This was different from male mice, which did not decrease in heart weight and showed a slight, but not significant, increase. In K-line mice, there was an increase in heart weight in male mice that was not significant. There was almost no change in heart weight in K-line female mice. We compared the relative changes that occurred in males versus females with exercise. There was no significant difference was found by means of the Wilcoxon signed-rank test, but a significant difference in the relative changes ($P < 0.05$) was found by the student t test. When comparing control sedentary mice of the same sex with either N-line or K-line transgenic mice, we found that

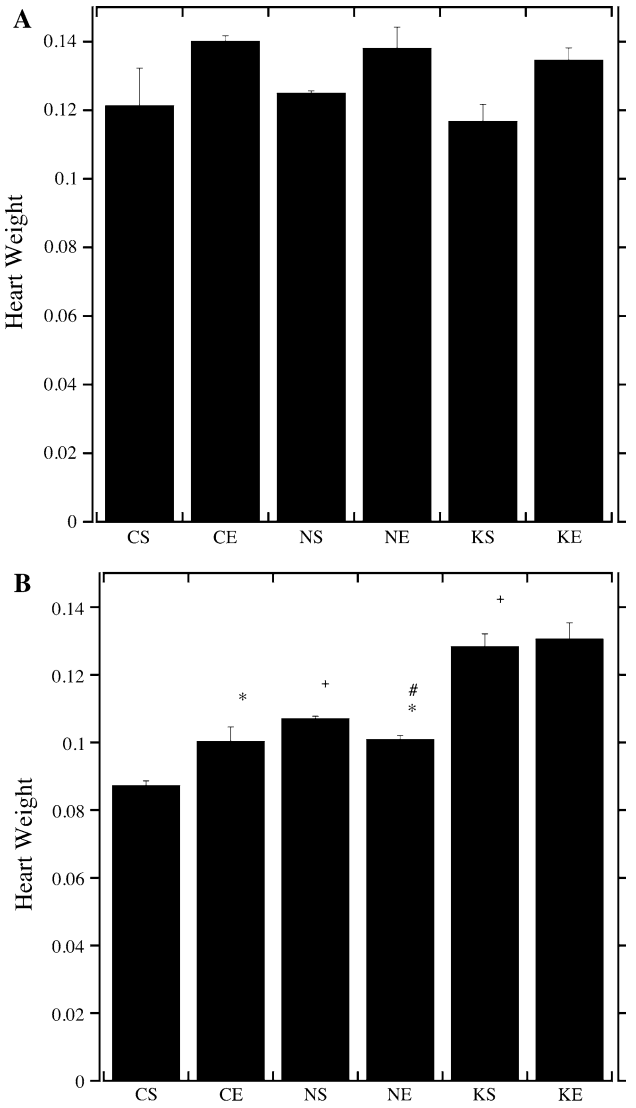

Fig. 1 Analysis of heart weight in male and female exercised and sedentary mice. Heart weights from control (sedentary *S*) or (exercised *E*) mice. Mice were wild type (control *C*) or N-line (*N*) or K-line (*K*). **a** male mice, **b** female mice. $^{*}P < 0.05$ versus sedentary mice of the same sex and group. $^{\#}$Indicates that the change versus sedentary mice is significantly different ($P < 0.05$) from that which occurred with male mice. $^{+}$Indicates significantly increased over control sedentary mice of the same sex

either of the transgenes caused an increase in heart weight in female mice, but not in male mice.

Figure 2a and b illustrate the body weight of male mice that were either sedentary or exercised. For the male mice, there were no significant differences between any of the groups; exercise did not change the body weight of the mice. There was a trend toward the reduction of body weight in N-line mice that was not statistically significant. In the female mice (Fig. 2b), all groups showed a trend toward reduction in body weight with exercise though this was only significant in the N-line and K-line mice. There

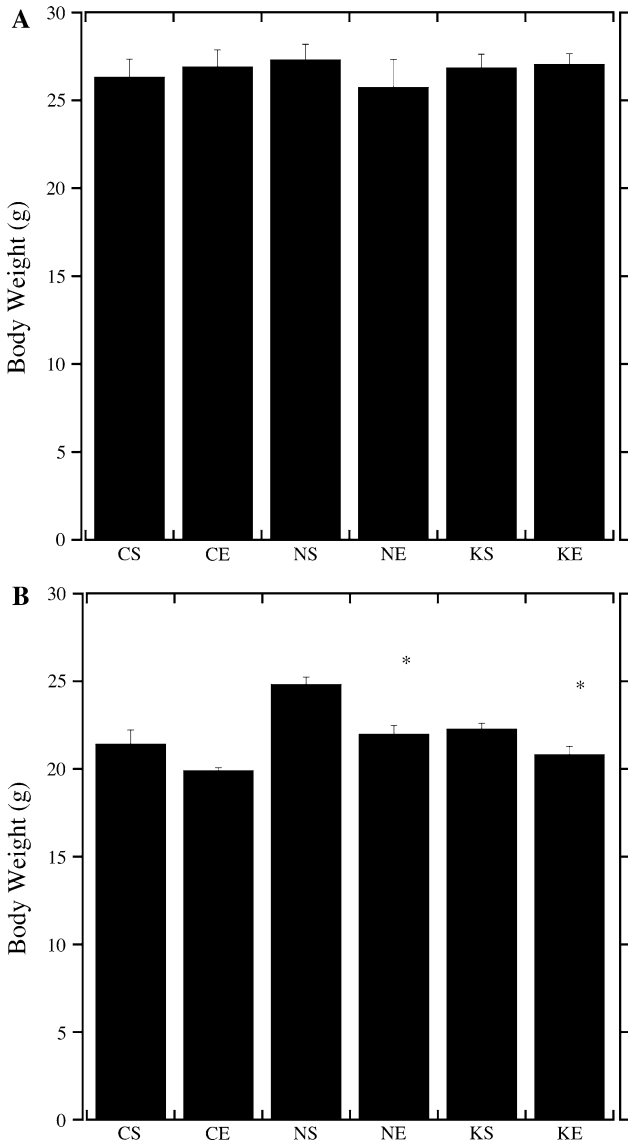

Fig. 2 Analysis of body weight in male and female exercised and sedentary mice. Body weights from control (sedentary *S*) or (exercised *E*) mice. Mice were wild type (control *C*) or N-line (*N*) or K-line (*K*). **a** male mice, **b** female mice. *$P < 0.05$ versus sedentary mice of the same sex and group

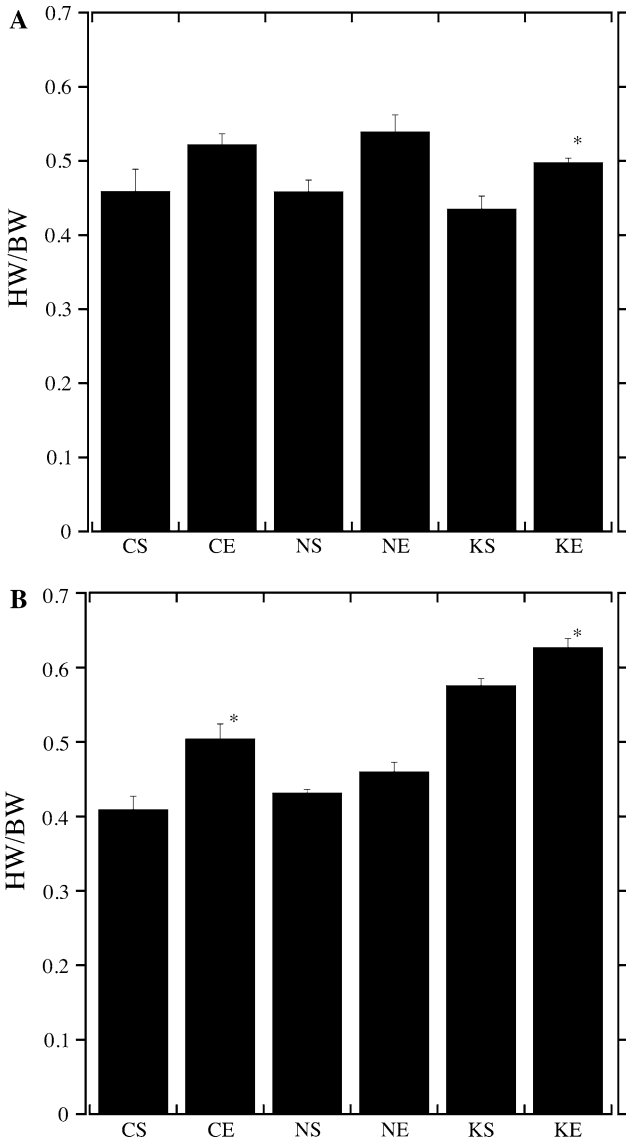

Fig. 3 Analysis of heart weight to body weight (HW/BW) ratios of male and female exercised and sedentary mice. Body weights from control (sedentary *S*) or (exercised *E*) mice. Mice were wild type (control *C*) or N-line (*N*) or K-line (*K*). **a** male mice, **b** female mice. *$P < 0.05$ versus sedentary mice of the same sex and group

were no significant differences in the relative changes that occurred in males versus females with exercise.

Figure 3a and b illustrate the HW/BW ratio of the male and female mice that were either sedentary or exercised. For the male mice, in all cases, there was a similar trend toward an increased HW/BW ratio, with exercise. However, this was only significant with the K-line mice. The amount of increase in the ratio was similar in all cases. For the female mice, in all cases, the HW/BW ratio increased with exercise though this was only significant for control and K-line mice. K-line mice had greatly increased HW/BW ratios, as reported earlier [18]. Surprisingly, exercise resulted in a further increase in this ratio. It should be noted, however, that this was principally due to a decrease in the body weight that declined significantly. Heart weight only increased marginally with exercise. There were no significant differences in the relative changes that occurred in males versus females with exercise.

To determine if the differences noted between male and female mice were due to varying levels of the NHE protein, we examined the levels of the NHE1 protein. Figure 4 examines the levels of NHE1 protein in control adult mice. Male and female NHE1 heart lysates were run on the same blot and immunoblotted for NHE1 using a commercial

antibody against NHE1. The upper panel is a representative western blot of female control heart lysates, represented in lanes 3–4, and male control heart lysates, represented in lanes 1–2. Quantification of a series of experiments showed no significant differences in NHE1 protein expression in heart lysates from female control mice versus heart lysates from male control mice (Fig. 4, bottom panel).

We next examined if expression of NHE1 varied between males and females in the two lines of transgenic mice. We examined the level of HA-tagged NHE1 protein and also used an antibody against the NHE1 protein itself to determine the total level of NHE1 protein. The results are shown in Fig. 5. Western blot analysis compared the levels of both HA-tagged NHE1 protein and the total NHE1 protein in sedentary mice. There were no significant differences between the male and female mice.

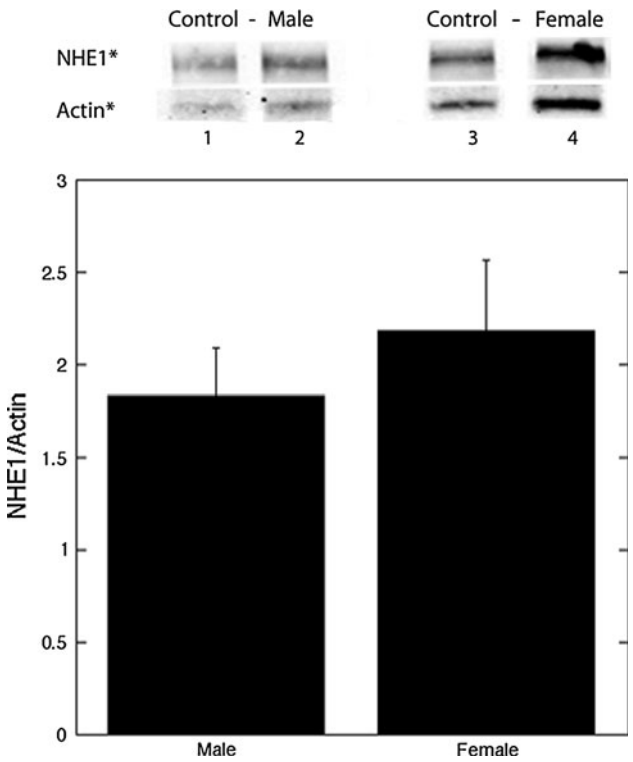

Fig. 4 Immunoblot analysis of total exogenous and endogenous NHE1 expression in adult hearts from control male and female mice. Total NHE1 protein expression was detected using mouse monoclonal anti-NHE1 antibody. *Upper panel* representative western blot of heart lysates from male and female control mice immunoblotted for NHE1 (90–110 kDa) and actin (40 kDa). *Lanes 1–2* represent heart lysates from male control mice and *lanes 3–4* represent heart lysates from female control mice. *Bottom panel* NHE1 protein levels were quantified and normalized to actin for each group. Results are expressed as a ratio of NHE/actin ± SEM ($n = 5$–7/group)

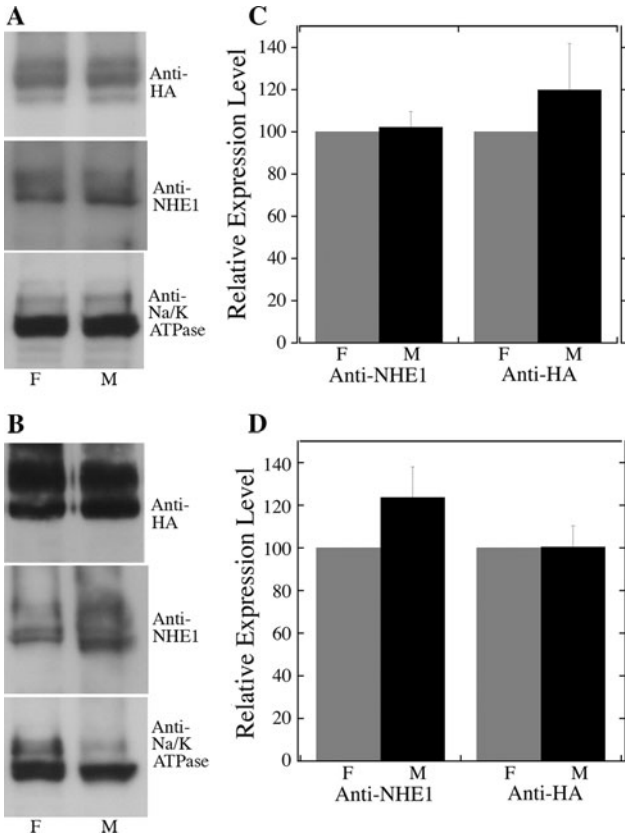

Fig. 5 Immunoblot analysis of NHE1 protein expression in 6 week old male versus female transgenic mice. **a**, **B** Western blot analysis of comparative NHE1 expression in N-line (**a**) or K-line (**b**) transgenic mice. *Upper panel* immunoblot with anti-HA antibody to detect exogenous NHE1 protein. *Middle panel* immunoblot with monoclonal anti-NHE1 antibody to detect total NHE1 protein. *Lower panel* immunoblot with anti Na^+/K^+ ATPase antibody for normalization of protein loading. **c**, **d** quantification of relative expression levels in male versus female N-line (**c**) and K-line (**d**) transgenic mice. The level of exogenous (HA-tagged) and total NHE1 protein was quantified as described in the materials and methods. Results are expressed as a ratio of NHE/Na^+/K^+ ATPase ± SEM ($n = 4$–6/group)

Discussion

In this study, we examined the effect of exercise on hearts in transgenic mice that expressed elevated levels of either wild type or activated NHE1 protein. We compared the effects that occurred in male mice with those that occurred in female mice. Previous studies have examined the effect of exercise on skeletal muscle NHE activity. Exercise of male rats increased the NHE activity of hind limb muscle [24]. However, it was not clear whether this effect was due to changes in protein expression levels or due to changes in regulation of the protein. Short term exhaustive swimming decreased sarcoplasmic reticulum calcium transport; however, this study only examined male rats and changes in the levels of proteins were not measured [25]. The effect of

prolonged exercise training on rats has shown that most parameters of cardiac sarcolemmal function are not affected with the exception of augmented calcium pump activity and 5′-nucleotidase activity [26]. However, neither NHE activity nor protein levels were examined in this study, and only male mice were characterized. In our study, we examined the differences that occurred between the two sexes with prolonged exercise. First, with regards to heart weight, exercise increased the heart weight in both male and female control mice. However, in the transgenic mice overexpressing the NHE, exercise always had a tendency to increase the heart weight in male mice, while there was no such tendency in the female mice and in fact a significant decrease in the size of the exercised hearts in female N-line mice occurred. If we compared the relative changes in the heart between male and female mice, we found that there was a significant difference in the effect of exercise on heart weight in male versus female N-line mice. This was not the case with K-line mice. The reason for this difference is not certain at this time. However, it may be that the female transgenic hearts were already elevated in size compared to the wild type hearts; thus, they may have reached a maximum attainable under these conditions. Male transgenic hearts were the same size as control animals, perhaps allowing for more plasticity in the heart remodeling. Studies have shown a surprising degree of variation in the effects of exercise on heart weight and body weight. Some studies have shown no increase in heart mass with exercise, while others show significant increase in cardiac mass with exercise in females. Age, type of exercise, and the strain of animals may influence these results (reviewed in [27]). It has been recently suggested that females have increased exercise capacity and increased hypertrophic capacity relative to males in response to exercise [27]. We did find that the maximal increases in heart size, relative to controls, were greater in female mice. However, this was apparent in the K-line transgenic mice only and was not a function of exercise. Ovariectomy and reintroduction of estrogen did not affect NHE1 protein levels [28]; however, it has been shown [29] that estrogen enhances cardiomyocyte hypertrophy through Erk-dependent activation of NHE1. It is thus possible that the greater effects noted in female mice were through an estrogen-dependent enhancement of NHE1 activity in the female mice. This may account for the greater effects that occurred in the female mice relative to the male mice.

Body weight of female mice was generally lower than that of male mice. An examination of the effects of exercise on body weight showed that it tended to decrease body weight in female transgenic mice, but not in male mice. This effect occurred with both N-line and K-line mice. Control mice showed no such significant differences though there was a trend toward reduced body weight in control female mice. Other studies have shown that exercise does not result in a decrease in body weight when mice or rats are eating ad libitum [27]. However, effects of exercise on mice are very strain dependent [30]. In our study, the reason that exercise decreased the body weight in the female transgenic mice is not yet clear. Comparative effects of equivalent amounts of exercise on male and female mice are few. In some studies, exercise has been shown to cause a decrease in body weight in female mice [31, 32]; with uncontrolled exercise, female mice do tend to run further and faster than male mice [30]. Recently, equivalent exercise has been shown to cause a greater cardiac hypertrophic effect in female mice compared with males [33]. In our study, males and females were exercised to the same degree. It was evident that the body weight of the female N-line mice was elevated compared to controls. Possibly, a slight amount of morbidity was associated with the transgene and this led to increased body weight. Forced exercise may have had a compensatory effect.

Analysis of the HW/BW further accentuated a pattern of greater effects of the transgene on female mice, versus the males. This was notable in the K-line mice that had much greater HW/BW ratios than controls, and much greater in females than the males. Exercise accentuated this effect. We have found this same effect on K-line female mice of another independently made mouse line indicating that it was not a function of the particular insertion site of the transgene (not shown). As noted above, we suggest that enhancement of NHE1 activity by estrogen [29] may be at least possibly responsible for this effect. We suggest that the partial enhancement of activity already present in the protein in K-line mice may be accentuated by estrogen-stimulated activation of the NHE1 protein. The exogenous protein added to K-line is activated by a mutation in the Ca^{2+}-calmodulin binding domain [19]. This is an independent mode of activation of estrogen, which acts through Erk-dependent activation of the NHE1 protein [29, 34]. We have shown earlier that K-line mice are more sensitive to hypertrophy that is induced by phenylephrine [18]. Phenylephrine also acts through Erk-dependent activation of NHE1 [35]. The present study is therefore consistent with the hypothesis that hormonal mechanisms activating NHE1 through Erk, such as estrogen, can accentuate hypertrophy in combination with activation of NHE1 through calmodulin. Exercise is also known to activate Erk-dependent pathways which could further activate NHE1 and exaggerate this effect [36, 37]. This hypothesis is summarized in Fig. 6. It should be noted that there were no significant differences in the level of NHE1 expression that could account for differences between the results shown in male and female mice (Fig. 5) so that the effect appears to be mediated through regulation and not through changes in protein levels. It is of significance that recent statistics have

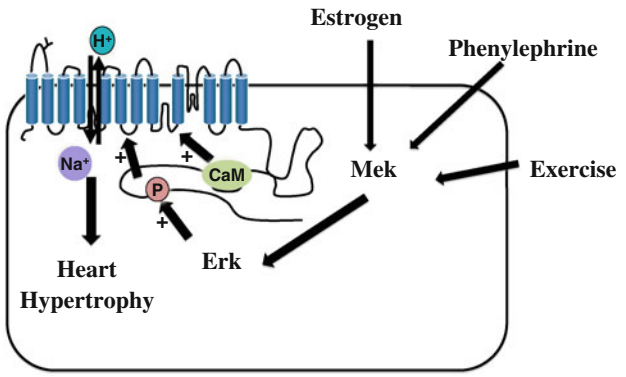

Fig. 6 Schematic diagram illustrating the converging effects of various stimuli on the Na^+/H^+ exchanger that can lead to heart hypertrophy. Estrogen [29], phenylephrine [35] and exercise [36] have been shown to activate Erk-dependent pathways which can lead to activation of the Na^+/H^+ exchanger [29, 35]. Activation by Erk occurs through phosphorylation of distal regions of the NHE1 cytoplasmic tail [34, 35]. K-line mice have a mutation in the CaM binding domain that also activates NHE1 via a different region of the cytosolic tail of NHE1 [19, 20]. Together these stimuli activate the NHE1 protein which has been shown to lead to heart hypertrophy [18, 39]

shown that women are more than twice at risk as men for developing heart failure after a myocardial infarct [38].

In summary, some of the principal differences we found between the sexes were that in female N-line mice, there was a significant decrease in the size of the exercised hearts which was different from what occurred with male mice. In addition, in sedentary female mice, both transgenic mouse lines showed significant increases in the heart size relative to controls. This did not occur in male mice. Finally, and perhaps most importantly, there was a greater tendency for female K-line mice to have elevated HW/BW ratios which was exaggerated by exercise. These results suggest that gender-specific activation of NHE1 may be critical and that NHE1 may play a more critical role in promoting some types of hypertrophy in females in comparison with males.

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