Cardioprotective effects of exercise training on myofilament calcium activation in ovariectomized rats

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EVIDENCE FROM A RECENT LANDMARK clinical study on hormone replacement therapy (HRT) in postmenopausal women has raised concerns about many risks associated with the therapy, especially heart diseases (42). Although steroid sex hormones have been hypothesized to play a significant role in myocardial function, the mechanisms of the effects of these hormones are not well understood. A myofibrillar Ca\(^{2+}\) hypersensitivity, a suppressed maximum myofibrillar ATPase activity, and a significant shift in myosin heavy chain (MHC) toward β-MHC isoform have been demonstrated in ovariectomized rat hearts (36, 37). These effects of ovarian sex hormones on myofilament Ca\(^{2+}\) activation were found to be cardiac specific (37). Furthermore, an upregulation of β-1-adrenergic receptors, which may partly contribute to changes in myofilament Ca\(^{2+}\) activation, was detected in ovariectomized hearts (32). Interestingly, differential cardioregulatory effects of the two ovarian sex hormones on the myofilament Ca\(^{2+}\) activation were demonstrated (38). Whereas either estrogen or progesterone supplementation could prevent the suppressed maximum myofibrillar ATPase activity, only estrogen could abolish the Ca\(^{2+}\) hypersensitivity of the myofilaments (38). It is the similarity in Ca\(^{2+}\) hypersensitivity detected in the ovariectomized hearts and in cardiomyopathic hearts reported earlier (14, 40, 41) that supports the beneficial effect of estrogen on cardiac myofilament Ca\(^{2+}\) activation. Although these data indicate a beneficial effect of HRT, controversies regarding the safety of HRT indicate the necessity to better understand the role of sex hormones and to apply other preventive or therapeutic alternatives.

Exercise training offers one approach to minimize changes in cardiac myofilament Ca\(^{2+}\) activation induced by ovarian sex hormone deprivation. Exercise training has been shown to elicit positive adaptations in the cardiovascular system that results in improved functional capacity and quality of life. A favorable beneficial outcome of exercise training has been indicated in patients with heart failure (3). Moreover, a number of studies examining the effect of exercise training on the contractile performance of cardiac muscle cells demonstrated the increased tension-generating capacity of the myocardium (7–9, 15, 39). Rats exposed to a regimen of treadmill exercise for 13 wk showed significant increases in indexes of cardiac function (15). The improved cardiac function after exercise training was detected without evidence of cardiac apoptosis and with a pattern of cardiac gene expression distinct from pathologic cardiac adaptation. In ovariectomized rats, swimming exercise was able to prevent the shift in cardiac myosin isoenzymes from a predominant V1, the highest Ca\(^{2+}\) -ATPase activity isozyme, to a predominant V3, the lowest Ca\(^{2+}\) -ATPase activity isozyme (19). These beneficial adaptations indicate the probability that the introduction of exercise training may be an alternative mode of prevention for those changes in cardiac myofilament Ca\(^{2+}\) activation induced by ovariectomy.

It is not yet clear how exercise training, a physical stress, improves or protects cardiac function. Generally, two major stress signals, including sympathetic outflow and heat production, are induced during exercise. It is the enhanced inotropic response to β-adrenergic stimulation that underlies the adaptive increase in ventricular performance after exercise training (29, 30). In addition, exercise training reverses the downregulation of β1-adrenergic receptors in chronic hypoxic hearts (12). For the heat signal, the heat-induced endogenous protective protein heat shock protein (HSP) 70 has been documented.
to be a cardioprotective factor (28). An increased expression of HSP72, the inducible form of HSP70, by exercise training has been reported (13). It was, therefore, of interest to investigate whether exercise training exerts cardioprotective effects on myofilament Ca$^{2+}$ activation in ovariectomized rats through possible alterations in expression of β$_1$-adrenergic receptors and/or HSP72.

Experiments reported here focused on two questions. 1) Can exercise training prevent changes in cardiac myofilament Ca$^{2+}$ activation as well as the switching of MHC isoforms in ovariectomized rat hearts? 2) Does exercise training affect the expression of β$_1$-adrenergic receptors and HSP in ovariectomized hearts? We approached these questions by subjecting the exercise groups of sham controls and ovariectomized rats 1 wk after surgery to a 9-wk treadmill-running program. Data from these groups were compared with sedentary controls. Results of our studies support the potential use of adequate exercise training as a preventive measure for the changes in cardiac myofilament Ca$^{2+}$ activation induced by ovariectomized sex hormone deficiency.

MATERIALS AND METHODS

Animal preparation. Female Sprague-Dawley rats weighing between 180 and 200 g (8–9 wk old) were sham operated or ovariectomized and then randomly divided into sedentary and exercise groups. Ovariectomy or a sham operation was performed through bilateral skin incision in the flank area of the lower back as previously described (36). Adequacy of ovariectomy was verified by uterine mass on the day the rats were killed. Individual rats were then housed in an 8 × 10 in. hanging cage with rat Chow and water ad libitum. The 9-wk running program on a motor-driven treadmill five times per week was introduced to the exercise groups 1 wk after surgery. The rats were trained to run during the first week at a fixed speed of 21 m/min with 0% grade, but running time was varied from 2 × 5 min of running with a 10-min resting interval on the day 1 to reach 2 × 25 min of running on the day 5. From the week 2 until the end of the running program, the rats were subjected to 2 × 30 min of running at a fixed speed of 21 m/min but with 7.5 and 5.5% grade for sham and ovariectomized groups, respectively. These grades were calculated for the work rate of 65–75% maximum oxygen consumption on the basis of the body weight of each group, as previously described (2). Adequacy of the exercise running program was determined by citrate synthase activity of plantaris muscle dissected on the day the rats were killed. The animal protocol was approved by the Experimental Animal Committee, Faculty of Science, Mahidol University, in accordance with National Laboratory Animal Centre, Thailand.

Cardiac myofibrillar actomyosin MgATPase activity. Ten weeks after surgery, hearts were rapidly removed from the rats under ether anesthesia and placed in ice-cold saline. Cardiac myofibrils were prepared from the left ventricles, as described by Pagani and Solaro (23). Ca$^{2+}$-dependent MgATPase activity of isolated myofibrils was assayed by determination of inorganic phosphate released in a 10-min linear reaction at 30°C in 2 mM Mg$^{2+}$, 60 mM imidazole, 5 mM Mg ATP$^{2-}$, pH 7.0, and ionic strength of 120 mM. Assays were run at various concentrations of Ca$^{2+}$ ranging from pCa 7.5 to 4.875. Total concentrations of CaCl$_2$, EGTA, KCl, MgCl$_2$, and ATP were calculated from the dissociation constants given by Fabiato (11). The concentration of inorganic phosphate was measured by the method of Carter and Karl (5).

Cardiac membrane preparation. Cardiac membrane was prepared from the left ventricle by the method of Baker and Potter (1) with modifications. Briefly, the left ventricle was homogenized in 10 mM ice-cold Tris-HCl, pH 8.0. The homogenate was incubated in 1 M KCl to dissolve the myofilament proteins and then filtered through layers of cheesecloth. The filtrate was centrifuged at 43,900 g at 4°C for 20 min. The pellet was resuspended in Tris buffer, homogenized, and resedimented. The final pellet was dispersed in ice-cold 50 mM HEPES buffer (pH 8.0) with a Teflon glass homogenizer and was immediately used for receptor binding assay after the protein content was determined by Bradford protein assay kit (Bio-Rad).

β$_1$-Adrenergic receptor binding assay. The binding assay for β$_1$-adrenergic receptors was conducted under equilibrium condition in various concentrations of $[^{3}H]$dihydroalprenolol, as previously described (32). Nonspecific binding was performed in a parallel set of experiments with addition of $[^{3}H]$alprenolol, a specific antagonist of β$_1$-adrenergic receptors. The saturation binding was determined from the relationships between the specific binding and the free ligand by nonlinear least-square regression analysis. Binding parameters, including the density and dissociation constant of the receptors, were determined from a linear transformation of data to the Scatchard plot of bound/free to bound form.

General methods and statistical analyses. Protein contents of β$_1$-adrenergic receptors and HSP72 in the left ventricular homogenate were examined by Western blot analysis with polyclonal antibodies of β$_1$-adrenoceptors (Affinity Bioreagents, Golden, CO) and HSP72 (Stressgen, Victoria, BC). MHC isoforms of left ventricular trabeculae were electrophoretically separated as previously described (37). The immunoblots and the silver stained gels were scanned by GS800 densitometer (Bio-Rad). Data are presented as means ± SE. All curve fittings were performed using GraphPad Inplot (ISI Software). The significance of differences among groups was analyzed by one-way ANOVA followed by the Student-Newman-Keuls test for multiple comparisons. A P value of <0.05 was set for the significant difference among groups.

Materials. All chemicals were purchased from Sigma Chemical (St. Louis, MO) and Fisher Scientific (Pittsburgh, PA). $[^{3}H]$dihydroalprenolol, enhanced chemiluminescence detection kit, and hyperfilm were obtained from Amersham Pharmacia Biotech (Buckinghamshire, UK).

RESULTS

Adequacy of ovariectomy or exercise induced hypertrophy of slow skeletal muscle of the hindlimb (soleus), as represented by percent soleus per body weight in the present study.

Results shown in Fig. 1 demonstrate a complete reversal of the suppressed maximum myofibrillar MgATPase activity detected in sedentary ovariectomized hearts by exercise training. The leftward shift in the pCa-ATPase activity relations with an increase in pC$_{30}$ demonstrated in ovariectomized hearts was also completely abolished by exercise training (Fig. 2). However, there was no change in the slope or Hill coefficient of the pCa-ATPase activity relations in these hearts (data not shown). Further analyses of MHC isoform shift associated with the changes in myofibrillar activity showed that exercise training was able to prevent the significant reduction in the relative amount of α-MHC in ovariectomized hearts (Fig. 3). Exercise training by itself induced no change in the maximum myofibrillar MgATPase activity, the myofibrillar Ca$^{2+}$ sensitivity,
and MHC isoforms in the sham hearts. These results indicate a beneficial role of exercise training in conserving the cardiac myofilbrillar function in the condition of ovarian sex hormone deprivation.

To understand better the significance of the preventive role of exercise training in ovariectomized hearts, we determined the possible preventive effect of exercise training on the upregulation of \(\beta_1\)-adrenergic receptors in ovariectomized hearts. As expected, deprivation of ovarian sex hormones for 10 wk induced a significant increase in the density of \(\beta_1\)-adrenoceptors (~25%) compared with that of sham controls (Fig. 4A). Although exercise training had no effect on \(\beta_1\)-adrenoceptors in hearts of sham rats, there was a prevention of the upregulation of the receptors in the hearts of exercise-ovariectomized rats (Fig. 4A) without any effect on the receptor binding affinity (Fig. 4B). Further determination of \(\beta_1\)-adrenoceptor proteins with the use of immunoblot analyses of the left ventricular homogenate confirmed the complete preven-

Table 1. Body, heart, uterine, and soleus weights and plantaris citrate synthase activities from sham controls and OVX rats of sedentary and exercise groups

<table>
<thead>
<tr>
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<th>Sham</th>
<th>OVX</th>
<th>Sham</th>
<th>OVX</th>
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<tr>
<td>Body weight, g</td>
<td>265 ± 5.1</td>
<td>336 ± 7.5*</td>
<td>280 ± 3.9*</td>
<td>324 ± 3.2*</td>
</tr>
<tr>
<td>Heart weight, g</td>
<td>0.80 ± 0.02</td>
<td>0.93 ± 0.01*</td>
<td>0.89 ± 0.02*</td>
<td>0.95 ± 0.02*</td>
</tr>
<tr>
<td>Uterine weight, g</td>
<td>0.56 ± 0.03</td>
<td>0.11 ± 0.00*</td>
<td>0.53 ± 0.04</td>
<td>0.10 ± 0.009*</td>
</tr>
<tr>
<td>Soleus weight, g</td>
<td>0.094 ± 0.001</td>
<td>0.128 ± 0.005*</td>
<td>0.118 ± 0.004*</td>
<td>0.118 ± 0.003*</td>
</tr>
<tr>
<td>% Heart/body wt</td>
<td>0.304 ± 0.004</td>
<td>0.278 ± 0.005*</td>
<td>0.318 ± 0.004*</td>
<td>0.293 ± 0.003†</td>
</tr>
<tr>
<td>% Soleus/body wt</td>
<td>0.037 ± 0.001</td>
<td>0.038 ± 0.001</td>
<td>0.039 ± 0.001</td>
<td>0.036 ± 0.000</td>
</tr>
<tr>
<td>Citrate synthase activity, (\text{mol/g protein}^{-1}\text{min}^{-1})</td>
<td>44.6 ± 1.9</td>
<td>44.4 ± 1.9</td>
<td>57.8 ± 1.9*</td>
<td>52.4 ± 1.6*</td>
</tr>
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Values are means ± SE of 8–9 rats. OVX, ovariectomized. Significant difference from sedentary sham controls and sedentary OVX rats (*\(P < 0.05\) and †\(P < 0.05\), respectively).
tion of receptor upregulation (Fig. 5). These results thus indicate the significant preventive role of exercise training in the expression of cardiac $\beta_1$-adrenoceptor proteins, which could further modify the myofibrillar activity, in ovariectomized hearts.

To test for a possible contribution of HSP in the cardioprotective role of exercise training in ovariectomized hearts, HSP72 was examined by blot analysis. There was a downregulation of HSP72 evident in hearts of ovariectomized rats compared with those of sham controls (Fig. 6). The level of HSP72 in hearts of shams was not affected by exercise training. On the other hand, exercise training in ovariectomized rats completely abolished the downregulation of HSP72 protein. These results imply a possibly similar mechanism of female sex hormones and exercise training in protecting changes in cardiac performance.

DISCUSSION

Results from the present study in ovariectomized rats provide important and novel evidence for exercise training as a...
cardioprotective alternative in ovarian sex hormone-deficient condition. Our study points to a possibility that regular running with moderate intensity could normalize the changes in myofilament Ca$^{2+}$ activation associated with ovariectomy, including suppression and Ca$^{2+}$ hypersensitivity of myofilament Ca$^{2+}$ activation, and shift MHC isofoms toward β-MHC. These cardioprotective effects of exercise training may be responsible by alterations in the expression of β$_1$-adrenergic receptors and HSP72.

It is theoretically possible that HRT provides the best risk-benefit profile for the prevention of cardiovascular disease in postmenopausal women. Many previous studies in animal models provide evidence supporting the idea that estrogen supplementation prevents cardiac contractile changes after chronic deprivation of female sex hormones (27, 32, 38). However, results from the clinical trial in an average 5.2-yr follow-up among healthy postmenopausal US women using a combined regimen of estrogen plus progestin indicate that the treatment is generally not beneficial (42). Our data indicate the potential importance of exercise training as a cost-effective alternative strategy.

Results presented here extend our understanding of the molecular adaptations in ovarian sex hormone-deprived hearts to moderate-intensity regular running. The American Heart Association Scientific Statement recently released for health professionals has summarized the evidence for the benefits of physical activity in the prevention and treatment of cardiovascular disease (33). Although there is sufficient evidence to encourage increased exercise and physical activity for the public and most patient groups, additional physiological and basic research is needed to provide the scientific rationale to support the importance of such a recommendation. Importantly, the underlying mechanisms by which exercise training reduces cardiovascular risk should be addressed as indicated in the present study.

Changes in the sympathetic control of the heart are well known to modify the myofilament Ca$^{2+}$ activation via many protein phosphorylations. Physiologically, adrenergic stimulation induces improvement of cardiac contraction and relaxation cycle. Pharmacologically, administration of β-adrenergic agonists has been shown to decrease survival of patients with chronic heart failure (22). It is presently accepted that chronic adrenergic signaling is a harmful compensatory mechanism to the heart (4, 10, 17). A short-lived improvement of cardiac function but a final production of cardiomyopathic phenotype with dilation and depressed contractile function has been demonstrated in transgenic overexpression of human β$_1$-adrenergic receptors in the heart (4, 10). Despite an unclear conclusion whether the upregulation of β$_1$-adrenoceptors in ovariectomized hearts was induced by a direct effect or an adaptive response of hormone deficiency, normalization of the receptor level should be a beneficial outcome to the cardiac performance. However, it is not known at this point how exercise training normalizes the upregulation of β$_1$-adrenergic receptors as well as the consequent activation of myofilament in ovariectomized hearts. Inasmuch as exercise training has no effect on the level of β$_1$-adrenergic receptors in sham hearts, as shown in the present study and in other previous reports (12, 26), additional studies are needed to better understand the underlying mechanisms of exercise training on the expression of β$_1$-adrenergic receptors.

Induction of HSP72 by exercise training was also suggested from our results to be a protective factor of molecular alterations in myofilament Ca$^{2+}$ activation in ovariectomized hearts. Upregulation of HSP synthesis is considered to be a powerful physiological route involved in crucial cellular homeostatic mechanisms against a number of stresses (28). The cytoprotective function of HSP in rat ventricle after a global ischemic insult has been demonstrated (6). Furthermore, a direct cardioprotective effect of HSP72 against myocardial trauma has been reported in studies using transgenic models (20, 25, 34). Sufficient enhancement of HSP synthesis in mammalian cells and tissues, including cardiomyocytes, by exercise training has been found (13, 18). It was also shown that female rats have twice as much HSP72 as male hearts and that estrogen is responsible for the sexual dimorphism in the expression of cardiac HSP72 (35). A single bout of exercise was also demonstrated to induce a significant increase in cardiac HSP72 expression in ovariectomized but not estrogen-positive rats (24). In addition, a decrease in Ca$^{2+}$ sensitivity of force generation in dog ventricular trabeculae after an application of HSP coinducer bimoclomol was also reported (31). Our laboratory’s previous reports (36, 37) regarding changes in myofilament Ca$^{2+}$ activation and the preventive effects of exercise training demonstrated in the present study thus point to the significance of HSP72 induction by exercise training in the molecular adaptations of cardiac myofilament in ovariectomized rats. However, it is not known at present exactly how exercise signals the upregulation of HSP72. Thermal stress (13) and/or cardiomyocyte stretch sensors may be involved (16).

Results in the present study support the preventive effects of exercise training on the molecular alterations in ovarian sex hormone-deprived rat hearts. Despite the effective outcomes in ovariectomized rats, it would be too early to suggest the use of exercise training for cardiac preventive treatment in postmenopausal women. Yet, prudent application of exercise training as an additional treatment in addition to the regularly used preventive regimen should do no harm.

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