study. They were fed Purita rat chow and water ad libitum except the pair-fed ovariectomized rats to which daily food intake was given in exactly the same amount as that consumed by sham-operated control group. In hormone supplement groups, ovariectomized rats were subcutaneously injected with estrogen (5 μg/rat), progesterone (1 mg/rat), or estrogen plus progesterone in a fixed volume of 0.1 ml three times a week starting two days after the operation. In control groups, sham-operated and ovariectomized controls, the rats were injected with corn oil (vehicle) in the same manner as in hormone-treated groups. After ten-week period, the rats were anesthetized with ether and the hearts were quickly excised, weighed, and placed in ice-cold saline for cardiac membrane preparations. Successful ovariectomy was verified by the absence of ovarian tissues and by decreased uterine weights at the time of sacrifice.

Cardiac Membrane Preparation

Cardiac membrane was prepared from the left ventricle using the method of Baker and Potter [14] with some modifications. Briefly, the left ventricle was homogenized using Omni Macro Homoginizer in 10 mM ice-cold Tris-HCl, pH 8.0. The homogenate was incubated on ice for 20 minutes in the presence of 1 M KCl to dissolve the myofilament proteins. The homogenate was filtered through layers of cheesecloth and the filtrate was then centrifuged at 43,900 g, 4 °C for 20 minutes. The pellet was resuspended in Tris buffer, homogenized, and resedimented by the same speed of centrifugation. The final pellet was dispersed in ice-cold 50 mM HEPES buffer (pH 8.0) using taflon-glass homogenizer. Protein content was determined by the method of Bradford [15] and was immediately used for receptor binding assay.

β_1-Adrenergic Receptor Binding Assay

The binding assay for β_1-adrenergic receptors was performed under an equilibrium condition following the procedure described earlier [16]. Approximately 250 μg/ml of the cardiac membrane protein in various concentrations (0.5–40 nM) of [^{3}H]-DHA, were incubated for 20 minutes at 25 °C in 50 mM HEPES buffer solution (pH 8.0) containing 4 mM $MgCl_2$. The bound and free ligands were separated by the addition of 4 ml ice-cold 25 mM HEPES buffer containing 4 mM $MgCl_2$, followed by rapid vacuum filtration through Whatman GF/B filters. The filters were rinsed twice and the radioactivity was then measured by liquid scintillation counter (LKB Wallac 1219 Rackbeta). Nonspecific binding was evaluated in a parallel set of experiments with addition of 20 μM (-)-alprenolol. The saturation binding curve was determined from the relations between the specific binding (fmole/mg protein) and the free ligand (nM). The binding parameters including the density (B_{max}) and dissociation constant (K_d) of the receptor were determined from a linear transformation of data to the Scatchard Plot of bound/free to bound form.

Western Blot Analysis of β_1-Adrenergic Receptor Protein

Protein contents of the β_1-adrenergic receptors in the left ventricular homogenate were determined following the procedure described earlier [17]. Briefly, the left ventricular homogenate was separated by 7.5% polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE) and then electrophoretically transferred onto polyvinylidene difluoride (PVDF) membrane. The non-

specific binding of the membrane was blocked with 5% skim-milk in TBST containing 50 mM Tris-base, 150 mM NaCl, 0.1% v/v Tween-20, pH 7.5 for 2 hour at room temperature. The membrane was then incubated in 1:2,000 dilution of polyclonal antibodies against β_1-adrenergic receptors overnight at 4 °C. The horseradish peroxidase (HRP)-linked anti-rabbit IgG with a dilution of 1:5,000 was used as the secondary antibody. Protein bands reacting with the antibody were visualized using the enhanced chemiluminescence (ECL) detection system and quantified by laser-scanning densitometry of the immunoblots.

General Methods and Statistics

All data analyses were performed with a microcomputer. Curves relating the specific binding and the free ligand concentration were fitted to the binding isotherm equation using non-linear least squares regression analysis. All of the curve fittings were performed using GraphPad Inplot, ISI software, version 4. Data were presented as mean ± standard error of mean (SE). The significant effect of ovarian sex hormone deficiency on the receptor binding was determined among the groups of ovariectomized, pair-fed ovariectomized, and sham-operated rats using one way analysis of variance (ANOVA) followed by a Student-Newman-Keuls test for multiple comparisons. ANOVA was also used for comparisons among the ovariectomized treated groups and the ovariectomized control rats. The p-value of less than 0.05 was set for the significant difference between groups.

Materials

All chemicals were purchased from Sigma, St. Louis, MO, and Fisher Scientific, Pittsburgh, PA. [^{3}H]-DHA with specific activity of 96 Ci/mmole and the ECL detection kit were obtained from Amersham Pharmacia Biotech, Piscataway, NJ. Polyclonal antibody of β_1-adrenergic receptor protein was purchased from Affinity Bioreagents, Golden, CO. The HRP-linked anti-rabbit IgG was obtained from Zymed Laboratories, San Francisco, CA.

Table 1
Body, heart, and uterine weights of sham-operated, pair-fed ovariectomized, ovariectomized and various hormone-treated ovariectomized rats

	n	BW (g)	HW (g)	% HW/BW	UW (g)
SHAM + OIL	8	263 ± 3.0	0.82 ± 0.02	0.312 ± 0.007	0.41 ± 0.03
PF-OVX	8	265 ± 6.1	0.77 ± 0.02	0.291 ± 0.005*	0.08 ± 0.01*
OVX + OIL	8	340 ± 7.3*	0.96 ± 0.02*	0.282 ± 0.005*	0.10 ± 0.01*
OVX + E	8	233 ± 4.9#	0.79 ± 0.01#	0.341 ± 0.007#	0.48 ± 0.03#
OVX + P	7	318 ± 11.6	0.94 ± 0.02	0.297 ± 0.008	0.10 ± 0.01
OVX + EP	8	235 ± 3.9#	0.77 ± 0.01#	0.327 ± 0.007#	0.52 ± 0.04#

Body weight (BW), heart weight (HW), % HW/BW, and uterine weight (UW) of sham-operated rats injected with corn oil (SHAM + OIL), pair-fed ovariectomized (PF-OVX) rats, ovariectomized rats injected with corn oil (OVX + OIL), and ovariectomized rats injected with estrogen (E), progesterone (P), or estrogen plus progesterone (EP). Each value represents the mean ± SE. n = number of rats.

*$P < 0.05$ = significantly different from sham-operated control using Student-Newman-Keuls Test after ANOVA.

#$P < 0.05$ = significantly different from ovariectomized control using Student-Newman-Keuls Test after ANOVA.

Results

As summarized in Table 1, adequacy of ovarian sex hormone deficiency in ovariectomized rats either with or without pair feeding was verified by significant reduction in the uterine mass of the animals as compared to those of sham-operated control. Supplementation with either estrogen or estrogen plus progesterone after ovariectomy could prevent the reduction in uterine weights. Similarly, the increase in body weights after ovariectomy was prevented by estrogen supplement. In contrast, replacement with progesterone in ovariectomized rats did not restore either body weight or uterine weight.

To test the significance of ovarian sex hormones in the expression of β_1-adrenergic receptors in the cardiac membrane, the receptor binding analysis was performed in ovariectomized hearts using [^{3}H]-DHA as a ligand. The saturation bindings of the β_1-adrenergic receptors from cardiac membrane preparations of both sham and ovariectomized hearts were obtained as shown in Fig. 1A. However, an

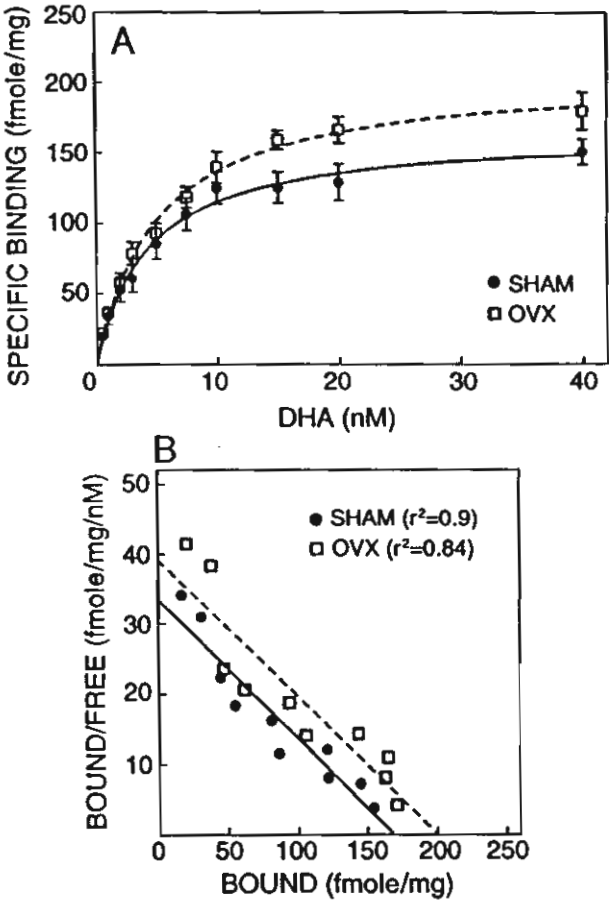

Fig. 1. Saturation binding of [^{3}H]-DHA to cardiac sarcolemmal membranes (A) prepared from sham (●) and ovariectomized (□) rat hearts. Assays were performed as described in the Methods. Values represent mean ± SE of eight preparations with one heart per preparation. The Scatchard Plots of one representative set of data from sham (●) and ovariectomized (□) rat hearts were depicted in (B).

upward shift of the binding isotherm from ovariectomized hearts was observed. A parallel upward shift of the Scatchard Plot from the ovariectomized hearts as compared to that of sham-operated control as shown by a representative data set was clearly indicated (Fig. 1B). Interestingly, in pair-fed ovariectomized rats with no body weight gain but with ovarian sex hormone deprivation, the upward shift was evident in the binding isotherm and the Scatchard Plot as shown by the representative data set (Fig. 2A,B). These data imply a significant change in the binding density but not the binding affinity of the receptors in ovariectomized hearts. When the binding parameters of each individual set of the experiments were calculated from the Scatchard Plot, significant increases in the density of β_1-adrenergic receptors were detected in both ovariectomized and pair-fed ovariectomized hearts as compared to that of sham control (Fig. 3A). In contrast, ovarian sex hormone deficiency induced no change in the binding affinity of the receptors as indicated by the dissociation constant summarized in Fig. 3B.

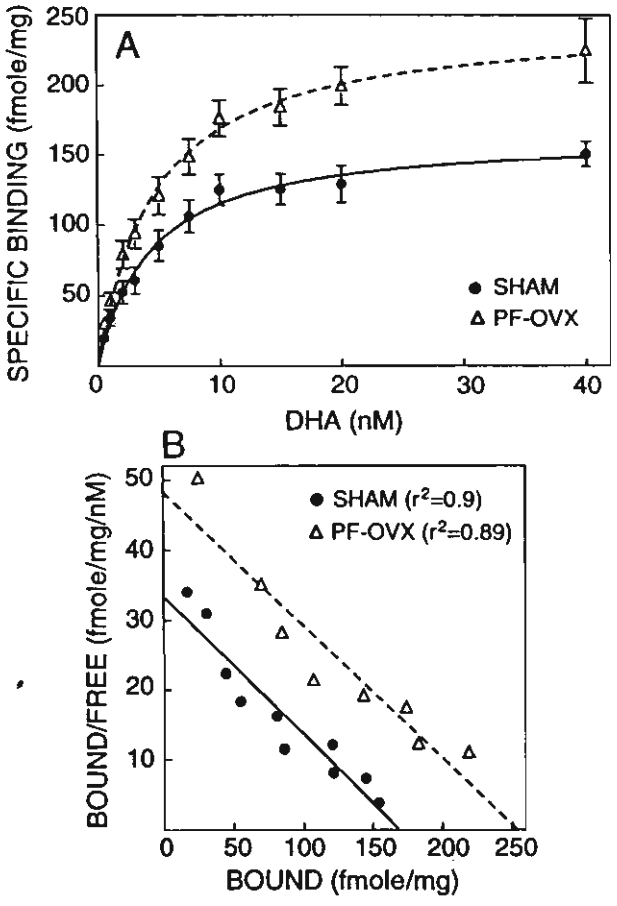

Fig. 2. Saturation binding of [^{3}H]-DHA to cardiac sarcolemmal membranes (A) prepared from sham (●) and pair-fed ovariectomized (△) rat hearts. Assays were performed as described in the Methods. Values represent mean ± SE of eight preparations with one heart per preparation. The Scatchard Plots of one representative set of data from sham (●) and pair-fed ovariectomized (△) rat hearts were presented in (B).

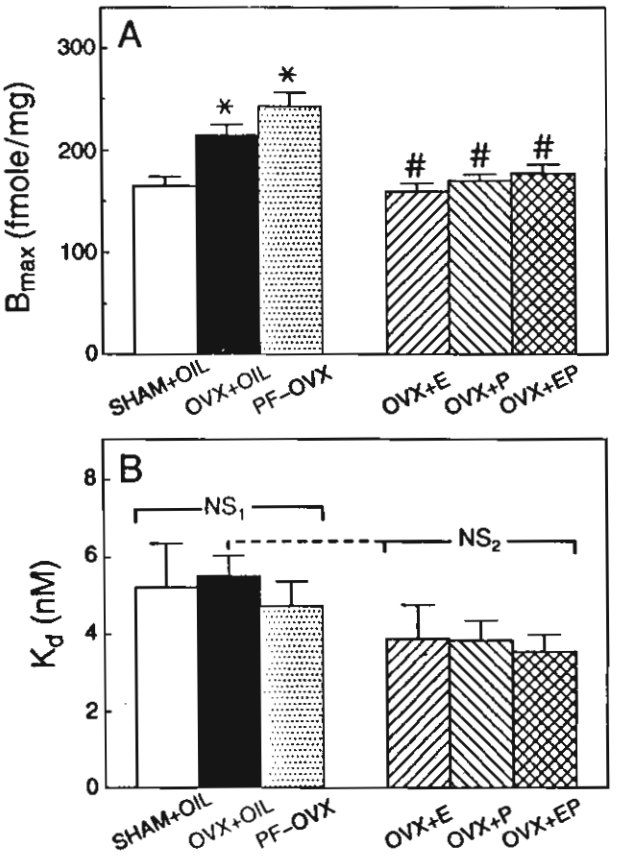

Fig. 3. The density (B_{max}, **A**) and dissociation constant (K_d, **B**) of β_1-adrenergic receptors in cardiac membrane preparations from sham control injected with oil (SHAM + OIL), pair-fed ovariectomized rats (PF-OVX), and various groups of ovariectomized rats injected with oil (OVX + OIL), estrogen (OVX + E), progesterone (OVX + P), or estrogen plus progesterone (OVX + EP). Values represent mean ± SE of 7–8 hearts. * $P < 0.05$ = significantly different from SHAM + OIL using Student-Newman-Keuls Test after ANOVA. # $P < 0.05$ = significantly different from OVX + OIL using Student-Newman-Keuls Test after ANOVA. NS = not significantly different among the groups.

These results clearly indicate a significant effect of ovarian sex hormone deprivation on the upregulation of the β_1-adrenergic receptors in cardiac myocyte membrane without any contribution of ovariectomy-induced body weight gain.

To identify the hormone that may be responsible for upregulating the β_1-adrenergic receptors in the cardiac myocytes, receptor binding assays using cardiac membrane preparations from various groups of ovariectomized rats supplemented with different ovarian sex hormones were compared. As compared to ovariectomized control hearts, replacement of estrogen significantly prevented the increase in β_1-adrenergic receptor density with no effect on the binding affinity of the receptor as shown in Fig. 3A and 3B, respectively. Interestingly, despite no preventive effects on the body and uterine weights (Table 1), replacement of progesterone after ovariectomy could still significantly prevent the increase in the receptor density. Moreover, this preventive effect of either estrogen or progesterone on the receptor density was not additive when the combined regimen of estrogen plus progesterone was given after

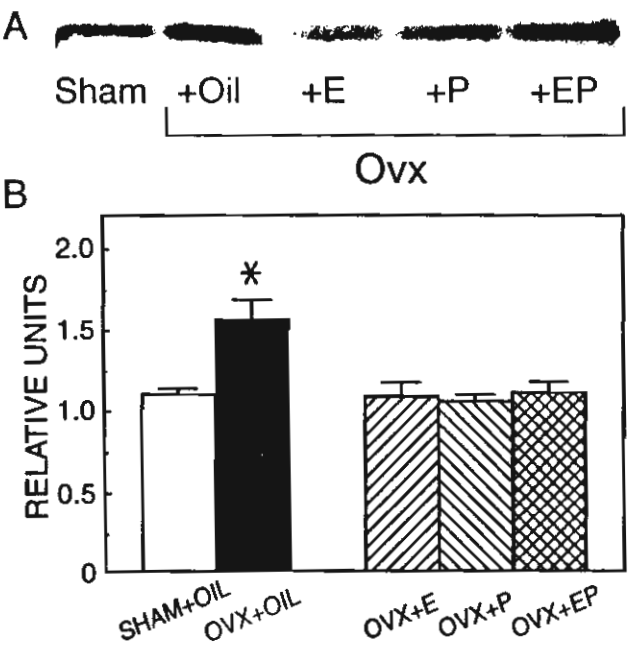

Fig. 4. Immunoblot of β_1-adrenergic receptor proteins from left ventricular homogenates of various groups of rat hearts (A) and summary of immunoblot analyses from each group (B). Data are presented as mean ± SE of 4–6 hearts. * $P < 0.05$ = significantly different from other groups using Student-Newman-Keuls Test after ANOVA.

ovariectomy (Fig. 3). The findings from receptor binding study were confirmed by the immunoblot analyses of the whole heart homogenates from various groups of the experimental rats (Fig. 4). These results thus indicate a significant suppressive effect of ovarian sex hormones on the expression of cardiac β_1-adrenergic receptors possibly via their common mechanism of genomic action.

Discussion

It is well accepted that changes in sympathetic control of the heart may lead to alterations in myofibrillar protein phosphorylation and, therefore, myofilament Ca^{2+} sensitivity. Results presented in this study provide the first evidence that long-term deprivation of ovarian sex hormones in rats induces an upregulation of cardiac β_1-adrenergic receptors without affecting the receptor binding affinity. The body weight gain after ovariectomy contributes no effect on the receptor activity. In addition, hormonal supplementations either with estrogen or progesterone could prevent this upregulation of the receptors.

The upregulation of the β_1-adrenergic receptor density is known to be toxic to the hearts [9], even though the downregulation of the receptors has been recognized as one characteristic of human heart failure [18]. It remains unclear whether the downregulation of β-adrenoceptors in the failing heart is a direct result of heart failure or a contributing factor in the progression of decompensation. However, a recent study suggested that the reduction in β-adrenergic receptors in the failing heart may be features of the decompensated state [19]. The norepinephrine spillover into the plasma of these patients [20] despite a decrease in receptor density does indicate the persistent adrenergic drive to the failing hearts. Many lines of

evidence have indicated that it is the increase in adrenergic drive to the heart that is initially supportive but then ultimately damaging to the failing heart [21–23]. In transgenic mice model, overexpression of human β_1-adrenergic receptors in the heart produces a short-lived improvement of cardiac function but it ultimately produces cardiomyopathic phenotype with dilation and depressed contractile functions [10,24]. These data indicate that chronic adrenergic signaling is a harmful compensatory mechanism to the heart. Nevertheless, they provide the fundamental basis for the use of antiadrenergic agents in the treatment of chronic heart failure [25]. Recently, β-blockers have been evaluated in many randomized controlled clinical trials involving more than 10,000 patients with mild, moderate, or severe heart failure [26–31]. The same conclusions have been drawn from these studies that the use of β-blockers was associated with reductions in morbidity and mortality in heart failure patients. Despite the relative resemblance of the effects of ovariectomy and cardiomyopathy on the cardiac function reported earlier [1] and also on the upregulation of β_1-adrenoceptors reported here, it would be premature to suggest the use of antiadrenergic agents for cardiac preventive treatment after ovarian sex hormone deprivation such as in menopause. Additional studies are needed to provide a better understanding of the underlying mechanisms of changes in cardiac myofilament activation induced after ovarian sex hormone deficiency before introducing the preventive approach for cardiovascular diseases in women.

It is unknown whether β_1-adrenoceptor upregulation in the ovariectomized heart demonstrated in this study is a result of ablating a direct effect of ovarian sex hormones on the receptor expression or a contributing factor in cardiac adaptations to the decreased contractile function. The presence of both estrogen and progesterone receptors in the myocardial tissues undoubtedly indicate the possible direct effect of these hormones on the hearts [32,33]. Although we did not measure the catecholamine levels in this study, both the norepinephrine and epinephrine levels have been reported to be unaltered in ovariectomized hearts but significantly increased in food-restricted ovariectomized hearts [13]. The increased receptor density with normal cathecholamine level simply implies increased adrenergic drive to the ovariectomized hearts. Inasmuch as the same magnitude of receptor upregulation was also detected in the pair-fed ovariectomized hearts in which the cathecholamine concentration was likely to be elevated, a possible suppressed expression of β_1-adrenoceptors by ovarian sex hormones can then be postulated. This assertion is supported by the fact that estrogen receptors may repress the transcription of selected target genes by interacting with corepressors [34]. If this effect was actually the case for β_1-adrenergic receptor upregulation after ovarian sex hormone deprivation, changes in myofilament activation demonstrated in the ovariectomized hearts should then more likely be a response rather than a contributing cause of the change in receptor levels. Dose-response and time course studies of the direct action of ovarian sex hormones on the gene and protein expressions of the β_1-adrenergic receptors using isolated cardiac myocyte preparations should provide more insights into a possible direct effect of the hormones.

It is not clear at this point how the upregulation of β_1-adrenergic receptors is associated with the increase in myofilament calcium responsiveness in ovariectomized hearts. However, a decrease in Ca^{2+} myofilament sensitivity following sympathetic stimulation on β-adrenergic receptors has been shown [35,36]. The specific phosphorylation of cardiac troponin I (cTnI) isoform expressed in cardiac tissue by PKA has been defined as the basis for the decrease in myofilament Ca^{2+} sensitivity after β-adrenergic stimulation [37]. Based on this information, it is speculated that an increase in myofilament sensitivity to Ca^{2+} in ovariectomized hearts may result from impaired sympathetic response with a lower level of phosphorylated cTnI. This possibility is indirectly supported by a report of reduced extent of cTnI phosphorylation by PKA in preparations from failing human hearts [38] in which the similar increase in calcium responsiveness has been demonstrated. Upregulation of β_1-adrenoceptors in the ovariectomized hearts simply implies

effects of ovarian sex hormone deficiency on sympathetic modulations at post-receptor level in the heart. Further studies in the post-receptor signaling process as well as cTnI phosphorylation should provide additional insight into the altered myofilament Ca^{2+} responsiveness following ovariectomy.

The differential effects of estrogen and progesterone on the regulation of cardiac myofilament Ca^{2+}-activation previously demonstrated in our laboratory were not observed in the present study of cardiac β_1-adrenoceptor expressions. It has been demonstrated that either estrogen or progesterone supplement could prevent the decrease in maximum myofilament ATPase activity in ovariectomized hearts [3]. In contrast, only the estrogen replacement could abolish the Ca^{2+} hypersensitivity of the myofilaments in these hearts. In the present study the upregulation of cardiac β_1-adrenoceptors could be completely reversed by either estrogen or progesterone supplement. These results indirectly imply that the steroid-mediated genomic regulation of estrogen and progesterone is able to modulate the transcription of the β_1-adrenergic receptor gene in the hearts. In addition, a practicably common mechanism by which these hormones exert their transcriptional regulation could also be suggested since no additive effect was observed. These results suggest a potential benefit of estrogen replacement on the cardiac myofibrillar activity as well as the expression of β_1-adrenergic receptors. Many information from recent clinical studies of hormone replacement therapy in postmenopausal women also indicate significant improvement in cardiac functions both at rest and during exercise [39–41]. Moreover, these results suggest that changes in the density of the β_1-adrenergic receptors may partly contribute to the changes in myofilament Ca^{2+} activation in ovariectomized hearts.

Conclusion

In the present study, a possible direct suppressive effect of ovarian sex hormones on the expression of cardiac β_1-adrenergic receptors was demonstrated. Long-term deprivation of ovarian sex hormones induced receptor upregulation which could in turn detrimentally affect the cardiac functions through over stimulation of the β-adrenergic activity.

Acknowledgements

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FINAL ACCEPTED VERSION

CARDIO-PROTECTIVE EFFECTS OF EXERCISE TRAINING ON MYOFILAMENT CALCIUM ACTIVATION IN OVARIECTOMIZED RATS

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Running head: Exercise training and ovariectomized rat hearts

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ABSTRACT

The risks associated with hormone replacement therapy (HRT) especially cardiac diseases in postmenopausal women have prompted extensive studies for other preventive or therapeutic alternatives. We investigated the cardio-protective effects of exercise training on the changes in cardiac myofilament Ca^{2+} activation in 10-wk ovariectomized rats. The exercise groups were subjected to a nine-wk running program on a motor-driven treadmill one week after surgery. The relation between pCa (-log molar free Ca^{2+} concentration) and myofibrillar MgATPase activity of exercise-sham myofibrils or exercise-ovariectomized myofibrils was the same and could not be distinguished from that of sedentary-sham control hearts. In contrast, a significant suppression in maximum MgATPase activity and a leftward shift of pCa_{50} (half-maximally activating pCa) in the pCa-ATPase activity relationship were detected in sedentary-ovariectomized rats. Exercise training also prevented the shift in myosin heavy chain (MHC) isoforms towards β-MHC in ovariectomized hearts. The upregulation of β_1-adrenergic receptors in the left ventricular membranes of ovariectomized rat hearts, as measured using receptor binding and immunoblot analyses, was no longer observed in exercise-ovariectomized hearts. Immuno-blot analyses of heat shock protein (Hsp) 72, an inducible form of Hsp70, demonstrated a significant downregulation in ovariectomized hearts. Exercise training in ovariectomized rats completely reversed the expression of Hsp72 to the same level as sham controls. Our results clearly indicate the cardio-protective effects of exercise training on changes in cardiac myofilament Ca^{2+} activation in ovariectomized rats. Alterations in expression of β_1-adrenergic receptors and Hsp72 may in part play a mechanistic role in the cardio-protective effects.

Keywords: myofilament Ca^{2+} activation, ovarian sex hormones, myosin heavy chain, β_1-adrenergic receptors, heat shock protein

INTRODUCTION

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). Even though steroid sex hormones have been hypothesized to play a significant role in myocardial function, the mechanisms of the effects of these hormones is not well understood. A myofibrillar Ca^{2+} hypersensitivity, a suppressed maximum myofibrillar ATPase activity, and a significant shift in MHC towards 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–adrenoceptors, which may partly contribute to changes in myofilament Ca^{2+} activation, was detected in ovariectomized hearts (32). Interestingly, differential cardio-regulatory 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 weeks showed significant increases in indices 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 pathological cardiac adaptation. In ovariectomized rats, swimming exercise was able to prevent the shift in cardiac myosin isoenzymes from a predominant V_1 to a predominant V_3 (19). These beneficial adaptations indicate the probability that introducing 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 β_1-adrenergic receptors in chronic hypoxic hearts (12). For the heat signal, the heat-induced endogenous protective protein, Hsp70, has been documented to be a cardio-protective 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 cardio-protective 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 one week after surgery to a nine-weeks of a treadmill-running program. Data from these groups were compared to 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 ovarian 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 sacrificed. Individual rats were then housed in 8"x10" hanging cage with rat chow and water *ad libitum*. The nine-wk running program on a motor-driven treadmill five times a week was introduced to the exercise groups one week after surgery. The rats were trained to run during the first week at a fixed speed of 21 m/min with 0% grade but varied running time from 5-min twice running with 10 min resting interval on the first day to reach 25 min twice running on the fifth day. From the second week till the end of the running program, the rats were subjected to 30 min twice running at 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 based on 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 sacrificed. 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 (Pi) 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 using a computer program generated from the dissociation constants given by Fabiato (11). The concentration of Pi was measured by the method of Carter and Karl (5).

Cardiac membrane preparation. Cardiac membrane was prepared from the left ventricle using the method of Backer 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, 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) using Teflon-glass homogenizer and was immediately used for receptor binding assay after determining the protein content 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 ([^{3}H]-DHA) as previously described (32). Nonspecific binding was performed in a parallel set of experiments with addition of -/- alprenolol, a specific antagonist of β_1-adrenergic receptors. The saturation binding was determined from the relations between the specific binding and the free ligand using non-linear least square regression analysis. Binding parameters including the density (B_{max}) and dissociation constant (K_d) 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 using polyclonal antibodies of β_1-adrenoceptors (Affinity Bioreagents, Golden, CO) and Hsp72

(Stressgen, Victoria, BC). Myosin heavy chain 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 were presented as means ± standard error of mean (SE). All curve fittings were performed using GraphPad Inplot, ISI software. The significance of differences among groups was analyzed using one way analysis of variance (ANOVA) followed by the Student-Newman-Keul test for multiple comparisons. A p-value of less than 0.05 was set for the significant difference among groups.

Materials. All chemicals were purchased from Sigma, St Louis, MO, and Fisher Scientific, Pittsburgh, PA. [^{3}H]-DHA, ECL detection kit and hyperfilm were obtained from Amersham Pharmacia Biotech, Buckinghamshire, England.

RESULTS

Adequacy of ovarian sex hormone deficiency was clearly demonstrated by a significant reduction in the uterine weight in ovariectomized groups when compared with that of sham controls (Table 1). Efficiency of the running program was verified by a significant increase in citrate synthase activities of plantaris muscles isolated from exercised rats. The cardiac hypertrophic effect of exercise training as determined by the ratio of heart to body weight was also demonstrated in both groups of exercised rats when compared with sedentary controls. In contrast, neither ovariectomy nor exercise induced hypertrophy of slow skeletal muscle of hind limb (soleus) as represented by % soleus/body weight in the present study.

Results shown in Fig. 1, A and B 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 pCa_{50} demonstrated in ovariectomized hearts was also completely abolished by exercise training (Fig. 2, A and B). 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, A and B). 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 myofibrillar 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 β_1-adrenergic receptors in ovariectomized hearts. As expected, deprivation of

ovarian sex hormones for 10 weeks induced a significant increase in the density of β_1-adrenoceptors (~25%) as compared to that of sham controls (Fig. 4A). While exercise training had no effect on β_1-adrenoceptors in hearts of sham rats, there was a prevention of the up-regulation 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 β_1-adrenoceptor proteins using immunoblot analyses of the left ventricular homogenate confirmed the complete prevention of receptor upregulation (Fig. 5). These results thus indicate the significant preventive role of exercise training in the expression of cardiac β_1-adrenoceptor proteins, which could further modify the myofibrillar activity, in ovariectomized hearts.

To test for a possible contribution of Hsp in the cardio-protective role of exercise training in ovariectomized hearts, Hsp72 was examined using blot analysis. There was a downregulation of Hsp72 evident in hearts of ovariectomized rats as compared to 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 cardio-protective 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 in MHC isoforms towards β-MHC. These cardio-protective 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-year 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 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 currently 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 involving in crucial cellular homeostatic mechanisms against a number of stresses (28). The

cyto-protective function of Hsp in rat ventricle after a global ischemic insult has been demonstrated (6). Furthermore, a direct cardio-protective 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 exercise was also demonstrated to induce a significant increase in the 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 a Hsp-coinducer, bimoclomol, was also reported (31). Our 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 the cardiomyocyte stretch sensors may be involved (16).

Results in the current 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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FIGURE LEGENDS

Fig. 1. **(A)** Effects of ovariectomy and exercise training on the Ca^{2+}-dependent MgATPase activities of cardiac myofibrils in various calcium concentrations ranging from pCa 7.5 to 4.875, pH 7.0. **(B)** Comparisons of maximum MgATPase activities between sham controls (SHAM) and ovariectomized rats (OVX) of sedentary and exercise groups. Data are means ± SE from 8-9 preparations with 2-3 rat hearts for each preparation. * $P<0.05$ = significantly different from sedentary-sham controls using Student Newman-Keuls test after ANOVA.

Fig. 2. **(A)** Effects of ovariectomy and exercise training on the % maximum MgATPase activities of cardiac myofibrils in various calcium concentrations ranging from pCa 7.5 to 4.875, pH 7.0. **(B)** Comparisons of pCa_{50} determinants between sham controls (SHAM) and ovariectomized rats (OVX) of sedentary and exercise groups. Data are means ± SE from 8-9 preparations with 2-3 rat hearts for each preparation. * $P<0.05$ = significantly different from sedentary-sham controls using Student Newman-Keuls test after ANOVA.

Fig. 3. **(A)** Myosin heavy chain (MHC) region of SDS gels on which were samples of left ventricular trabeculae from sham controls (SHAM) and ovariectomized rats (OVX) of sedentary and exercise groups. **(B)** Relative amount of α-MHC (as a percentage of total MHC) of left ventricular trabeculae from each group. Data are means ± SE from 8 hearts. * $P<0.05$ = significantly different from sedentary-sham controls using Student Newman-Keuls test after ANOVA.

Fig. 4. Comparisons of density (B_{max}, **A**) and dissociation constant (K_d, **B**) of cardiac β_1-adrenergic receptors in left ventricular membrane preparations from sham controls (SHAM) and ovariectomized rats (OVX) of sedentary and exercise groups. Data are means ± SE of 8 hearts. * $P<0.05$ = significantly different from sedentary-sham controls using Student Newman-Keuls test after ANOVA.

Fig. 5. Region of β_1-adrenergic receptor proteins on immunoblots on which were samples of left ventricular homogenates from sham controls (SHAM) and ovariectomized rats (OVX) of sedentary and exercise groups **(upper panel)** and comparisons of the relative intensity unit from densitometry of immunoblots from each group **(lower panel)**. Data are means ± SE from 4 hearts. * $P<0.05$ = significantly different from sedentary-sham controls using Student Newman-Keuls test after ANOVA.

Fig. 6. Region of Hsp72 and calsequestin on immunoblots on which were samples of left ventricular homogenates from sham controls (SHAM) and ovariectomized rats (OVX) of sedentary and exercise groups **(upper panel)** and comparisons of the relative Hsp72 to the relative calsequestin intensity from densitometry of immunoblots from each group **(lower panel)**. Data are means ± SE from 4 hearts. * $P<0.05$ = significantly different from sedentary-sham controls using Student Newman-Keuls test after ANOVA.

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

	SEDENTARY		EXERCISE	
	SHAM	OVX	SHAM	OVX
Body weight, g	265 ± 5.1	336 ± 7.5*	280 ± 3.9*	324 ± 3.2*
Heart weight, g	0.80 ± 0.02	0.93 ± 0.01*	0.89 ± 0.02*	0.95 ± 0.02*
Uterine weight, g	0.56 ± 0.03	0.11 ± 0.00*	0.53 ± 0.04	0.10 ± 0.009*
Soleus weight, g	0.094 ± 0.001	0.128 ± 0.005*	0.118 ± 0.004*	0.118 ± 0.003*
% Heart/body	0.304 ± 0.004	0.278 ± 0.005*	0.318 ± 0.004*	0.293 ± 0.003#
% Soleus/body	0.037 ± 0.001	0.038 ± 0.001	0.039 ± 0.001	0.036 ± 0.000
Citrate synthase activity (μmole/g protein/min)	44.6 ± 1.9	44.4 ± 1.9	57.8 ± 1.9*	52.4 ± 1.6*

Values are means ± SE of 8-9 rats. * $P < 0.05$ and # $P < 0.05$ represent significant difference from sedentary-sham controls and sedentary-ovariectomized rats, respectively.

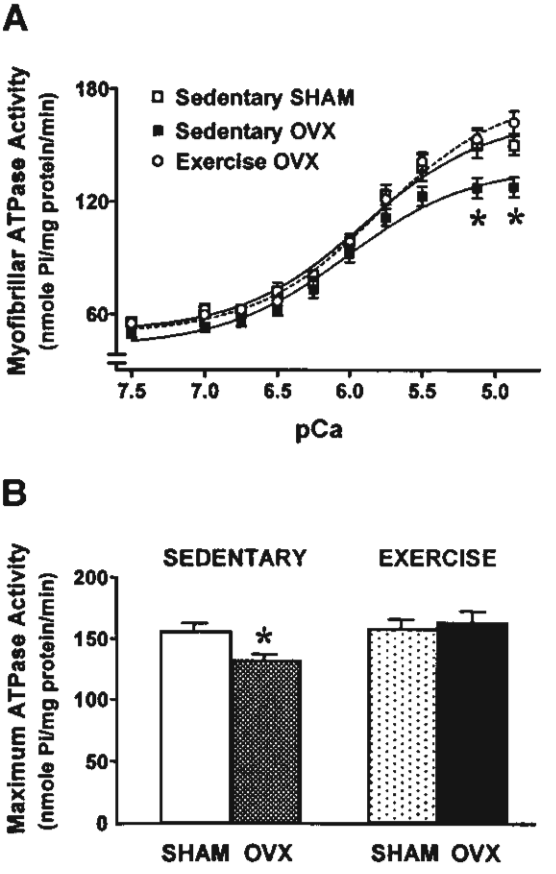
A
Myofibrillar ATPase Activity
(nmole Pi/mg protein/min)
180
120
60
Sedentary SHAM
Sedentary OVX
Exercise OVX
*
*
7.5
7.0
6.5
6.0
5.5
5.0
pCa
B
Maximum ATPase Activity
(nmole Pi/mg protein/min)
200
150
100
50
0
SEDENTARY
EXERCISE
*
SHAM OVX
SHAM OVX

Fig. 1

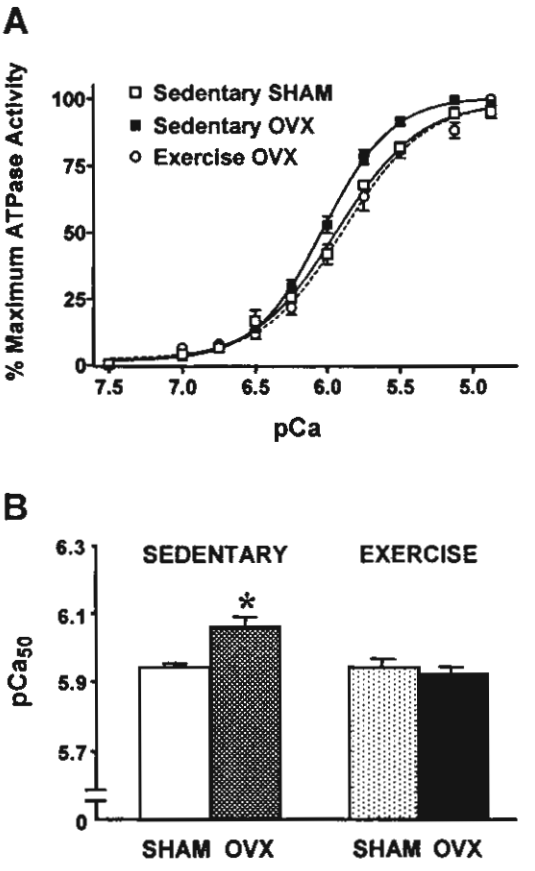
A
% Maximum ATPase Activity
100
75
50
25
0
Sedentary SHAM
Sedentary OVX
Exercise OVX
7.5
7.0
6.5
6.0
5.5
5.0
pCa
B
pCa50
6.3
6.1
5.9
5.7
0
SEDENTARY
EXERCISE
*
SHAM OVX
SHAM OVX

Fig. 2

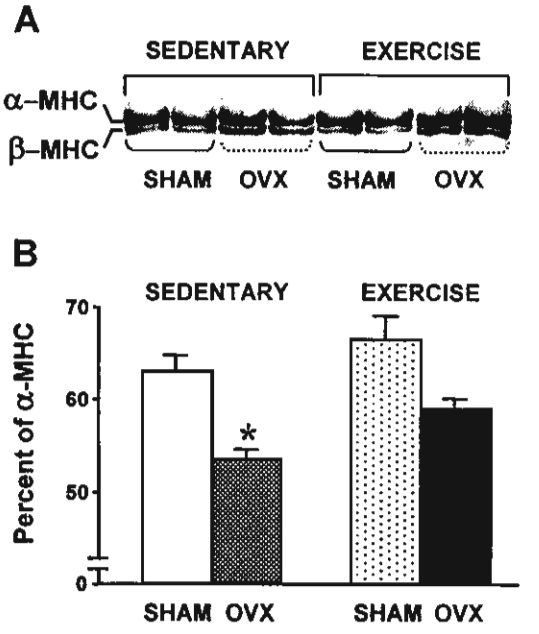
A
SEDENTARY
EXERCISE
α-MHC
β-MHC
SHAM
OVX
SHAM
OVX
B
SEDENTARY
EXERCISE
Percent of α-MHC
70
60
50
0
*
SHAM
OVX
SHAM
OVX

Fig. 3

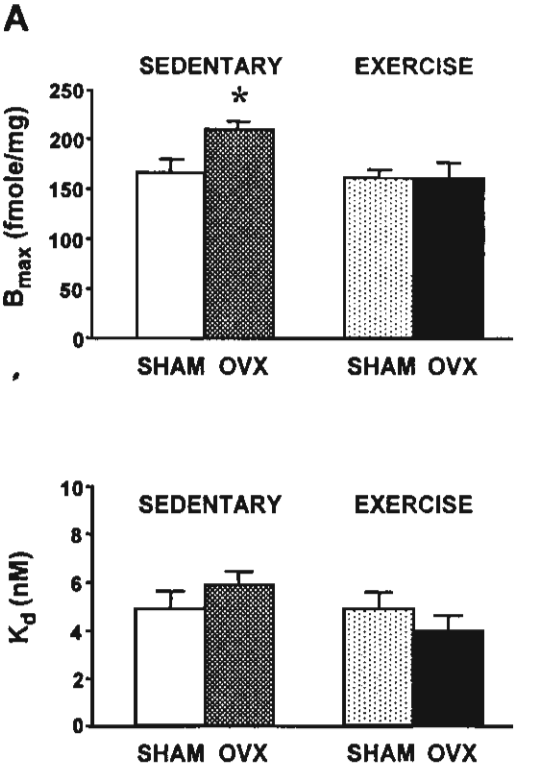
A
SEDENTARY
EXERCISE
B_{max} (fmole/mg)
250
200
150
100
50
0
*
SHAM
OVX
SHAM
OVX
SEDENTARY
EXERCISE
K_d (nM)
10
8
6
4
2
0
SHAM
OVX
SHAM
OVX

Fig. 4

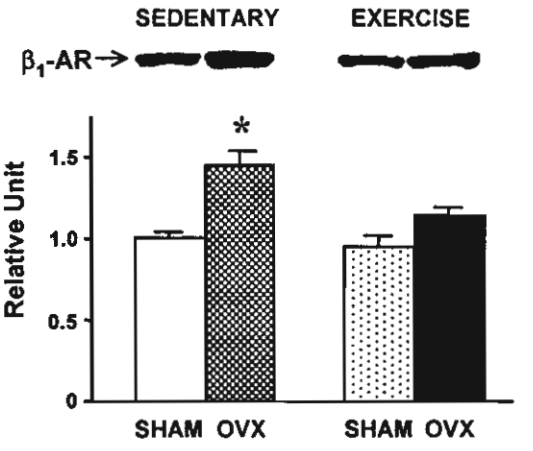
SEDENTARY
EXERCISE
β1-AR→
*
Relative Unit
1.5
1.0
0.5
0
SHAM OVX
SHAM OVX

Fig. 5

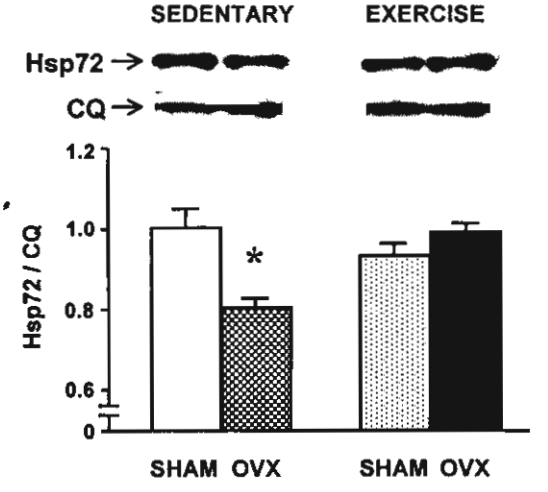
SEDENTARY
EXERCISE
Hsp72 →
CQ →
1.2
1.0
0.8
0.6
0
Hsp72 / CQ
*
SHAM OVX
SHAM OVX

Fig. 6