Exercise Increases Cystathionine-γ-lyase Expression and Decreases the Status of Oxidative Stress in Myocardium of Ovariectomized Rats

Zhiping Tang,† BSc, Yujun Wang,† BSc, Xiaoyan Zhu,‡ PhD, Xin Ni,‡ PhD, and Jianqiang Lu,† PhD

SUMMARY

Exercise could be a therapeutic approach for cardiovascular dysfunction induced by estrogen deficiency. Our previous study has shown that estrogen maintains cystathionine-γ-lyase (CSE) expression and inhibits oxidative stress in the myocardium of female rats. In the present study, we investigated whether exercise improves CSE expression and oxidative stress status and ameliorates ischemic cardiac damage in ovariectomized (OVX) rats. The results showed that treadmill training restored the ovariectomy-induced reduction of CSE and estrogen receptor (ER)α and decrease of total antioxidant capacity (T-AOC) and increase of malondialdehyde (MDA). The level of CSE was positively correlated to T-AOC and ERα while inversely correlated to MDA. OVX rats showed increases in the serum levels of creatine kinase (CK) and lactate dehydrogenase (LDH) and the percentage of TUNEL staining in myocardium upon ISO insult compared to sham rats. Exercise training significantly reduced the serum levels of LDH and CK and the percentage of TUNEL staining in myocardium upon ISO insult in OVX rats. In cultured cardiomyocytes, ISO treatment decreased cell viability and increased LDH release, while overexpression of CSE increased cell viability and decreased LDH release in the cells upon ISO insult. The results suggest that exercise training improves the oxidative stress status and ameliorates the cardiac damage induced by oxidative stress in OVX rats. The improvement of oxidative stress status by exercise might be at least partially due to upregulation of CSE/H₂S signaling. (Int Heart J 2016; 57: 96-103)

Key words: Hydrogen sulfide, Estrogens, Estrogen receptor

Menopause is a risk factor for many cardiovascular diseases. Cross-sectional data show that the incidence of cardiovascular diseases is much higher in post-menopausal women compared with women of the same age who are pre-menopausal. Many studies in animal models have demonstrated that a lack of ovary hormones, in particular estrogens, has detrimental effects on various organs including the cardiovascular system. Exercise replacement therapy (HRT) has therefore been recommended to postmenopausal women. However, the controversies regarding the safety of HRT have drawn attention to new therapies for postmenopausal women.

Exercise training is one of the therapies used to prevent postmenopausal syndrome. Numerous epidemiological studies have convincingly shown that physical exercise has a beneficial effect on cardiovascular disease outcomes. Exercise reduces heart rate and blood pressure, augments myocardial oxygen uptake, and regulates circulating blood volume as well as various metabolic processes. Furthermore, it has been shown that postmenopausal women who engage in intermittent, moderate-intensity physical training have a decreased risk of cardiovascular complications.

Oxidative stress is considered one of the main causative factors in various cardiovascular disorders including postmenopausal cardiovascular disorders. Ovarian steroid deprivation can induce oxidative stress in various tissues including myocardium. Moreover, administration of estrogens attenuates the generation of reactive oxygen species (ROS) and reduces apoptosis by anti-oxidative stress following ischemia/reoxygenation in myocardium.

Hydrogen sulfide (H₂S), a newly characterized gasotransmitter, has recently been shown to have cardioprotective effects. Endogenous H₂S is synthesized in the mammalian body from L-cysteine by the activity of three enzymes, cystathionine-γ-lyase (CSE), cystathionine-β-synthetase (CBS), and 3-mercaptopruvate sulfurtransferase (3-MST). In myocardium, H₂S is generated predominantly by CSE. Although the mechanisms by which H₂S exerts its cardioprotective ef-
fecteds remain to be elucidated, it has been demonstrated that anti-oxidative stress of H2S contributes to its cardioprotection. More recently, we have demonstrated that the level of oxidative stress was inversely correlated to CSE level in the myocardium of ovariectomized (OVX) rats, suggesting that CSE is associated with oxidative stress state in myocardium in vivo.

In the present study, we investigated whether chronic exercise impacts the oxidative stress state and the key factor of anti-oxidative stress CSE in myocardium of OVX rats, analyzed the correlation between estrogen receptors (ERs) and CSE in myocardium, and explored whether chronic exercise has protective actions against the oxidative insult in OVX rats.

**METHODS**

**Animals:** A total of 32 female Sprague-Dawley (SD) rats at 10 weeks of age were obtained from Shanghai SLAC Laboratory Animal Co. (Shanghai, China) and housed at controlled room temperature. Food and water were available ad libitum. All animal protocols were approved by the Ethical Committee of Experimental Animals of Shanghai University of Sport.

**Surgical procedure:** A total of 60 rats were used in the present study and were randomly assigned to 4 groups; sham, sham with exercise (Sham-EX), OVX, and OVX with exercise (OVX-EX). Two groups underwent ovariectomy and two groups a sham operation. Bilateral ovariectomy and sham operation were performed under anesthesia with sodium pentobarbital (60 mg/kg, ip).

**Experimental protocol and collection of tissues:** Two weeks after the operation, 1 OVX group and 1 sham-operated group underwent endurance training, which consisted of continuous running on a motor-driven rodent treadmill 5 times/week for 8 weeks. The rats ran progressively from 15 minutes/day at 15 meters/minute, on a 0% slope for one week, to 60 minutes/day at 18 meters/minute on a 0% slope for 7 weeks. One OVX group and 1 sham-operated group were assigned to sedentary rats. Thirty-two rats were sacrificed after training. Myocardium biopsies were obtained from the left ventricular wall and stored at -80°C for subsequent protein studies.

After exercise training, 28 rats from the 4 groups were administered isoproterenol (ISO, Sigma-Aldrich) subcutaneously (100 mg/kg) twice with an interval of 24 hours. The dosage of isoproterenol was chosen based on the literature and preliminary optimized experiments. The animals were sacrificed 24 hours after the second dose of isoproterenol. Serum and myocardial tissues were collected. The tissues were fixed in 10% buffered formalin.

In order to confirm that the ovariectomy was successful, uterus weight and serum 17β-estradiol (E2) level were determined. A significant reduction in serum E2 level accompanied by a significant decrease in uterus weight was observed in OVX rats compared to sham rats (data not shown), indicating that ovariectomy was successful.

**Western blot analysis:** Protein extraction and Western blot analysis were performed as described previously. Briefly, rat heart tissues were homogenized in cold T-Per lysis buffer (Pierce). Lysates were quickly sonicated in an ice bath and boiled for 5 minutes at 95°C. Protein samples (50 μg) were separated by 10% SDS-PAGE and subsequently transferred to nitrocellulose membranes. The nitrocellulose filters were incubated with primary antibody (1:500) for CSE (Santa Cruz Biotechnology), ERα (Santa Cruz), ERβ (Santa Cruz), or GAPDH (Santa Cruz) at 4°C overnight. After washing, the filters were incubated with a secondary horseradish peroxidase-conjugated IgG (1:1000) for 1 hour at room temperature. Immunoreactive proteins were visualized using an enhanced chemiluminescence Western blotting detection system (Santa Cruz). The staining intensity of the bands was measured using a densitometer (Syngene, Braintree, UK) together with Genesnap and Genetools software (Syngene). To control sampling errors, the ratio of band intensity to GAPDH was obtained to quantify the relative protein expression level.

**Measurement of total antioxidant capacity, activities of superoxide dismutase and catalase, and content of malondialdehyde:** Myocardium tissue (100 mg) was homogenized in cold saline. Homogenates were then centrifuged at 1000 g for 10 minutes. The total antioxidant capacity (T-AOC), superoxide dismutase (SOD), catalase (CAT) activity, and malondialdehyde (MDA) content in myocardium tissue were determined according to the kit manufacturer's protocol (Winching, Nanjing, China) as described previously. The mean intra-assay coefficients of variation for T-AOC, SOD, CAT, and MDA were 3.2%, 1.7%, 1.9%, and 2.6%, respectively. The mean inter-assay coefficients of variation for T-AOC, SOD, CAT, and MDA were 6.83%, 3.52%, 4.94%, and 4.35%, respectively.

**TUNEL staining:** The myocardial tissues were embedded in paraffin. The tissue blocks were then cut into 10-μm-thick slides, and 5 slides were obtained from each tissue block. Apoptotic cardiomyocytes were detected by TdT-mediated dUTP nick-end labeling (TUNEL) staining using an apoptosis detection kit (Beyotime Institute of Biotechnology, Shanghai, China) for apoptotic cell nuclei, while a Hoechst 33258 kit (Beyotime Institute of Biotechnology) was used to stain all cell nuclei, both according to the manufacturer's protocols. The fluorescent signals were observed with a fluorescence microscope, and in each slide, nuclei were counted in 8 microscopic fields by image J software. Apoptosis was determined as the ratio of the number of TUNEL-positive nuclei (red signals) to total nuclei (blue signals) in each field.

**Primary cardiomyocyte culture:** Ventricle cardiomyocytes were isolated from 1-2 day old SD rats. The isolation and culture protocol was described previously. Briefly, ventricle tissues were minced in dissociation buffer (in mmol/L: 116 NaCl, 20 HEPES, 0.8 Na2HPO4, 5.6 glucose, 5.4 KCl, 0.8 MgSO4, PH7.35). Serial digestions up to 10 times were performed with 0.1% trypsin and 0.05% collagenase type II (Worthington Biochemical) in dissociation buffer at 37°C. Cell pellets were collected and then resuspended in DMEM containing 10% fetal bovine serum and placed in culture dishes at 37°C for 1 hour to allow selective attachment of non-myocytes (primarily cardiaco fibroblasts). Cardiomyocyte-enriched fraction (> 95% cardiomyocytes as determined by immunocytochemistry staining) were then seeded in 12-well culture plates (Corning, Inc. Costar Corp., Cambridge, MA) at a density of 1 × 104 cells/cm2 and cultured in DMEM containing 15 mmol/L HEPES, 10% FBS, 0.1 mmol/L bromodeoxyuridine (BrdU), and antibiotics (100 U/mL penicillin and 100 mg/mL streptomycin) for 72 hours.

**Overexpression of CSE in cardiomyocytes:** Adenovirus encoding CSE (Gene bank No. NM 017074) was constructed by Genechem Corporation (Shanghai, China) using the AdEasyTM adenoviral vector system (Strategene, La Jolla, CA, USA). The
overexpression of CSE was achieved by infecting cultured primary cardiomyocytes with the recombinant adenovirus (multiplicity of infection [MOI] = 10). An empty adenoviral construct used at the same titer served as control. After 24 hours of infection, the cells were treated with isoproterenol (10 μM) for 24 hours.

Measurement of creatine kinase (CK) and lactate dehydrogenase (LDH) activity: CK activity in serum was determined by an automatic analyzer using a commercial CK-NAC test kit (Roche Diagnostics Shanghai Co. Ltd, Shanghai), and a spectrophotometric kit (Roche Diagnostics) was used to assess LDH activity in serum and culture media.

MTT assay: Cell viability was evaluated by 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) assay based on the reduction of MTT (Sigma-Aldrich) by functional mitochondria to formazan, as described previously.[23] Statistical analysis: Data are expressed as the mean ± SEM. For illustration purposes, some data are presented as the mean percent control ± SEM. All data were tested for homogeneity of variance using the Bartlett test. The results indicated that the data were normally distributed. The significance was then estimated by two-way analysis of variance (ANOVA) followed by the Student-Newman-Keuls test. Pearson’s correlation was used to analyze the correlation between CSE levels and the level of oxidative stress markers or ERs. A P value < 0.05 was considered statistically significant.

RESULTS

Exercise training increases CSE expression in myocardium: As expected, ovariectomy resulted in a significant decrease in CSE expression in myocardium (P < 0.01 versus sham). After 8-week exercise training, the CSE protein level was significantly up-regulated in OVX-exercise (OVX-ex) rats compared to OVX without exercise (OVX) rats (P < 0.01). There was no significant difference in CSE expression in myocardium between the sham and sham-exercise (sham-ex) groups (Figure 1).

Effect of exercise on oxidative status in the myocardium: The oxidative status in the myocardium was investigated by determining the markers of the anti-oxidative defence system, including T-AOC, MDA, and the activities of two important antioxidant enzymes CAT and SOD. As shown in Figure 2, ovariectomy resulted in a decrease in T-AOC (P < 0.05) while exercise significantly increased the level of T-AOC in OVX rats (P < 0.05). Exercise did not affect the T-AOC level in sham rats. The MDA level was significantly increased in OVX rats compared to sham rats (P < 0.05). Exercise training significantly decreased MDA level in OVX rats (P < 0.05). CAT and SOD levels were significantly decreased in OVX rats compared to sham rats (P < 0.05). Exercise could decrease CAT and SOD activity in sham rats (P < 0.05), but did not affect CAT and SOD activity in OVX rats.

Correlations between CSE and level of anti-oxidative biomarkers: We next explored the relationship between CSE expression and oxidative status in OVX rats. As shown in Figure 3, significant positive correlations were found between CSE and T-AOC in OVX rats (P < 0.01). CSE inversely correlated to MDA content in OVX rats (P < 0.01). There was no correlation between CSE and SOD or CSE and CAT.

ERα expression correlates to CSE level and activity of T-AOC and MDA: As shown in Figure 4, OVX resulted in a decrease in ERα expression in myocardium (P < 0.05 versus sham). Exercise could increase ERα expression in OVX rats (P < 0.05) but did not affect ERα expression in sham rats. OVX and exercise did not affect ERβ expression in myocardium.

As shown in Figure 5, the level of CSE correlated to ERα level in OVX rats (P < 0.01). CSE expression did not correlate to ERβ.

The activities of T-AOC and MDA were also correlated to ERα level in OVX rats (P < 0.01). SOD activity and CAT activity were not correlated to ERα (Figure 6). ERβ expression was not correlated to the activity of T-AOC, MDA, SOD, or CAT (data not shown).

Exercise ameliorates ISO-induced cardiac damage: It was reported that the synthetic catecholamine ISO at large doses induced cardiac damage.[23] Therefore we investigated the effects of OVX and exercise on ISO-induced cardiac damage. As shown in Figure 7, the serum levels of LDH and CK were significantly increased in OVX rats compared to the sham group in response to ISO treatment (P < 0.01). The percentage of TUNEL staining was also significantly increased in OVX rats compared with the sham group upon ISO insult (P < 0.01). Exercise training significantly reduced the serum levels of LDH and CK and the percentage of TUNEL staining in myocardium upon ISO insult in OVX rats (P < 0.01 versus OVX group).

Overexpression of CSE prevents isoproterenol-induced injury in primary cardiomyocytes: As shown in Figure 8A, infection of cardiomyocytes with CSE adenovirus resulted in CSE overexpression. ISO treatment significantly decreased cell viability and increased LDH release (Figure 8B and C). Overexpression of CSE increased cell viability and decreased LDH release in
Figure 2. Effects of exercise training and ovariectomy on oxidative status in the myocardium of OVX rats. Bilateral ovariectomy or sham-operation was performed under anesthesia. The rats then underwent treadmill training or not for 8 weeks. T-AOC (A), MDA content (B), CAT (C) and SOD (D) activities in myocardium were determined as described in the “Materials and Methods”. The sham rats without exercise training served as control. Data are expressed as the mean percentage control ± SEM (n = 8/group). *P < 0.05, #P < 0.05, ##P < 0.01.

Figure 3. Correlation between CSE level and oxidative stress state in myometrium of OVX rats. Pearson’s correlation was applied to analyze the relationship between myocardial CSE expression and T-AOC (A), MDA content (B), CAT activity (C), and SOD activity (D).
the cells upon ISO treatment.

**Discussion**

The present study has demonstrated that chronic exercise training can improve oxidative stress status, ameliorate oxidative stress-induced damage, and increase CSE expression in the myocardium of OVX rats.

Oxidative stress is considered one of the main causative factors in various cardiovascular disorders including postmenopausal cardiovascular disorders. Our previous study demonstrated that OVX resulted in a decrease in anti-oxidative biomarkers. In the present study, we confirmed our previous findings by showing that chronic exercise training can improve oxidative stress status, ameliorate oxidative stress-induced damage, and increase CSE expression in the myocardium of OVX rats.

**Figure 4.** Effects of ovariectomy and exercise training on ERα and ERβ protein levels in myocardium of female rats. Bilateral ovariectomy or sham-operation was performed under anesthesia. Two weeks after operation, the rats underwent treadmill training or not for 8 weeks. The rats were then sacrificed and myocardium tissues were collected. ERα(A) and ERβ(B) protein levels were determined by Western blotting. Sham rats without exercise training served as control. Data are expressed as mean percentage control ± SEM (n = 8/group). The represented Western blot bands are on the top of the graph. *P < 0.05, #P < 0.05 versus OVX.

**Figure 5.** Correlation between ERα or ERβ and CSE level in myometrium of OVX rats. Pearson’s correlation was applied to analyze the relationship between ERα and CSE expression (A) or ERβ and CSE level (B) in myocardium. The results showed that CSE correlated to ERα but not ERβ.
Figure 6. Correlation between ERα and oxidative stress state in myometrium of OVX rats. Pearson’s correlation was applied to analyze the relationship between myocardial ERα expression and T-AOC (A), MDA content (B), CAT activity (C), and SOD activity (D).

Figure 7. Serum CK and LDH levels and TUNEL staining in myocardium upon ISO insult. ISO insult was performed on sham, sham-EX, OVX and OVX-EX rats as described in the Materials and Methods. A: level of LDH, B: CK level, C: representative images showing the TUNEL-positive nuclei (red) and total nuclei (blue), D: cumulative data showing the percentage of TUNEL staining in sham, sham-EX, OVX and OVX-EX rats. Data are presented as the mean ± SEM (n = 7 in each group). *P < 0.05 and **P < 0.01 versus Sham. ##P < 0.01 versus OVX.
findings and also showed that OVX caused an increase in content of MDA, an oxidative biomarker, in myocardium. The CSE/H$_2$S system has been implicated as one of the endogenous anti-oxidative stress factors in cardiomyocytes.\textsuperscript{19,24} It has been shown that H$_2$S acts as a direct scavenger of reactive oxygen species (ROS) and peroxynitrite and enhances mitochondrial Mn-SOD activity.\textsuperscript{25,26} One of our previous studies demonstrated that the level of CSE and H$_2$S generation correlated to T-AOC in myocardium of OVX rats.\textsuperscript{20} In the present study, we not only confirmed our previous findings, but also showed that the level of CSE inversely correlates to MDA in myocardium. Moreover, we also found that overexpression of CSE could prevent ISO-induced damage in cultured cardiomyocytes.

A number of studies have suggested that exercise training is beneficial for the maintenance of cardiovascular homeostasis after ovarian hormone deprivation. For instance, it has been demonstrated that exercise attenuates cardiac hypertrophy in OVX spontaneously hypertensive rats\textsuperscript{27} and can reduce oxidative stress-mediated destruction in the vasculature of OVX rats.\textsuperscript{28} One of the causative factors for ISO-induced cardiac damage is the generation of highly cytotoxic free radicals through autooxidation of catecholamines.\textsuperscript{23} The present study demonstrated that exercise training not only decreased the oxidative stress induced by ovariectomy, but also improved the activity of CAT and SOD in the myocardium of OVX rats. In the present study, we showed that exercise training could not improve the activity of CAT and SOD, and that CSE did not correlate to CAT and SOD activity in OVX rats with or without exercise, suggesting that the level of CAT and SOD in myocardium might not be associated with the CSE/H$_2$S system in female rats \textit{in vivo}.

Estrogens exert their effects via ERs. In myocardium, two classic nuclear ERs, ER$\alpha$ and ER$\beta$, have been identified. Our previous study has demonstrated that ER$\alpha$ mediates the estrogen protection of cardiomyocytes against hypoxia/reperfusion insults in female rats.\textsuperscript{21} The present study showed that the level of CSE was correlated to ER$\alpha$ in the myocardium of female rats, suggesting that estrogen regulation of CSE might be via ER$\alpha$. Consistently, the oxidative biomarker T-AOC and MDA content correlate to ER$\alpha$ but not ER$\beta$ level. This leads us to believe that ER$\alpha$ might play a predominant role in anti-oxidative stress in myocardium in female rats.

The mechanisms underlying the estrogenic-like effects of exercise remain largely unknown. Some studies have demonstrated that exercise training can increase the level of ERs, which may account for the estrogenic-like effects of exercise.\textsuperscript{29} The present study showed that the level of ER$\alpha$ in myocardium was significantly downregulated in OVX rats, and exercise training could restore the ER$\alpha$ expression in OVX rats. This suggests that exercise-induced CSE expression might be due to the upregulation of ER$\alpha$.

As mentioned before, the cardioprotective effects of estrogens have been well documented. However, the long-term
use of estrogen has numerous side effects such as increased risks of breast cancer and thromboembolic diseases. The data from the present study demonstrated that exercise training can ameliorate oxidative stress status in the myocardium of OVX rats, which supports that exercise can be an alternative treatment strategy for postmenopausal cardiac disorders.

**Conclusion:** Exercise training increases the anti-oxidative marker T-AOC and decreases the oxidative marker MDA in the myocardium and ameliorates the cardiac damage induced by oxidative stress in OVX rats. Improvement of oxidative stress status by exercise is associated with CSE expression in myocardium. Our study findings suggest that the improvement of oxidative stress status by exercise might be at least partially due to the upregulation of CSE expression.

**DISCLOSURE**

**Conflict of interest statement:** All authors have nothing to disclose.

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