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ORIGINAL ARTICLE

Resistance exercise training increase activation of AKT-eNOS and Ref-1 expression by FOXO-1 activation in aorta of F344 rats

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Meng Li, Wei Li, Jin-Hwan Yoon, Byeong Hwa Jeon and Sang Ki Lee. Resistance exercise training increase activation of AKT-eNOS and Ref-1 expression by FOXO-1 activation in aorta of F344 rats. *JENB*., Vol. 19, No. 3, pp.165-171, 2015 **[Purpose]** This study investigated the effects of resistance exercise on the Akt-eNOS, the activation of antioxidant protein and FOXO1 in the aorta of F344 rats. **[Methods]** Male 7 week-old F344 rats were randomly divided into 2 groups: a climbing group (n = 6) and a sedentary group (n = 6). H&E staining and western blotting were used to analyze the rat aortas and target proteins. **[Results]** Resistance exercise training did not significantly affect aortic structure. Phosphorylation of AKT and eNOS and expression of MnSOD and Ref-1 were significantly increased while FOXO1 phosphorylation was significantly decreased in the resistance exercise group compared with the sedentary group. **[Conclusion]** We demonstrate that resistance exercise activates the Akt-eNOS and Ref-1 protein without changes to aortic thickness via FOXO-1 activation in the aorta of F344 rats. **[Key words]** resistance exercise, aorta structure, FOXO1, oxidative stress, MnSOD, Ref-1

INTRODUCTION

The risk factors of cardiovascular disease include unhealthy lifestyle habits such as smoking, alcohol consumption, and high-fat diet consumption as well as sedentary lifestyles, diabetes and aging [1]. Endothelial cells play a crucial role in maintaining vascular function and structure. Aging and an unhealthy lifestyle impair endothelial cells, which contributes to the pathogenesis of several cardiovascular diseases. This manifests in its earliest form as an attenuation of endothelium-dependent dilator responses as a consequence of the alteration in the expression or activity of endothelial nitric oxide synthases (eNOS) [2]. Endothelial nitric oxide synthases, which play a pivotal role in vasorelaxation, are derived from nitric oxide (NO) and are phosphorylated by the PI3K/Akt activating signaling pathway via the increase of shear stress at the cellular level and in isolated blood vessels [3]. Exercise is a power stimulus that increases blood flow and shear stress in the vascular bed, which in turn improves NO production and/or NO bioactivity. It was well established that exercise induces vascular eNOS expression, through which exercise stimulates NO production, and also that insufficient phosphorylated eNOS induces oxidative stress and endothelial dysfunction [4]. Recent evidence suggests that high levels of reactive oxygen species (ROS) and the subsequent oxidative stress affect the availability and/or balance of key-regulators of vascular homeostasis in addition to favoring the development of cardiovascular disease [5]. Physical activity is able to alleviate oxidative stress, which has important public health implications and may facilitate compliance with exercise recommendations [6]. A recent prospective study demonstrated that the lack of exercise was related to higher mortality rates and shorter life expectancies in both men and women [7]. Hence, a small change in oxidative stress brought about by resistance exercise could have important benefits for health.

Several factors are involved in the regulation of oxidative stress and anti-oxidation such as forehead transcription factors, apurinic/apyrimidinic (AP) endonuclease1/redox factor-1

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(APE1/Ref-1) and manganese superoxide dismutase (MnSOD). Ref-1 is a multifunctional transcription factors with both DNA repair and redox functions that affect distinct sites and residues [8]. Ref-1 overexpression suppresses H2O2- induced apoptosis and improves oxidative stress by redox activity [9]. However, there is no paper that presents evidence related to exercise and Ref-1 expression.

The FOXO families of forkhead transcription factors include FOXO1, FOXO3, FOXO4, and FOXO6, which play key roles in the regulation of a variety of cellular, pathological, physiological and biological processes such as oxidative stress, cell metabolism and cardiovascular diseases [10,11]. FOXO1 transcription factors contribute in different types of tissues including the liver, heart, and vessels. Phosphorylated (inactivated) FOXO1 proteins are triggered by oxidative stress and contribute to ROS-induced cell damage and apoptosis [12]. In addition, reactive oxygen species are generated by different types of intracellular signaling pathways principally located in the cytoplasm and in the mitochondria [1]. Evidence shows that activation of FOXO transcription factors reduce the level of oxidative stress by generating enzymes that break down ROS such as MnSOD and catalase. MnSOD, an anti-oxidative enzyme, enhance catalase activity and down regulate oxidative stress [14]. Based on published data and evidence, FOXOs have considerable effect on branches of the PI3K/AKT signaling pathway, and are inactivated upon AKT-mediated phosphorylation [15,16]. This suggests that increasing the number of FOXOs may be an important protective pathway to regulate the balance of oxidative stress and anti-oxidative functions. However, it is unknown whether this protective pathway is activated by resistance exercise. The purpose of the present study was to examine the effect of resistance exercise on balancing the oxidative stress regulated by FOXO transcription factors in the F344 rat model.

METHODS

Animals and Housing

7-week-old male F344 rats were used in this study. Rats were housed under controlled temperature (24℃ ± 3℃), humidity (56% ± 3%), and light conditions (12 : 12h light-dark cycle) and were provided food and water ad libitum. Rats were randomly assigned to a Tower climbing exercise group (n = 6) or a sedentary (control) group (n = 6). Rats were anesthetized with an intraperitoneal injection of Ketamine (100mg/kg) / xylazine (80mg/kg). All experimentation was conducted using aseptic procedures. The Animal Care Committee of Chungnam National University approved all animal care and experimental procedures in this study.

Table 1. Resistance training protocol

Reps	Weight [%1RM or 1RM +7α (30g)	Duration
1	50% 1RM	12 Weeks
2	75% 1RM	
3	90% 1RM	
4	100% 1RM	
5	100% 1RM + 30 g	
6	5 + 30 g	
7	6 + 30 g	
8	7 + 30 g	

Training Protocol

The training protocol is described in a published paper [17]. Briefly, training was performed using a ladder with length 135 cm, grid step 2.5 cm (diameter 0.5 mm) and grade 60 degree. Rats became familiar with the ladder by practicing climbing from the bottom of the ladder to the top of the cage over a period of 3 days. Afterward, the resistance training regimen was initiated. Exercise intensity and duration are listed in Table 1. We used cylinder as a weigh load attached to the tail. The initial weight load was 50% of the rat's body weight. Rats were allowed to rest for 2 min once they finished a bout of exercise (bottom-top). After the next circle of exercise, additional weight was placed in the cylinder. Additional weights were added to the cylinder in 30 g increments for each subsequent climb if the rats were able to climb the ladder with the existing load. Training was stopped when rats refused to climb.

H&E staining

Hematoxylin & Eosin staining was used to measure the aortic ring. Aortic rings were fixed in 4% formaldehyde and paraffin-embedded. Serial cross-sections (5 μm thick) of the aorta were stained with hematoxylin and eosin (MHS-32, Sigma, USA). A DP70 camera (Olympus, Tokyo) and TSView version 7 (Fuzhou Tucsen Image Technology, Japan) were used to measure the planimetry of the intima, media and lumen to calculate the cross-sectional area of the intima-media (IM) and lumen [18,19].

Western blotting

Aortas were isolated and homogenized on ice with a tissue homogenizer (Biospec Products Inc., USA) using lysis buffer

containing 20 mM Tris HCl, 0.5% NP-40, 250 mM NaCl, 3 mM EDTA, 3 mM EGTA, 2 mM DTT, 0.5 mM phenylmethylsulfonylfluoride, 2 mM b-glycerophosphate, 1 mM sodium vanadate, and 1 lg/ml leupeptin at pH 7.5. The homogenized tissues were centrifuged at 10,000 g for 30 min at 4℃ and the supernatants were used for the determination of total protein concentration with the Bradford protein assay.

Forty micrograms of protein were separated by 7.5-10% SDS-PAGE and then transferred onto a polyvinylidene difluoride (PVDF) membrane. Next, the membranes were blocked for 1h in 5% skim milk solution, after which they were incubated with antibodies of AKT, p-AKT, eNOS, p-eNOS, FOXO1, p-FOXO1, Ref-1 and MnSOD.

Protein expression was detected using an enhanced chemiluminescence kit (Amersham Pharmacia Biotech) [20]. The band intensities were quantified by densitometry using a Bio-Rad image analysis system (Quantity One, Bio-Rad, USA), and normalized against the b-actin protein. Each experiment was repeated 3 times with each animal.

Statistical analysis

All data were expressed as Mean ± SD. The statistical evaluation was performed using t-test and $p < .05$ was considered statistically significant.

RESULTS

Effect of resistance exercise on vessel structure

We compared the aortic rings in the control group and in the resistance exercise group (Fig. 1). Microscopic images showed there was no significant difference in the aortic rings of the control and resistance exercise groups under 40x and 200x magnification. For the cross-sectional area of the IM, no significant differences were found in the control and resistance groups.

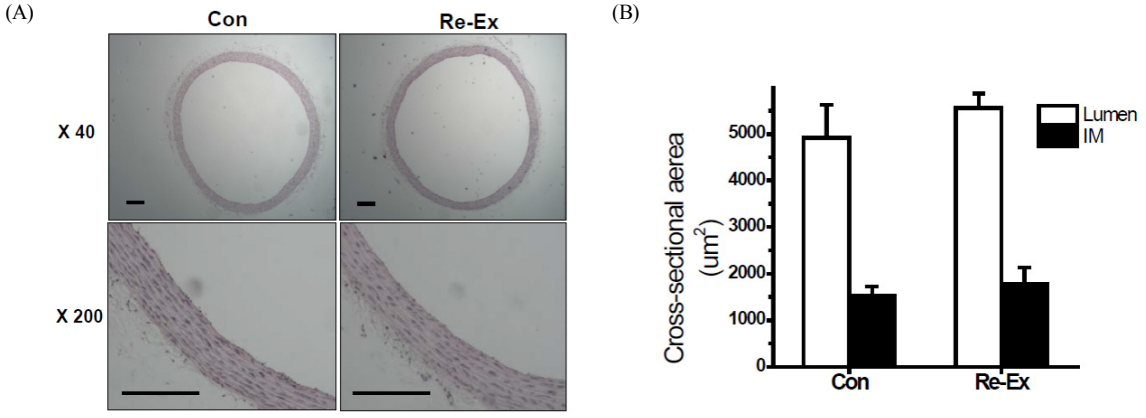

Fig. 1. Effect of resistance exercise on aortic ring thickness in F344 rats. Microscopic images of the effects of resistance exercise on aortic ring thickness are shown (n = 6). Thickness of cross-sectional area (2um) was quantified by planimetry. (A) Result of H&E staining in aortic rings and (B) in areas of the lumen and intima-to-media (IM) were calculated in aortic rings. Con, Sedentary control; Re-Ex, Resistance exercise. Data represent Mean ± SD (n = 6). Microscopes at 40X (upper panel) and 200X (lower panel) magnification were used for visualization. Scale bar are 100 um.

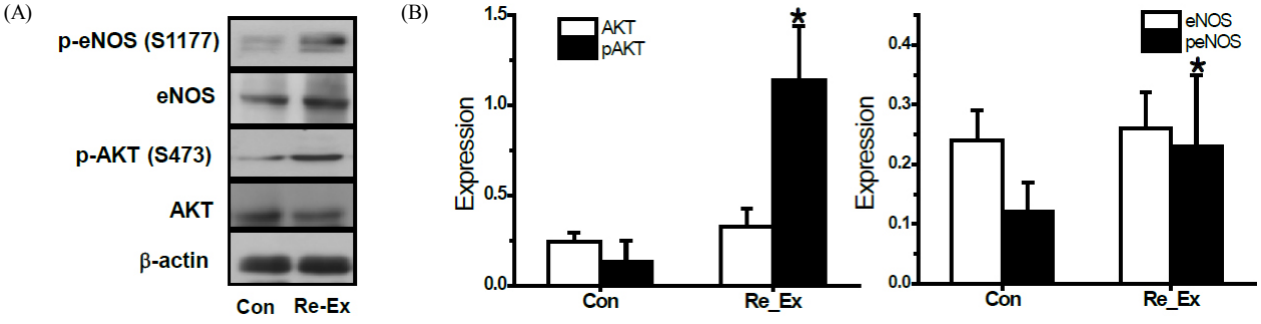

Fig. 2. Resistance exercise increased phosphorylation of eNOS and AKT in aorta of F344 rats. (A) Result of western blot and (B) Densitometry analysis for expression and phosphorylation of eNOS and AKT. Con, Sedentary control; Re_Ex, Resistance exercise. Data represent Mean ± SD (n = 6). * $p < 0.05$ vs. Con.

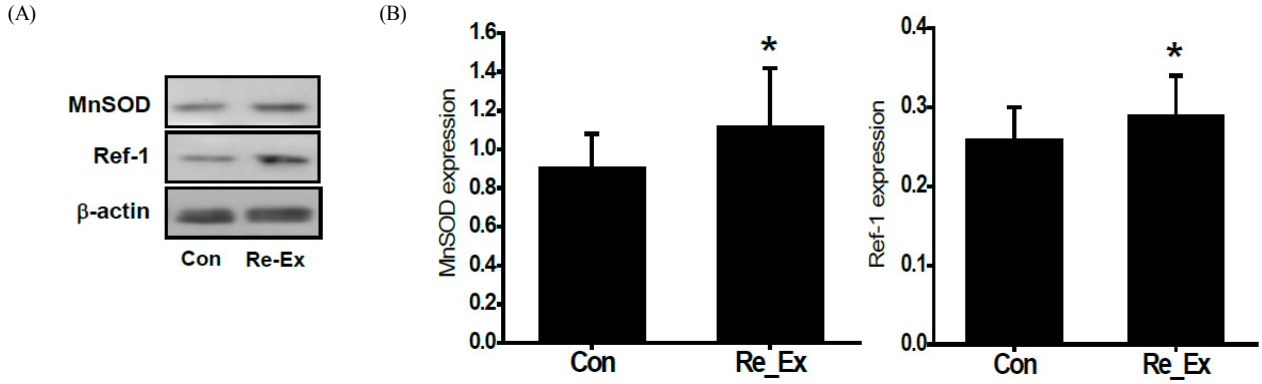

Fig. 3. Resistance exercise increased expression of MnSOD and Ref-1 in aorta of F344 rats. (A) Result of western blot and (B) Densitometry analysis for expression of MnSOD and Ref-1. Con, Sedentary control; Re_Ex, Resistance exercise. Data represent Mean ± SD (n = 6). * $p < 0.05$ vs. Con.

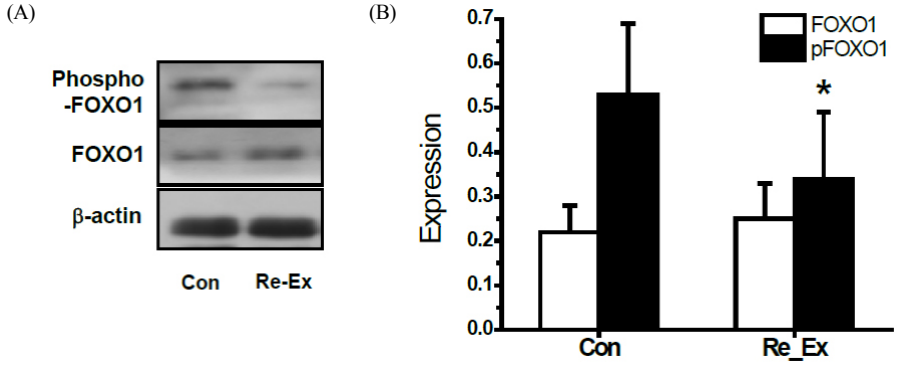

Fig. 4. Resistance exercise decreased FOXO1 phosphorylation, but FOXO1 expression did not change in aorta of F344 rats. (A) Result of western blot and (B) Densitometry analysis for expression and phosphorylation of FOXO1. Con, Sedentary control; Re-Ex, Resistance exercise. Data represent Mean ± SD (n = 6). * $p < 0.05$ vs. Con.

Effect of resistance exercise on eNOS, p-eNOS-ser1177, AKT, p-AKT-Ser473 expression

We examined the expression and phosphorylation of eNOS and AKT in the aorta (Fig. 2). The results of the western blot showed that phosphorylation of eNOS and AKT were significantly higher in the resistance exercise group compared to the control group (Fig. 2A). But the expressions of eNOS and AKT did not change in the resistance exercise group compared with the control group.

Effect of resistance exercise on Expression of MnSOD and Ref-1

Western blot was used to detect the expression of MnSOD and Ref-1 in the control and resistance exercise groups (Fig. 3). In the resistance exercise group, both MnSOD and Ref-1 were highly expressed and significantly increased compared with the control group.

Effect of resistance exercise on p-FOXO, FOXO, and Redox factor-1 expression

We examined the expression and phosphorylation of FOXO1 in the aorta (Fig. 4). FOXO1 phosphorylation was significantly decreased in the resistance exericse group compared with the control group. However, FOXO1 expression was not affected by resistance exercise.

DISCUSSION

In this study, we present three findings: Resistance exercise significantly increased AKT-eNOS phosphorylation in the aorta of rats; Resistance exercise significantly increased the expressions of MnSOD and Redox factor-1 in the aorta of rats; Resistance exercise significantly increased FOXO1 expression in the aorta of rats.

Treadmill exercise can reduce aortic wall thickness in high-fat diet-induced hypertensive rats [21]. Aortic wall thickness is highly related with vascular tone functions inclu-

ding vasodilation and vasoconstriction. Excessive aortic wall thickness confers a higher risk of atherosclerosis and also causes vascular tone dysfunction [22]. A published study showed that treadmill exercise with low to moderate intensity did not change aortic wall thickness in young healthy rats [23]. Another study reported that 4 months of moderate intensity resistance exercise had no effect on carotid artery wall thickness in older male subjects [24]. Many previous studies focused on the relationship between aerobic exercise and arterial walls in locations such as the carotid and femoral artery in healthy or middle-age subjects. But there is no evidence showing a correlation between resistance exercise and aortic wall thickness in the aging rat model. In our research, we did not find that the climbing tower significantly reduced the thickness of the aortic wall in healthy F344 rats compared with the control group. (Fig. 1). Different types of exercise, intensity and duration, which might contribute to diverse molecular signaling pathways in normal and pathological situations, may explain our results and those of previous studies.

To investigate the function of the aorta, we assessed the expression and phosphorylation of eNOS. There was a significant difference between the exercise and control groups. These results indicate that resistance exercise may stimulate significantly higher expression of phosphorylated eNOS through activated AKT (Fig. 2). Our data provide evidence that the function of the aorta can be improved by resistance physical activity.

Some studies have indicated that activation of the PI3K/AKT signalling pathway causes FOXOs to translocate from the nucleus to the cytoplasm, phosphorylated and inactivated [25]. In the nucleus, activated FOXOs bind directly to the MnSOD promoter to enhance its expression, up regulate catalase activity and alleviate oxidative stress [25-27].

A study revealed that long-term exercise training could up regulate mitochondrial and cardiac MnSOD activity in aging rats [29,30]. Activation of MnSOD in the mitochondria protects cells from ROS-mediated damage by converting superoxide radicals to oxygen and hydrogen peroxide (H_2O_2). Enzymes catalyze further breakdown of H_2O_2 into water and oxygen [26,27].

Our data indicated that Akt phosphorylation was significantly higher in the resistance exercise group (Fig. 2). We also demonstrated that resistance exercise resulted in an increase of MnSOD activity in the aorta (Fig. 3).

A body of evidence underscored the critical role of oxygen free radicals in endothelial injury, aging and cardiovascular disease [5]. Aging shares several molecular features with cardiovascular disease and metabolic syndrome such as dysfunction of vasodilation, coronary artery disease and hyperlipidemia. The free radical theory of aging is wildly accepted as the reference concept describing the mechanism underline aging and aging-related disease [29].

The intracellular levels of ref-1 reflect the balance between oxidative stress, anti-oxidative enzymes and the amount of ROS. A number of studies reported correlations between exercise and antioxidant functions in both animal models and human subjects. A review article concluded that an acute single bout of exercise training and endurance exercise training elevated the levels of antioxidant enzymes including SODs, glutathione (GSH) and AP-1 [30]. FOXOs enhance MnSOD and catalase activity in both endothelial cells and vascular smooth muscle cells in addition to playing a considerable role in removing ROS [31].

AP endonuclease/ Redox factor-1 (APE/Ref-1) was reported to have the ability to inhibit endothelial apoptosis and to suppress TNF-alpha induced expression of VCAM-1 [20] as well as the subsequent monocyte adhesion [34]. VCAM-1 and monocyte adhesion plays a critical role in the development of atherosclerosis. In a balloon injury-induced atherosclerosis rat model, APE/Ref-1 inhibited neointimal formation in carotid arteries. Another study demonstrated that APE/Ref-1 could suppress protein kinase C-mediated phosphorylation of p66shc, a protein related to oxidative stress, in endothelial cells and also restrained vasoconstriction in rat aorta [32]. These results suggest that APE/Ref-1 not only plays a role in the antioxidant scene but is also a key factor involved in anti-inflammation processes.

As we know, there is no article demonstrating a connection between resistance exercise, antioxidant enzyme activity, and FOXO expression. To investigate the activity of antioxidant enzymes, we evaluated Ref-1 and MnSOD expression in the aorta. In our data, we found that resistance exercise stimulated Ref-1 expression in rat aorta (Fig. 3). This suggests that resistance exercise can protect the balance of oxidative stress and antioxidant function by increasing the level of Ref-1. Guan *et al*. [33] indicated that decreasing the levels of APE/Ref-1 reduced the activity of NF-kappaB, enhancing TNF-alpha-induced endothelial apoptosis in Ref-1+/- (hemizygous transgenic mice harbouring a single allele of Ref-1) mice aorta. Overexpression of APE/Ref-1 inhibited TNF-alpha and hypoxia-induced endothelial apoptosis in vitro through NF-kappaB dependent and independent signaling pathways [34].

FOXO proteins are important in the development of atherosclerosis because shear stress induces the activation of AKT, eNOS and AMPK (AMP activated protein kinase) [35].

They are also associated with complicated signaling pathways. As a downstream regulator of the PI3K/AKT pathway, FOXO1 participates in the regulation of the cardiovascular system in a variety of diseases such as hypertension, cardiac hypertrophy and atherosclerosis. In a previous study, we demonstrated that FOXO1 was significantly increased by exercise in a balloon-injury induced rat model [19].

The results suggested that endurance exercise could inhibit neointimal formation via FOXO1 activation. In a hepatocytic FOXO1 knockout rat model, blood pressure was significantly decreased by the inhibition of angiotensinogen compared with wild type mice [36]. Activated FOXOs suppress angiogenesis in endothelial cells and inhibit excessive growth in cardiomyocytes [22].

In this study, we measured changes in the expression and phosphorylation of FOXO1 by resistance exercise in the aorta of rats. Resistance exercise decreased FOXO1 phosphorylation but did not affect FOXO1 expression (Fig. 4). Our results indicated that resistance exercise may inhibit FOXO1 translocation into the cytosol from the nucleus.

The function of the FOXO signal transduction pathway varies in difference types of cells. Since phosphorylation of FOXO results in its inactivation, it is important to investigate whether exercise leads to the phosphorylation of FOXOs. In further studies, we should determine the relationship between phosphorylated FOXOs and different types of exercise, taking into account the intensity and duration. We should also investigate FOXO signaling pathways both in cardiovascular disease animal models and healthy subjects.

In conclusion, our data suggest that resistance exercise activates Akt-eNOS and ref-1 expression by FOXO-1 activation.

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