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

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

Table 1. Resistance training protocol

Committee of Chungnam National University approved all animal care and experimental procedures in this study.

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

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-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.

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.

Aortic wall thickness is highly related with vascular tone functions inclu-
Correlating resistance exercise and aortic wall thickness in the aging rat model has been investigated. A study revealed that long-term exercise training could upregulate 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 (H2O2). Enzymes catalyze further breakdown of H2O2 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 hepatocyte 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 different 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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REFERENCES

[1] Oellerich MF, Potente M. FOXOs and Sirtuins in vascular growth, maintenance, and aging. Circ Res. 2012;110:1238-1251.
[2] Kolluru GK, Siamwala JH, Suvo Chatterjee S. eNOS phosphorylation in health and disease. Biochimie. 2010;92:1186-1198.
[3] North BJ, Sinclair DA. The intersection between aging and cardiovascular disease. Circ Res. 2012;110:1097-1108.
[4] Lakatta EG, Levy D. Arterial and Cardiac Aging: Major Shareholders in Cardiovascular Disease Enterprises Part II: The Aging Heart in Health: Links to Heart Disease. Circulation. 2003;107:346-354.
[5] Camici GG, Cosentino F, Tanner FC, Lüscher TF. The role of p66Shc deletion in age-associated arterial dysfunction and disease states. J Appl Physiol. 2008;105:1628-1631.
[6] Park JH, Miyashita M, Takahashi M, Kawanishi N, Bae SR, Kim HS, Suzuki K, Nakamura Y. Effects of low-volume walking programme and vitamin E supplementation on oxidative damage and health-related variables in healthy older adults. Nutr Metab. 2013;10:38.
[7] Reimers CD, Knapp G, Reimers AK. Does Physical Activity Increase Life Expectancy? A review of the literature. J Aging Res. 2012;2012:243958.
[8] Mark RK. Millie MG, Melissa LF. APE/Ref-1 role redox signaling: translational applications of targeting the redox function of the DNA repair/Redox protein APE/Ref-1. Curr Mol Pharmac. 2012;5:36-53.
[9] Sung Y, Frank LM. Apurinic/Apyrimidinic Endonuclease/Redox Effector Factor-1(APE/Ref-1): A Unique Target for the Prevention and Treatment of Human Melanoma. Antioxid Redox Signal. 2009;11:639-650.
[10] Tuteja G, Kaestner KH. Snapshot: forkhead transcription factors I. Cell. 2007;130:1160.
[11] Tuteja G, Kaestner KH. Snapshot: forkhead transcription factors II. Cell. 2007;131:192.
[12] Bhaskar Ponugoti, Guangyu Dong, Dana T. Graves. Role of forkhead transcription factors in diabetes-induced oxidative stress. Exp Diabetes Res. 2012, 2012:939751.
[13] Kops GJ, Dansen TB, Polderman PE, Saarloos I, Wirtz KW, Coffer PJ, Huang TT, Bos JL, Medema RH, Burgering BM. Forkhead transcription factor FOXO3a protects quiescent cells from oxidative stress. Nature. 2002;419:316-321.
[14] Sulih DA, Brunet A. FoxO transcription factors in the maintenance of cellular homeostasis during aging. Curr Opin Cell Biol. 2008;20:126-136. 
[15] Tzivion G, Dobson M, Ramakrishnan G. FoxO transcription factors; Regulation by AKT and 14-3-3 proteins. Biochim Biophys Acta. 2011;1813:1938-1945.
[16] Calnan DR, Brunet A. The FoxO code. Oncogene.
[17] Lee S, Barton ER, Sweeney HL, Farrar RP. Viral expression of insulin-like growth factor-I enhances muscle hypertrophy in resistance-trained rats. J Appl Physiol. 2004;96:1097-104.

[18] Lee HM, Jeon BH, Won KJ, Lee CK, Park TK, Choi WS, Bae YM, Kim HS, Lee SK, Park SH, Irani K, Kim B. Gene transfer of redox factor-1 inhibits neointimal formation: involvement of platelet-derived growth factor-beta receptor signaling via the inhibition of the reactive oxygen species-mediated Syk pathway. Circ Res. 2009;104:219-227.

[19] Li W, Jeong JH, Park HG, Lee YR, Li M, Lee SK. Endurance exercise training inhibits neointimal formation via enhancement of FOXOs expression in balloon-induced atherosclerosis rat model. J Exerc Nutr Biochem 2014;18:105-110.

[20] Song YJ, Lee JY, Joo HK, Kim HS, Lee SK, Lee KH, Cho CH, Park JB, Jeon BH. Tat-APE1/ref-1 protein inhibits TNF-alpha-induced endothelial cell activation. Biochem Biophys Res Commun. 2008;368:68-73.

[21] Pinheiro AR, Cunha AR, Aguila MB, Mandarim-de-Lacerda CA. Beneficial effects of physical exercise on hypertension and cardiovascular adverse remodeling of diet-induced obese rats. Nutr Metab Cardiovasc Dis. 2007;17:365-75.

[22] Cavalcante JL, Lima JA, Redheuil A, Al-Mallah MH. Aortic stiffness, current understanding and future directions. J Am Coll Cardio. 2011;57:1511-1522.

[23] Niederhoffer N, Kieffer P, Desplanches D, Lartaud-Idjouadiene I, Sornay MH, Atkinson J. Physical exercise, aortic blood pressure, and aortic wall elasticity and composition in rats. Hypertension. 2000;35:919-924.

[24] Miyachi M, Kawano H, Sugawara J, Takahashi K, Hayashi K, Yamazaki K, Tabata I, Tanaka H. Unfavorable effects of resistance training on central arterial compliance: a randomized intervention study. Circulation. 2004;110:2858-2863.

[25] Puthanveetil P, Wan A, Rodrigues B. FoxO1 is crucial for sustaining cardiomyocyte metabolism and cell survival. Cardiovasc Res. 2013;97:393-403.

[26] Balaban RS, Nemoto S, Finkel T. Mitochondria, Oxidants, and Aging. Cell. 2005;120:483-95.

[27] Van der Horst A, Burgering BM. Stressing the role of FoxO proteins in lifespan and disease. Nat Rev Mol Cell Biol. 2007;8:440-50.

[28] Lawler JM, Kwak HB, Kim JH, Suk MH. Exercise training inducibility of MnSOD protein expression and activity is retained while reducing prooxidant signaling in the heart of senescent rats. Am J Physiol Regul Integr Comp Physiol. 2009;296:R1496-502.

[29] Dröge W. Free radicals in the physiological control of cell function. Physiol Rev. 2002;82:47-95.

[30] Ji LL. Exercise-induced Modulation of Antioxidant Defense. Ann N Y Acad Sci. 2002;959:82-92.

[31] Kim CS, Son SJ, Kim EK, Kim SN, Yoo DG, Kim HS, Ryoo SW, Lee SD, Irani K, Jeon BH. Apurinic/apyrimidinic endonuclease1/redox factor-1 inhibits monocyte adhesion in endothelial cells. Cardiovasc Res. 2006;69:520-526.

[32] Lee SK, Chung JJ, Park MS, Joo HK, Lee EJ, Cho EJ, Park JB, Ryoo S, Irani K, Jeon BH. Apurinic/apyrimidinic endonuclease 1 inhibits protein kinase C-mediated p66shc phosphorylation and vasoconstriction. Cardiovasc Res. 2011;91:502-509.

[33] Guan Z, Basi D, Li Q, Mariash A, Xia YF, Geng JG, Kao E, Hall JL. Loss of redox factor 1 decreases NF-kappaB activity and increases susceptibility of endothelial cells to apoptosis. Arterioscler Thromb Vasc Biol. 2005;25:96-101.

[34] Kabe Y, Ando K, Hirao S, Yoshida M, Handa H. Redox Regulation of NF-kappaB Activation: Distinct Redox Regulation Between the Cytoplasm and the Nucleus. Antioxid Redox Signal. 2005;7:395-403.

[35] Mattagajasingh I, Kim CS, Naqvi A, Yamamori T, Hoffman TA, Jung SB, DeRicco J, Kasuno K, Irani K. SIRT1 promotes endothelium-dependent vascular relaxation by activating endothelial nitric oxide synthase. Proc Natl Acad Sci. 2007;104:14855-60.

[36] Qi Y, Zhang K, Wu Y, Xu Z, Yong QC, Kumar R, Baker KM, Zhu Q, Chen S, Guo S. Novel mechanism of blood pressure regulation by forkhead box class O1-mediated transcriptional control of hepatic angiotensinogen. hypertension. 2014;64:1131-40.