Long-term treadmill training ameliorates endothelium-dependent vasorelaxation mediated by insulin and insulin-like growth factor-1 in hypertension

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1Graduate Institute of Clinical Medical Science and 2Department of Physical Therapy, Graduate Institute of Rehabilitation Science, China Medical University, Taichung, Taiwan; 3Department of Healthcare Administration, Asia University, Taichung, Taiwan; 4Department of Occupational Therapy, College of Medicine, Fu Jen Catholic University, Taipei, Taiwan; 5Department of Physiology, College of Medicine, Kaohsiung Medical University, Kaohsiung, Taiwan; 6Orthopaedic Research Center, College of Medicine, Kaohsiung Medical University Hospital, Kaohsiung Medical University, Kaohsiung, Taiwan; and 7Graduate Institute of Exercise Science, University of Taipei, Taipei, Taiwan

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Long-term treadmill training ameliorates endothelium-dependent vasorelaxation mediated by insulin and insulin-like growth factor-1 in hypertension. *J Appl Physiol* 119: 663–669, 2015. First published July 16, 2015; doi:10.1152/japplphysiol.01062.2014.—Dysfunction of insulin and insulin-like growth factor-1 (IGF-1) is associated with the pathophysiology of hypertension. The influence of long-term exercise on vascular dysfunction caused by hypertension remains unclear. We investigated whether long-term treadmill training improved insulin- and IGF-1-mediated vasorelaxation in hypertensive rats. Eight-week-old male spontaneously hypertensive rats (SHR) were randomly divided into sedentary and exercise (SHR-EX) groups. The SHR-EX group was trained on a treadmill for 60 min/day, 5 days/wk, for 8 wk. Wistar-Kyoto rats (WKY) were used as the normal control group. After training, aortic insulin- and IGF-1-mediated vasorelaxation was evaluated in organ baths. Additionally, the roles of phosphatidylinositol 3-kinase (PI3K), nitric oxide synthase (NOS), and aortic protein expression were examined in the three groups. Compared with sedentary SHR and WKY groups, insulin- and IGF-1-mediated vasorelaxation was significantly enhanced in the SHR-EX group. After endothelial denudation, blunted and comparable vasorelaxation was found among the three groups. Pretreatment with selective PI3K and NOS inhibitors attenuated insulin- and IGF-1-mediated vasorelaxation, and no significant difference was found among the three groups after the pretreatment. The aortic protein levels of the insulin receptor (IR), IGF-1 receptor (IGF-1R), insulin receptor substrate-1 (IRS-1), and endothelial NOS (eNOS) were also significantly increased in the SHR-EX group compared with the other two groups. These results suggested that treadmill training elicited the amelioration of endothelium-dependent insulin/IGF-1-mediated vasorelaxation partly via the increased activation of PI3K and NOS, as well as the enhancement of protein levels of IR, IGF-1R, IRS-1, and eNOS, in hypertension.

INSULIN AND INSULIN-LIKE GROWTH FACTOR-1 (IGF-1) have been known as regulatory agents for the maintenance of cardiovascular function (17, 27, 30). Mounting evidence demonstrates that dysfunction of insulin and IGF-1 is involved in the pathophysiology of cardiovascular disorders, such as hypertension and diabetes (1, 18, 32). Insulin and IGF-1 modulate normal vasorelaxation by binding to its receptors and further activating phosphatidylinositol 3-kinase (PI3K), which phosphorylates endothelial nitric oxide synthase (eNOS) at Ser1177, subsequently resulting in the production of NO (20, 21, 37). A few researchers have indicated that vascular function mediated by insulin and IGF-1 is negatively affected in hypertension and obesity (18, 32, 33). In addition, a lower IGF-1 level has been found to be associated with a higher risk of endothelial dysfunction and cardiovascular mortality (2, 25). However, the roles of insulin and IGF-1 in regulating vascular function and related mechanisms in cardiovascular disorders, such as hypertension, have not been fully investigated.

Exercise training is a well-known regime for the prevention and treatment of cardiovascular disease, such as hypertension (7, 12, 16, 26). Many studies have suggested that treadmill training elicits multiple beneficial effects on cardiovascular function, such as lowering blood pressure, improving endothelial dysfunction, and decreasing risk of cardiovascular morbidity (8, 19, 31). Moreover, exercise training can improve arterial compliance and endothelium-dependent acetylcholine (ACH)-induced vasorelaxation in normal and hypertensive subjects (9-11, 31, 34). Recently, we found that acute moderate-intensity exercise significantly improved insulin- and IGF-1-induced vasorelaxation in hypertensive rats (35). However, what the influence is of long-term exercise training on hypertension with regard to the roles played by insulin and IGF-1 remains unclear. In the present study, we investigated whether long-term treadmill training elicits accumulative effects for the enhancement of vasorelaxation mediated by insulin and IGF-1 in hypertension. We clarified the possible roles of PI3K and NOS involved in the NO-dependent vasorelaxant pathway by using selective inhibitors. Finally, aortic protein expression related to insulin- and IGF-1-mediated vasorelaxation, including insulin receptor (IR), IGF-1 receptor (IGF-1R), insulin receptor substrate-1 (IRS-1), and endothelial NOS (eNOS), was examined after the treadmill training. Our hypothesis proposes that long-term treadmill training could ameliorate insulin- and IGF-1-mediated vasorelaxation in hypertensive rats, which were dysfunctional because of hypertension.

MATERIALS AND METHODS

*Animals.* The spontaneously hypertensive rat (SHR) is commonly used as a model for human hypertension. The SHR with an increase in peripheral vascular resistance develops high blood pressure, vas-
Resting blood pressure and heart rate. All rats were loosely restrained when measuring the resting blood pressure and heart rate. By using a tail-cuff pressure meter system (LE5001, Panlab, Wood Dale, IL), the systolic/diastolic blood pressure (SBP/DBP), mean arterial pressure. *P < 0.05, significant differences from WKY group; #P < 0.05, significant differences between SHR and SHR-EX groups.

<table>
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<th>General characteristics</th>
<th>WKY</th>
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<td>147 ± 2*#</td>
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<td>Citrate synthase activity</td>
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<td>2.68 ± 0.03</td>
<td>2.96 ± 0.03*#</td>
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</table>

Values are means ± SE. Three groups: Wistar-Kyoto rats (WKY), spontaneously hypertensive rats (SHR), and SHR with exercise training (SHR-EX). SBP, systolic blood pressure; DBP, diastolic blood pressure; MAP, mean arterial pressure. *P < 0.05, significant differences from WKY group; #P < 0.05, significant differences between SHR and SHR-EX groups.

Citrate synthase activity. The effect of aerobic exercise training is commonly confirmed by an increase in citrate synthase activity. Citrate synthase activity was measured as described previously (34). Briefly, soleus muscle samples were homogenized in five times volume of homogenizing buffer (0.1 M Tris buffer containing 0.1% Triton X-100). The enzyme activity was measured by spectrophotometer (Beckman Coulter DU-800) at 412 nm absorbance and expressed as micromoles of substrate utilized per minute per gram of the wet tissue.

Vasoreactivity experiments. The vasorelaxant responses of isolated aortic rings (3-mm length) were recorded isometrically by force displacement transducers (Grass Instrument, West Warwick, RI) and submerged in organ chambers containing the Krebs-Ringer buffer (118 mM NaCl, 4.8 mM KCl, 2.5 mM CaCl2, 1.2 mM MgSO4, 1.2 mM KH2PO4, 24 mM NaHCO3, 0.03 mM Na2-EDTA, and 11 mM glucose) oxygenated with 95% O2 and 5% CO2 at 37°C. The isolated aortic rings were stretched to the optimal passive tension (i.e., 2 g) and equilibrated for at least 60 min before drug administration. After equilibration, they were precontracted with phenylephrine (10⁻⁷ mol/l, Sigma Chemical, St Louis, MO) and exposed to various concentrations of insulin (3 × 10⁻⁸ to 3 × 10⁻⁶ mol/l, Sigma Chemical), IGF-1 (10⁻⁹ to 10⁻⁷ mol/l, CytoLab, Rehovot, Israel), and sodium nitroprusside (3 × 10⁻¹¹ to 3 × 10⁻⁹ M, Merck, Darmstadt, Germany) to evoke dose-dependent vasorelaxant responses. In the endothelium-denuded rings, these vasorelaxant responses were also evaluated among the three groups. To examine the roles of PI3K and NOS in the insulin- and IGF-1-mediated vasorelaxant responses, the selective inhibitors wortmannin (3 × 10⁻⁷ M; an inhibitor of PI3K; Sigma Chemical) and nitro-l-arginine methyl ester (l-NAME) (10⁻⁶ M; a NOS inhibitor; Sigma Chemical), were used as pretreatments for 15 min before the administration of phenylephrine to endothelium-intact rings (33).

Western immunoblot. Aortic tissue extracts were obtained by homogenization at 4°C in tissue lysis buffer (20 mM Tris, 2 mM EDTA, 0.03 M NaCl containing 1% Triton X-100). The tissue extracts were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to nitrocellulose membrane. The membranes were immunoblotted with the primary antibodies and then with the secondary antibodies. The immunoreactive bands were visualized by chemiluminescence.
10% glycerol, 50 mM 2-mercaptoethanol, pH 7.4) supplemented with complete protease and phosphatase inhibitors (Roche Applied Science, Mannheim, Germany). The homogenates were sequentially centrifuged at 12,000 g for 20 min and the supernatant collected. Protein concentration of the aortic tissue extract supernatant was determined by the Bradford method (Bio-Rad Laboratories, Hercules, CA) with bovine serum albumin as a standard. Protein samples (50 μg/lane) were separated by 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with a minigel apparatus (Bio-Rad Laboratories) and subsequently transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, MA). Primary antibodies, including anti-insulin receptor (Cell Signaling Technology, Boston, MA), anti-IGF-1 receptor (Cell Signaling), anti-IRS-1 (Sigma Chemical), and anti-eNOS (Cell Signaling), were diluted to 1:500 in antibody binding buffer overnight at 4°C. Actin (Millipore) was used as the loading control. After incubation with the appropriate primary antibody, the peroxidase-conjugated secondary antibodies (1:5,000; Cell Signaling) were then incubated at room temperature for 1 h. The immunoblotted proteins were detected by enhanced chemiluminescence by using enhanced chemiluminescence detection reagents in the Gel Doc XR System (Bio-Rad Laboratories).

Statistical analysis. All data were presented as means ± SE. Multiple group comparisons were performed by using one-way analysis of variance (ANOVA), followed by Duncan post hoc analysis. Dose responses of vasorelaxation were analyzed by ANOVA with a repeated measures design. In all cases, \( P < 0.05 \) was considered statistically significant.

Fig. 2. Vasorelaxant responses of insulin-like growth factor-1 (IGF-1) (10^{-9} to 10^{-7} M) at cumulative concentration-response curves for endothelium-intact (A) and endothelium-denuded (B) aortic rings in WKY, SHR, and SHR-EX groups. * \( P < 0.05 \), significant differences from WKY group; # \( P < 0.05 \), significant differences between SHR and SHR-EX groups; \( + P < 0.05 \), pre- vs. postinhibition with wortmannin or L-NAME; \( n = 8 \) in each group.

Fig. 3. Vasorelaxant responses of insulin (3 × 10^{-9} M) (A) or IGF-1 (10^{-8} M) (B) in the presence of wortmannin (3 × 10^{-7} M) or nitro-l-arginine methyl ester (l-NAME) (10^{-6} M) in WKY, SHR, and SHR-EX groups. * \( P < 0.05 \), significant differences from WKY group; # \( P < 0.05 \), significant differences between SHR and SHR-EX groups; \( + P < 0.05 \), pre- vs. postinhibition with wortmannin or l-NAME; \( n = 8 \) in each group.

Fig. 4. Vasorelaxant responses of sodium nitroprusside (SNP) (3 × 10^{-11} to 3 × 10^{-9} M) at cumulative concentration-response curves in WKY, SHR, and SHR-EX groups. \( n = 8 \) in each group.
RESULTS

General characteristics. After 8 wk of training, body weight was similar among the WKY, SHR, and SHR-EX groups. However, the SBP and MAP were significantly ($P < 0.05$) decreased in the SHR-EX group compared with the SHR group, which was found only after 8 wk of training (Table 1). In addition, the level of citrate synthase activity was significantly ($P < 0.05$) enhanced in the SHR-EX group, but not in the WKY and SHR groups, indicating that our training program was aerobic based and effective (Table 1).

Vasorelaxation mediated by insulin and IGF-1. Figure 1 shows the cumulative dose-response curves for vasorelaxation mediated by insulin in the WKY, SHR, and SHR-EX groups after the treadmill training. Compared with sedentary SHR and WKY groups, insulin- and IGF-1-mediated vasorelaxation was significantly ($P < 0.05$) enhanced to a nearly normal level in the SHR-EX group (Fig. 1A). However, in the endothelium-denuded rings, insulin-mediated vasorelaxation was blunted, and no significant difference was found among the three groups (Fig. 1B).

Similarly, Fig. 2 shows the vasorelaxation mediated by IGF-1 in the three groups after training. This indicated that the IGF-1-mediated vasorelaxation was significantly ($P < 0.05$) enhanced in the SHR-EX group compared with the other two groups (Fig. 2A). After the endothelium was denuded, no significant difference was found in the IGF-1-mediated vasorelaxation among the three groups (Fig. 2B).

Roles of PI3K and NOS in insulin- and IGF-1-mediated vasorelaxation. To verify the roles of PI3K and NOS in insulin- and IGF-1-mediated vasorelaxation, the selective inhibitors wortmannin and L-NAME were pretreated to evaluate vasorelaxation (Fig. 3). Before wortmannin or L-NAME was added, the vascular responses to insulin ($3 \times 10^{-7}$ M) and IGF-1 ($10^{-8}$ M) were significantly ($P < 0.05$) higher in the SHR-EX group than in the SHR group. However, if either wortmannin or L-NAME were added, the insulin- and IGF-1-mediated vasorelaxation was significantly ($P < 0.05$) diminished in the three groups, and thus the group differences were absent (Fig. 3, A and B).

Vasorelaxation mediated by sodium nitroprusside. As shown in Fig. 4, the cumulative dose-response curves of sodium nitroprusside-mediated vasorelaxation were also evaluated in the WKY, SHR, and SHR-EX groups. We found that...
the administration of sodium nitroprusside, a direct vasodilator of vascular smooth muscle, caused a concentration-dependent vasorelaxation in all three groups. However, there was no significant difference in endothelium-independent sodium nitroprusside-mediated vasorelaxation among the three groups.

**Aortic protein expression.** The aortic protein expression involved in the insulin- and IGF-1-mediated vasorelaxation, including IR, IGF-1R, IRS-1, and eNOS, was evaluated among the three groups after the treadmill training. Figure 5 shows that the proteins of IR, IGF-1R, IRS-1, and eNOS were significantly (*P < 0.05*) decreased in the SHR group compared with the WKY group, whereas these proteins were significantly (*P < 0.05*) increased in the SHR-EX group, compared with the SHR group.

**DISCUSSION**

To the best of our knowledge, this is the first study to examine the long-term effects of exercise intervention on insulin- and IGF-1-mediated vasorelaxation in hypertension. We found that the 8-wk treadmill training significantly improved the insulin- and IGF-1-mediated vasorelaxation, which was dysfunctional because of hypertension, to a nearly normal level in hypertensive rats. These improvements were mediated partly by the increased activation of PI3K and NOS in an endothelium-dependent manner. In addition, the aortic protein levels of aortic IR, IGF-1R, IRS-1, and eNOS were significantly enhanced in hypertensive rats after the treadmill training. However, the treadmill training did not affect the endothelium-independent sodium nitroprusside-mediated vasorelaxation in hypertension.

Numerous studies have reported that regular exercise training induces positive effects on cardiac and vascular function in several cardiovascular diseases, such as hypertension, atherosclerosis, and stroke (7, 14, 24, 29). Clinically, it has been regarded as an efficient nonpharmacological treatment for hypertensive patients (5, 7, 10, 19). Exercise training can efficiently ameliorate high blood pressure and endothelium-dependent ACh-induced vasorelaxation in hypertension (9, 11, 31, 34). However, what the influence is of long-term exercise training on hypertension with regard to the roles played by insulin and IGF-1 remains unclear. Our previous findings indicated that chronic exercise significantly enhanced both insulin- and IGF-1-mediated vasorelaxation in normal animals (34). In addition, we found that single-bout moderate exercise significantly improved these vasorelaxant responses in hypertensive animals (35). The effect of long-term exercise training in hypertension was further highlighted by the present study. Our results indicate that 8 wk of treadmill training-induced cumulative effects and improved insulin- and IGF-1-induced vasorelaxation in hypertensive rats to a near-normal level. Also, these exercise-induced improvements in hypertensive rats have been found in an endothelium-dependent manner by using the endothelium-denuded vessels.

In addition to glucose metabolism, insulin and IGF-1 both modulate specific cardiovascular responses, such as an increase in skeletal muscle blood flow and a decrease in vascular resistance (28, 30). Several studies have noted that insulin- and IGF-1-mediated vascular function are impaired in hypertensive and diabetic animal models (1, 18, 32, 35). Specifically, insulin and IGF-1 exert normal vasorelaxant responses which are via PI3K and eNOS pathways in normotensive WKY rats, but these responses were dysfunctional in SHR (18, 35). Consistent with previous findings, the present study indicates that the insulin- and IGF-1-mediated vasorelaxant property was impaired in hypertensive rats. Furthermore, the preincubation of PI3K or NOS inhibitor almost completely inhibited insulin- and IGF-1-mediated vasorelaxation in hypertensive rats. These results support that the impairment of insulin- and IGF-1-mediated vasorelaxation in hypertension was partly through the alteration of PI3K and NOS activation.

Inhibitors of PI3K and NOS were administrated to study the roles of PI3K and NOS in the exercise-induced improvements of insulin- and IGF-1-mediated vasorelaxation in hypertensive rats. We found that the exercise-induced improvements of insulin- and IGF-1-induced vasorelaxation were significantly diminished after the administration of PI3K or NOS inhibitor, and that differences among the three groups were absent. This suggested that the exercise-induced protective effects were related to the increased activation of PI3K and NOS, and partly resulting in the improvement of insulin- and IGF-1-induced vasorelaxation in hypertensive rats. One previous study demonstrated that insulin and IGF-1 stimulated NO production and vasorelaxation by the PI3K/Akt/eNOS pathway (20). Another
study indicated that vascular Akt signaling contributed importantly to eNOS phosphorylation and activation during treadmill running (37). However, the role of Akt was not explored in the present study, and further study should be required to clarify its role in hypertension following exercise.

Sodium nitroprusside is a potent vasodilator and induces endothelium-independent vasorelaxation (11, 34, 35). In this study, we examined sodium nitroprusside-induced vasorelaxation to determine whether exercise training affects the endothelium-independent vasorelaxant responses in hypertensive rats. Similar to previous studies, we found that the treadmill training did not affect the sodium nitroprusside-induced vasorelaxation in hypertension. Also, there was no discrepancy of the sodium nitroprusside-induced vasorelaxation among CON, SHR, and SHR-EX groups. Therefore, we suggest that endothelium-independent vasorelaxation would not be changed by hypertension or exercise training. Since the upregulation of insulin/IGF-1 receptors and downstream proteins, such as IRS-1 and eNOS, are considered to be the important factors involved in exercise-enhanced vasorelaxation, we also examined these protein expressions. Our results show that hypertension significantly decreased the protein expressions of IR and IGF-1, IRS-1, and eNOS, whereas exercise training significantly enhanced these protein expressions in the aortas of hypertensive rats. Previous studies have indicated that single-bout or long-term exercise improves endothelium-dependent vasorelaxation, which could be due to its receptor upregulation and eNOS expression (4, 9, 35). In the current study, we report that 8 wk of treadmill training ameliorated the vasorelaxant responses to insulin and IGF-1, mainly through the upregulation of their receptors, IRS-1, and eNOS proteins in hypertension. However, the upstream signals which stimulate the protein upregulation for these exercise-induced improvements in the vasorelaxant responses need to be further clarified. Several studies have indicated that exercise-induced increases in laminar shear stress have beneficial effects on vascular function and reactivity (8, 15, 23, 37). Physiologically, exercise training upregulates eNOS phosphorylation and enzyme activity, which is a response to increased shear stress during exercise (8, 23, 37). In vitro studies show that laminar shear stress affects multiple signaling pathways, including intracellular protein kinases Akt, PI3K, and endothelium-derived NO pathways (3, 13, 36, 37). Therefore, shear stress-mediated improvements in vascular endothelial function could provide one plausible explanation for the exercise-induced cardiovascular protection.

In conclusion, our study clearly demonstrates that the 8-wk treadmill training significantly improved the insulin- and IGF-1-mediated vascular function in an endothelium-dependent manner, which was dysfunctional in hypertension. These improvements were associated with the increased activation of PI3K and NOS, as well as the enhancement of protein levels of IR, IGF-1, IRS-1, and eNOS. However, the endothelium-independent vasorelaxation was not affected by the treadmill training in hypertension. Our findings contribute to parts of the theoretical base for the amelioration of hypertension-induced vascular dysfunction through long-term exercise intervention (as shown in Fig. 6). Further clinical and experimental studies are required to clarify the possible therapeutic applications in hypertension.


