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Chronic Exercise Enhances Vascular Responses to Clonidine in Rats by Increasing Endothelial α_2-Adrenergic Receptor Affinity

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Abstract

Chronic exercise increases endothelium-dependent vasodilating responses. To investigate whether endothelial α_2-adrenergic receptor upregulation is involved in the enhancement of clonidine-induced vasorelaxation by chronic exercise, 4-week-old male Wistar rats were used. They were divided into control and exercise groups. The trained animals ran on a treadmill at a moderate intensity for 60 min per day, 5 days per week for 10 weeks in total. Resting heart rates were measured by a tail-cuff method to confirm training effects. After training, rings of the thoracic aorta were prepared to evaluate vasodilating responses to clonidine, an α_2 agonist. Released endothelium-derived relaxing factors were pharmacologically identified by treatment of N^{ω}-nitro-L-arginine, a nitric oxide (NO) synthase inhibitor, or tetraethylammonium chloride, an endothelium-derived hyperpolarization factor (EDHF) inhibitor. Receptor binding assays were performed by using ^{3}H-labeled clonidine as a tracer. We found that chronic exercise enhanced vascular responses to clonidine by stimulating the release of both NO and EDHF. It also increased the binding affinity of endothelial cell α_2 receptor without changing the number of binding sites. Therefore, the elevated vasorelaxing responses to clonidine after chronic exercise may be partially resulted from an increase in endothelial α_2 receptor binding affinity.

Key Words: adrenergic agonist, arteries, endothelial receptors, nitric oxide, Wistar rats

Introduction

Endothelium can regulate vascular tone by releasing endothelium-derived vasoactive mediators (3, 20, 21). Several reports have indicated that α-adrenergic agonists evoke endothelium-dependent vasorelaxation and depress vasocontractile responses if endothelium is intact (11, 14, 17, 19, 25). We and other investigators have found that exercise training alters vasoreactivity to norepinephrine-induced vasocontraction and α-adrenergic agonists-induced vasorelaxation by releasing greater amounts of relaxing factors (4, 7, 13). In addition, we also noticed that only receptor-mediated vasodilating responses, but neither the basal release of endothelium-derived relaxing factors nor the responses to nonreceptor-mediated vasodilators, were altered by chronic exercise (4, 7). Our previous study has shown that acute exercise upregulates endothelial α_2 receptors (10). Whether chronic exercise can upregulate α receptors on endothelial cell membranes and lead to greater vascular responses to α-adrenergic agonists is unknown. Since clonidine (CLO, an α_2 agonist), but not phenylephrine (an α_1 agonist), could induce endothelium-dependent vasodilation in thoracic aortae of Wistar rats (10), we therefore conducted this study to investigate 1) the effect of chronic exercise on vascular responses to CLO; 2) the possibility of receptor upregulation in exercise-enhanced vasorelaxing responses to CLO. In addition, the relaxing factors involved in the CLO-induced vasorelaxation were clarified by treatment of N^{ω}-nitro-L-arginine, a nitric oxide (NO) synthase inhibitor, or tetraethylammonium chloride, an endothelium-derived hyperpolarization factor (EDHF) inhibitor.

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Materials and Methods

Animals and Exercise Protocol

The investigation confirms with the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). Four-week-old male Wistar rats were purchased from the Animal Center of National Cheng-Kung University Medical College, Tainan, Taiwan, ROC. All rats were housed in an environmentally controlled room (temperature 25±1°C; 12 hr light/12 hr dark cycle) and fed with a standard rat chow. They were randomly divided into control and exercise groups. After one week of familiarization, rats in the exercise group were trained by running on a motor-driven drum exerciser (Drex, Columbus Instrument, Columbus, OH, USA) at a speed of 0.2 m/sec for 20 min on the first day. This exercise intensity was approximately 60% of predetermined peak oxygen consumption as described in detail previously (5). On the subsequent days of training, the running time was extended by 10 min/day until a running time of 60 min/day was reached. The training speed was increased 0.05m/sec every 2 weeks. These animals were trained for 5 days/week for 10 weeks. The sedentary control animals were placed in the drum exerciser for 10 min/day without running for 10 weeks.

Measurement of Resting Heart Rates

Resting heart rates (HR) were measured by a tail cuff method (NARCO Bio-Systems, Houston, TX, USA) to assess the training effects. The animals were initially restrained in the measuring cages for 30 min/day to avoid novel effects. Following 1 week of familiarization, their resting HR were determined weekly. The results between control and trained groups were compared at the end of this experiment.

Vessel Preparation

To avoid the acute effects of exercise, animals were killed at least 48 hours after training by decapitation. Rings of thoracic aortae (3 mm long) were carefully excised and submerged in organ chambers containing oxygenated (95%O_2-5%CO_2) Krebs-Ringer solution which had the following composition (in mM):118.0 NaCl, 4.8 KCl, 2.5 $CaCl_2$, 1.2 $MgSO_4$, 1.2 KH_2PO_4, 24 $NaHCO_3$, 0.03 Na_2-EDTA, and 11.0 glucose (37°C, pH 7.4). After the vessel rings had been mounted on a force transducer (model FT03, Grass), they were progressively stretched to the optimal passive tension (ie, 1.8 g).

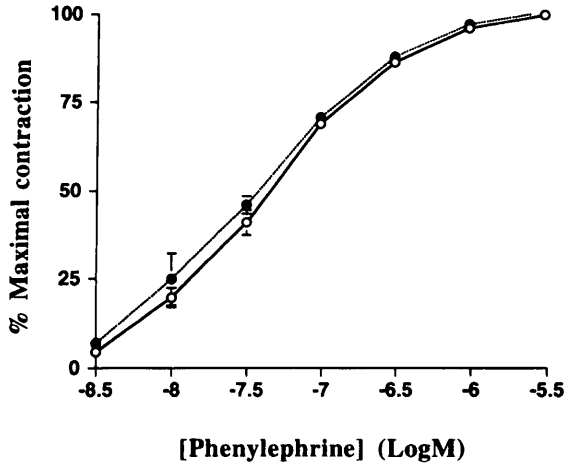

Fig. 1. Comparison of dose-response relations of PHE-induced vasocontraction between control (open circle, n=16) and exercise (closed circle, n=16) groups. It is noticed that chronic exercise does not influence vasocontractile responses to PHE (p=0.27, ANOVA with repeated measures design).

Functional integrity of the endothelium was confirmed by the fact that acetylcholine could induce at least 80% relaxation in norepinephrine-precontracted vessel rings. Experiments of vascular responses were carried out using vessel rings equilibrated for 120 min at its optimal passive tension.

Vascular Responses to CLO

Since vascular responses to phenylephrine (PHE) were unaltered by chronic exercise (Fig. 1), the vessel rings were precontracted with PHE (10^{-7} M). Dose responses of CLO-induced vasorelaxation were assessed by adding CLO cumulatively to the chamber solution (the range of final concentrations was from 3×10^{-9}~10^{-5} M). The vascular sensitivity to CLO was evaluated by its median effective dose (ED_{50}), which was obtained by logistic curve fitting of the semilog dose-response relationship for each vessel ring.

Characterization of CLO-Induced Endothelium-Derived Relaxing Factors

In order to clarify which types of relaxing factors are involved in CLO-induced vasorelaxation, vascular responses to CLO (10^{-7} M) were evaluated in the presence or absence of N^{ω}-nitro-L-arginine (LNNA, 10^{-4} M), an NO synthase inhibitor, or tetraethylammonium chloride (TEA, 10^{-2} M), an EDHF blocker.

Endothelial Membrane Preparation

Endothelial membrane fraction of thoracic

aortae was prepared for receptor binding assays as described in a previous study (10). The thoracic aortae from 24 rats of each group were pooled and placed in a cold Tris/EDTA buffer containing 50 mM Tris-HCl, 5 mM EDTA, and 0.1 mM phenylmethylsulphonyl-fluoride (PMSF) (pH 7.4). The lumen of the aorta was flushed with cold buffer to remove blood clots. After the aortae were stripped clean of fat and connective tissue, they were cut into half (about 1.5 cm each), everted over Pasteur pipettes, and immersed in a buffer with 50 mM Tris-HCl, 1% EDTA, 0.1 mM PMSF (pH 7.4) on ice for 15 min. Then the preparations were put into an ice water bath, sonicated (L&R ultrasonics, Quantrex 280H, NJ) for 25 min and vortexed for 3 min. To rule out the possibility of vascular smooth muscle contamination in our samples, we observed the remaining vessel preparation under scanning electron microscope (SEM) as described in our previous report (8). Selective rupture and removal of the endothelium under these conditions were found, as shown in our previous study (10). The cloudy solution of ruptured endothelial cells was centrifuged at 40,000 g for 30 min, and washed with a buffer containing 50 mM Tris-HCl, 0.1 mM PMSF (pH 7.4). The final pellet was resuspended in this assay buffer and its protein content was determined by Lowry assay (18), using bovine serum albumin as a standard. These experiments were repeated twice with a total of 48 rats used in each group.

Radioligand Binding Assay for Endothelial α_2 Receptors

The method used for α_2 receptor binding assays was adopted from Bockman, et al (1). For saturation studies, aliquots of endothelial membrane preparations (~35 μg of protein) were incubated in an assay buffer with various concentrations of ^{3}H-labeled CLO (ranged from 0.1 to 10 nM; specific activity 61.9 Ci/mmol, Du Pont NEN) in a final volume of 500 μl. After incubation for 45 min at room temperature, the bound and free 3H-labeled CLO were separated by vacuum filtration (cell harvester, FH225V, Hoefer Scientific Instruments) through 0.2% polyethylene imine pretreated Whatman GF/B glass fiber filters. The filter discs were washed three times with ice-cold binding buffer and counted for trapped radioactivity. The nonspecific binding was determined in a similar manner in the presence of 100 times excess of cold CLO. Radioactivity retained on the filter was determined by liquid scintillation counter (Beckman, LS5000TA). Affinity (K_d) and maximal binding sites (B_{max}) were calculated from a Scatchard plot by using a linear regression analysis of binding data (22).

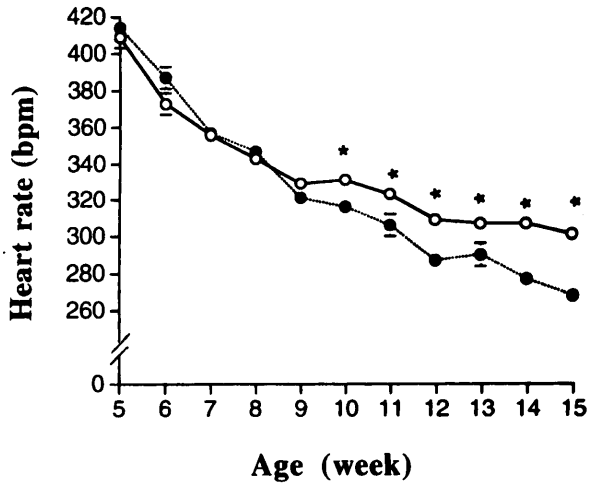

Fig. 2. Chronic exercise lowers resting heart rates in male Wistar rats (n=19 for each group). Open circle is for the control group, closed circle is for the trained group. *p<0.05.

Reagents

All chemicals for the preparation of Krebs-Ringer solution were purchased from Merck (Darmstadt, Germany). Polyethylene imine was purchased from Fluka (Buchs, Switzerland), and GF/B filter discs were from Whatman (Maidstone, England). ^{3}H-labeled CLO was purchased from NEN, Dupont (Boston, USA). Other reagents were obtained from Sigma Chemical Co (St. Louis, USA).

Statistical Analysis

Data were expressed as mean ± SEM. Unpaired Student's *t* test was used to compare the results between control and exercise groups. Comparison of dose-response curves was evaluated by analysis of variance with a repeated-measures design. Differences would be considered significant at $p<0.05$.

Results

Resting HR

Figure 2 showed that resting HR were lowered after moderate exercise training. This physiological parameter indicated that our training protocol indeed had training effects.

Vascular Response to CLO

We found that chronic exercise shifted the dose-response curve of CLO-induced vasorelaxation to the left (Fig. 3). In addition, the vascular sensitivity to CLO, indicated by ED_{50}, was enhanced by exercise

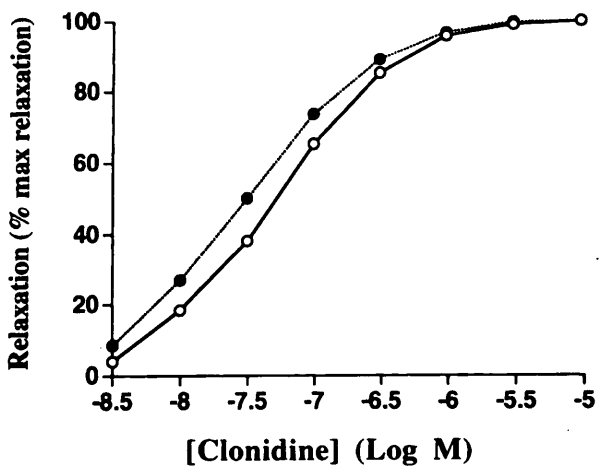

Fig. 3. Comparison of dose-response relations of CLO-induced vasorelaxation between control (open circle, n=12) and exercise (closed circle, n=10) groups. Chronic exercise shifted the curve to the left ($p<0.01$, ANOVA with repeated measures design). Note: the error bars in this figure are too small to be plotted clearly.

training (-7.29±0.04 for the control and -7.52±0.03 for the exercise group, respectively; $P<0.05$).

Relaxing Factors induced by CLO

Table 1 shows that LNNA or TEA partially inhibits CLO-induced vasorelaxing responses, while co-administration of LNNA and TEA blocks these responses completely. Therefore, both NO and EDHF are involved in CLO-induced vasorelaxation.

In addition, we noticed that the extent of relaxation in the trained group was greater than the control (Table 1). The difference was abolished by LNNA or TEA pretreatment. It implies that chronic exercise enhances CLO-induced vasorelaxation is due to an increase in NO/EDHF release.

Radioligand Binding Assay for Endothelial α_2 Receptors

The Scatchard plot of CLO binding assay was linear as shown in our previous study (9), indicating a single type of adrenergic receptors in rat aortae. This is consistent with a previous study on porcine coronary arteries (1). Table 2 showed that endothelial α_2 receptors in the exercise-trained group had higher affinity to CLO than the control. In contrast, there was no significant difference in B_{max}.

Discussion

Our results indicated that in the thoracic aorta of male Wistar rats, 1) the mediators for CLO-induced vasorelaxation were NO and EDHF; 2) chronic exercise enhanced CLO-evoked vasodilating responses by increasing the release of these two endothelium-derived relaxing factors (ie, NO and EDHF); 3) chronic exercise upregulated endothelial α_2 receptors, indicated by enhanced affinity to CLO.

In this study, we found that in male Wistar rats, chronic exercise enhanced CLO-induced vasorelaxation (Fig. 4). This finding is consistent with our previous reports in rabbits or spontaneously hypertensive rats (4, 7). As exercise does not change vascular responses to sodium nitroprusside, an NO donor, in denuded vessels (6), it is unlikely that the increase in CLO-induced vasorelaxing responses after chronic exercise was due to an increased sensitivity of vascular smooth muscles to relaxing factors.

Our results in Table 1 clearly show that co-administration of LNNA and TEA, but not either one alone, completely abolished CLO-evoked vasorelaxation. These observations suggest that this vasorelaxation is totally mediated by the release of NO and EDHF, and that both relaxing factors are involved in the exercise effect. It is interesting that either LNNA or TEA alone can eliminate about 70%

Table 1. Vascular Responses to CLO (10^{-7} M) in Control and Trained Vessels

Treatment	Relaxation (% contraction)	
	Control	Trained
LNNA:	(n=6)	(n=7)
−	52±6	68±3*
+	14±2#	12±2#
TEA:	(n=6)	(n=7)
−	57±3	69±4*
+	17±7#	21±6#
LNNA+TEA:	(n=5)	(n=5)
−	56±2	72±5*
+	0#	0#

−: without treatment; +: with treatment
* $p<0.05$ (trained vs. control); # $p<0.05$ (posttreatment vs. pretreatment) n: sample size

Table 2. Comparison of ^{3}H-CLO Binding Results in Aortic Endothelial Membranes between Control and Trained Groups

Parameters	Control	Trained
Bmax (fmol/mg Protein)	11.2±0.7	9.3±0.9
Kd (nM)	4.1±0.4	0.5±0.1*

Data are expressed as mean ± SEM of two experiments (24 rats were pooled together for each group per experiment).
*$p<0.05$ (trained vs. control)

of CLO-induced vasodilating responses. Since LNNA is a specific NO synthase inhibitor, NO should be the dominant mediator of CLO-induced vasorelaxation. On the other hand, in addition to stimulating guanylate cyclase, NO has been reported to be able to activate potassium channels in vascular smooth muscle cells and cause their hyperpolarization as well (2, 24). Therefore, TEA pretreatment inhibits not only EDHF effect but also part of NO effect. This can explain why TEA-caused inhibition is about 70%, not 30%.

Previous studies showed that only receptor-mediated vasodilating responses, but not the basal release of endothelium-derived relaxing factors or responses to nonreceptor-mediated vasodilators, were altered by chronic exercise (4, 6, 7). We therefore postulated that chronic exercise might upregulate endothelial receptors. The present study supports this viewpoint by demonstrating that chronic exercise upregulates endothelial α_2 receptors, as indicated by an increase in the affinity of these receptors (Table 2). However, a previous study did not show receptor upregulation of endothelial muscarinic receptors after chronic exercise (9). This discrepancy implies that some mechanisms other than the receptor upregulation may be involved in the exercise effects as well.

Although receptor upregulation is one possible mechanism for the enhancement of CLO-induced vasorelaxation by chronic exercise, the possibility of modifying some downstream signal transduction pathways of α_2 receptors cannot be ruled out. One potential candidate in this regard is the upregulation of endothelial nitric oxide synthase (eNOS). Recent studies have demonstrated that exercise training increases eNOS gene expression in rats, dogs, or pigs (12, 23, 26). Although these studies support the eNOS upregulation be a possible mechanism for the elevation of agonist-induced vasorelaxation, they can not explain the fact that there is no change in basal NO release after training (4, 7). The receptor upregulation mechanism, on the other hand, is consistent with the functional observations.

Our present results demonstrate that chronic exercise enhances CLO-stimulated NO/EDHF release (Table 1). In addition to an enhancement of endothelial α_2 receptor binding affinity, there may be some other alterations in the downstream intracellular signaling components, such as intracellular calcium levels, responsible for these exercise effects. This part of study is ongoing in our laboratory.

Previous studies have reported that membrane fluidity change or receptor conformational change would alter receptor affinity (15, 16). Whether the increase in affinity of endothelial α_2 receptors after chronic exercise is due to membrane fluidity change or receptor conformational change needs to be further investigated. It is interesting to note that acute exercise in rats also enhances receptor-mediated endothelium-dependent vasodilation (10). Moreover, this latter effect can be partially explained by receptor upregulation as well (10). In our hands, both acute and chronic exercises increase endothelial α_2 receptor binding affinity. Nevertheless, chronic exercise does not influence α_2 receptor numbers, whereas acute exercise decreases these receptor numbers, possibly due to agonist-promoted receptor internalization as plasma catecholamine level is elevated after acute exercise. Apparently various forms of exercise may affect vascular function via similar, but not necessarily the same, receptor upregulation mechanisms.

In conclusion, 10 weeks of running exercise training in male Wistar rats can enhance CLO-induced vasorelaxing response of thoracic aortae. This alteration is, at least in part, caused by endothelial α_2 receptor upregulation.

Acknowledgments

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