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Effects of Chronic Exercise on Muscarinic Receptor-Mediated Vasodilation in Rats

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Abstract

Previous studies have demonstrated that vascular responses to acetylcholine (ACh), an endothelium-dependent vasodilator, are enhanced in exercise-trained animals. In order to see if chronic exercise upregulates endothelial muscarinic (M) receptor, the subtype of M receptors responsible for ACh-induced vasorelaxation in the thoracic aorta of male Wistar rats was characterized first, then a receptor assay was performed. These animals were divided into exercise and control groups. The trained rats ran on a treadmill with a moderate intensity for 60 min per day, 5 days per week. After 10 weeks of training, rats were decapitated and their thoracic aortae were isolated. The subclass of M receptor in endothelium was pharmacologically identified on the basis of selective affinity of antagonists; ie, pirenzepine for M_1, gallamine for M_2, and 4-diphenylacetoxy-N-methylpiperidine methiodide for M_3. Our results showed that in the thoracic aorta of Wistar rats, 1) ACh-induced vasorelaxation was mediated by M_3 receptor; 2) chronic exercise enhanced ACh-evoked vasodilating responses. However, this alteration was not caused by receptor upregulation, as maximal binding sites and affinity of M_3 receptor were not changed by chronic exercise. Other possible mechanisms need to be further studied.

Key Words: nitric oxide, M_3 receptor, receptor assay

Introduction

Endothelium can regulate vascular tone by releasing several endothelium-derived vasoactive mediators (5, 14, 16). Our previous studies and other reports have shown that exercise training enhances acetylcholine (ACh)-induced vasorelaxation by releasing greater amounts of relaxing factors (2, 4, 7). In addition, we also noticed 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 (2, 4). Our recent studies found that endothelial receptors, including muscarinic and α_2-adrenergic receptors, were upregulated by a single bout of exercise (unpublished data). Whether chronic exercise can upregulate muscarinic receptors on endothelial cell membranes and lead to greater vascular responses to ACh is still unknown. We therefore conducted this study to investigate the possible role of receptor upregulation in chronic exercise-increased vasodilating responses to ACh.

In 1992, mRNA transcripts for 3 subtypes of muscarinic receptors have been identified in freshly isolated bovine endothelial cells (22). Other previous studies have demonstrated that ACh-induced vasodilating responses in rat pulmonary artery (12), feline middle cerebral artery (6), rabbit intrapulmonary arteries (1), and guinea-pig coronary artery (10) are mediated by M_3 subtype of endothelial receptors. In order to examine whether M_3 receptor is also responsible for ACh-induced vasorelaxation in rat thoracic aorta, specific muscarinic antagonists, ie, pirenzepine, gallamine, and 4- diphenylacetoxy-N-methylpiperidine methiodide (4-DAMP), were tested for their inhibitory effects on the ACh-induced vasorelaxation. The antagonist potency, indicated by the values of pA_2 (ie, the negative logarithm of the molar concentration of antagonist that produces a dose ratio of 2), for these antagonists were also determined (21). After endothelial muscarinic

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receptor subtype in rat thoracic aortae was identified, tritium-labeled antagonists were used for receptor binding assays. Results from control and trained groups were then compared to see if endothelial M_3 receptor upregulation plays a role in the alteration of vascular responses to ACh after chronic exercise.

Materials and Methods

Animals and Exercise Protocol

This study was conducted in confirmity with the policies and procedures detailed in the "Guide for the Care and Use of Laboratory Animals". 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 and fed with a standard rat chow. They were divided into control and exercise groups. After one week of familiariza-tion, rats in the exercise group were trained by running on a motor-driven drum exerciser (Drex, Columbus Instrument, Columbus, OH, USA) at an intensity of 60% of predetermined peak oxygen consumption ($\dot{V}_{O2}$peak), as described in detail previously (3), for 20 min on the first day. 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 loads were readjusted every 2 weeks according to the animal's newly determined $\dot{V}_{O2}$peak. These animals were trained for 5 days/week for 10 weeks. The sedentary control animals were placed in the drum exerciser without running for 10 min/day until sacrifice.

Measurement of Resting Systolic Blood Pressure and Heart Rates

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

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, pH7.4). After the vessel rings had been mounted on a force transducer (model FT03, Grass), they were progressively stretched to the optimal passive tension at which the contractile response evoked by norepinephrine (NE) was maximal. Functional integrity of the endothelium was confirmed by the fact that ACh could induce at least 80% relaxation in NE-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 ACh

After the vessel rings were prepared and equilibrated for 120 min, they were precontracted with NE (3×10^{-8}M). Dose responses of ACh-induced vasorelaxation were assessed by adding ACh cumulatively to the chamber solution (the range of final concentrations was from 10^{-9}M~10^{-5}M). The vascular sensitivity to ACh was evaluated by its median effective dose (ED_{50}), which was obtained by logistic curve fitting of the semilogarithmic dose-response relationship for each vessel ring.

Characterization of Muscarinic Receptor Subtypes on Endothelial Cells in Rat Thoracic Aorta

To identify which muscarinic receptor subtypes were responsible for ACh-induced vasodilating responses, different muscarinic receptor antagonists were used; ie, pirenzepine (10^{-6}M, for M_1 receptor), gallamine (10^{-5}M, for M_2 receptor), and 4-DAMP (3×10^{-8}M, for M_3 receptor). Our preliminary results showed that gallamine did not inhibit ACh-evoked vasodilating response. Therefore, we measured the values of pA_2 for pirenzepine and 4-DAMP to further verify the subtype of endothelial muscarinic receptors in the following experiments.

To determine pA_2 values of muscarinic antagonists, dose responses of ACh-induced vasore-laxation were performed under the following conditions: 1) without antagonist treatment as a control; 2) with pirenzepine pretreatment (10^{-7}, 10^{-6} or 10^{-5}M) for 30 min; 3) with 4-DAMP pretreatment (10^{-9}, 10^{-8} or 10^{-7}M) for 30 min. The values of pA_2 were then obtained by Schild regression (21).

Endothelial Membrane Preparation

Endothelial membrane fraction of thoracic aortae

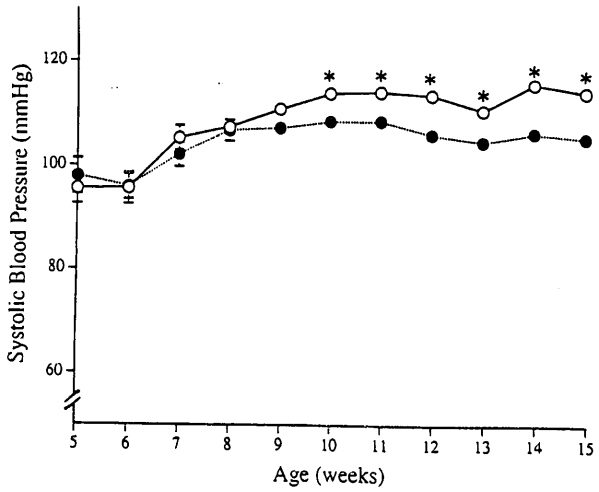

Fig 1. Chronic exercise lowers SBP in male Wistar rats. Open circle is for the control group, closed circle is for the trained group. * P<0.05.

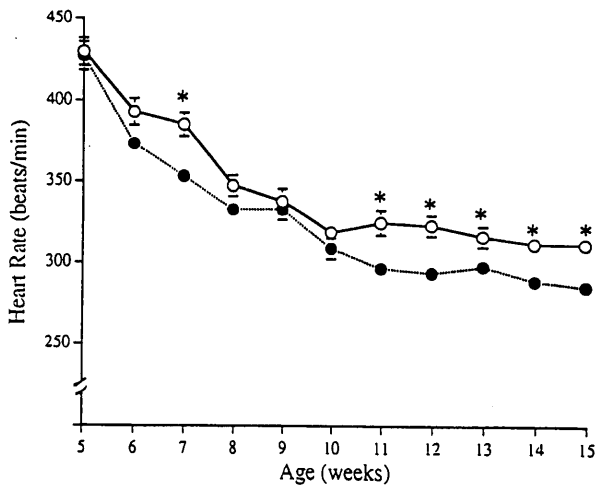

Fig. 2. Chronic exercise lowers resting heart rates in male Wistar rats. Symbols are the same as in Fig. 1.

was prepared for receptor binding assays by modifying the methods of Sim and Manjeet (20). 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 phenylme-thylsulphonyl-fluoride (PMSF) (pH7.4). 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 (pH7.4) 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. Selective rupture and removal of the endothelium from aortae without smooth muscle cells contamination occurred under these conditions. 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 (pH7.4). The final pellet was resuspended in this assay buffer and its protein content was determined by Lowry assay (11), using bovine serum albumin as a standard. These experiments were repeated three times with a total of 72 rats used in each group.

Radioligand Binding Assay for Endothelial M_3 Receptors

The method used for muscarinic receptor binding assays was adopted from Michel et al (13). 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 4-DAMP (ranged from 0.1 to 10 nM; specific activity 75.4 Ci/mmol, Du Pont NEN) in a final volume of 500 µl. After incubation for 45 min at room temperature, the bound and free ^{3}H-labeled 4-DAMP were separated by vacuum filtration (cell harvester, FH225V, Hoefer Scientific Instruments) through 0.2% polyethylene imine pretreated Whatman GF/B glass fibre 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 500 times excess of cold 4-DAMP. Specific binding was the difference between total and nonspecific binding. 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 nonlinear regression analysis of binding data (17).

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 4-DAMP 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 were evaluated by analysis of variance with a repeated-measures design. Differences would be considered significant at $p<0.05$.

Results

Resting SBP and HR

Figures 1 and 2 showed that resting SBP and HR were lowered after moderate exercise training. These physiological findings indicated that our training protocol indeed had training effects.

Vascular Response to ACh

We found that chronic exercise shifted the dose-response curve of ACh-induced vasorelaxation to the left (Fig. 3). In addition, the vascular sensitivity to ACh, indicated by ED_{50}, was enhanced by exercise training (-7.61±0.06 for the control and -7.86±0.05 for the exercise group, respectively).

Characterization of Muscarinic Receptor Subtypes on Endothelial Cells in Rat Thoracic Aorta

Table 1 shows that gallamine had little effect on ACh-induced vasodilating responses. In contrast, pirenzepine and 4-DAMP inhibited ACh-induced vasorealxation to various extents; ie, 4-DAMP had greater inhibitory effects than pirenzepine. To further characterize which subtype was the major muscarinic receptors on endothelial cells, pA_2 of these 2 antagonists were determined. Figure 4A demonstrates

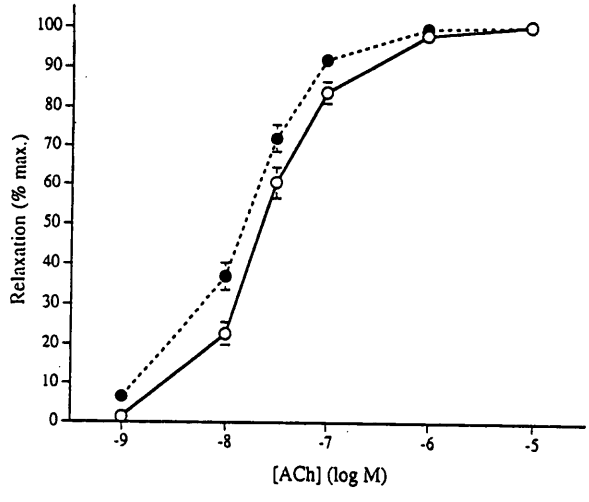

Fig. 3. Comparison of dose-response relations of ACh-induced vasorelaxation between control (open circle) and exercise (closed circle) groups. Chronic exercise shifted the curve to the left ($P<0.05$, ANOVA with repeated measures design).

Table 1. Inhibitory Effects of Various Muscarinic Antagonists on ACh (10^{-6}M)-Induced Vasorelaxation

Antagonist	% Inhibition
Pirenzepine (10^{-6}M)	14.7±2.3
Gallamine (10^{-5}M)	3.9±2.1
4-DAMP (3×10^{-8}M)	60.9±6.6

% inhibition = (pre-post)/pre × 100%
n=5 for each experiment

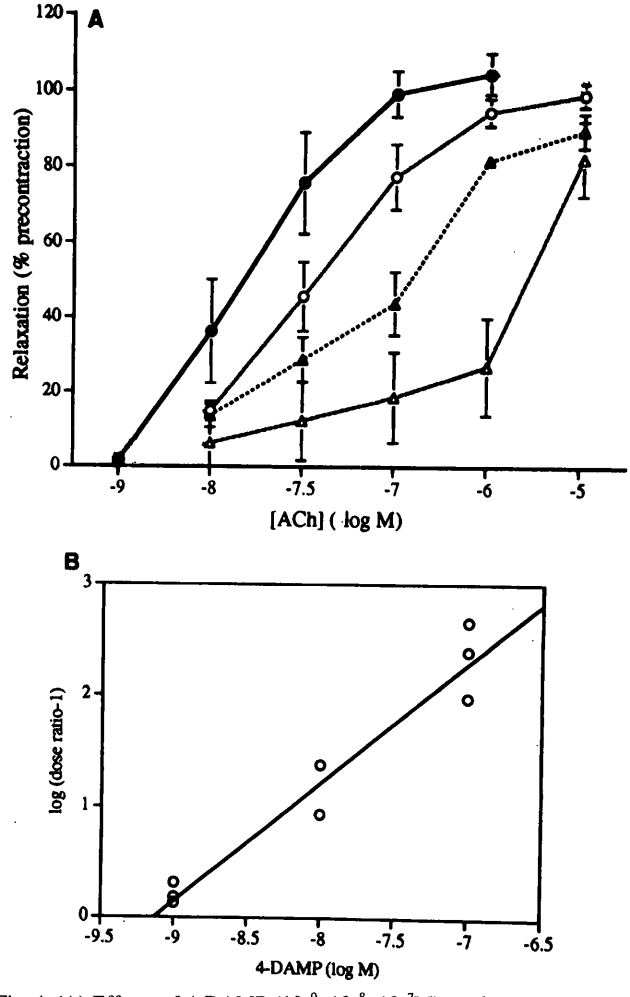

Fig. 4. (A) Effects of 4-DAMP (10^{-9}, 10^{-8}, 10^{-7}M) on dose-response curves of ACh-induced vasorelaxation. Symbols: closed circle- control; open circle- 10^{-9} M 4-DAMP; closed triangle- 10^{-8} M 4-DAMP; open triangle- 10^{-7} M 4-DAMP. (B) Schild plot to determine pA_2 of 4-DAMP. The abscissa is the concentration of antagonist(s), while the ordinate is log(dose ratio-1). When dose ratio is equal to 2, the intercept on the abscissa (ie, log K_A) is equal to "-pA_2".

that in the control group, 4-DAMP shifted dose-response curves of ACh-evoked vasorelaxtion to the right in a dose-dependent manner. Its pA_2 value was

Table 2. The Values of pA_2 for Pirenzepine and 4-DAMP in Control and Trained Groups

Antagonist	Control		Trained	
	pA_2	slope	pA_2	slope
Pirenzepine	6.8±0.2	1.1±0.1	6.8±0.2	1.1±0.1
4-DAMP	9.1±0.1	1.1±0.1	8.9±0.1	1.1±0.1

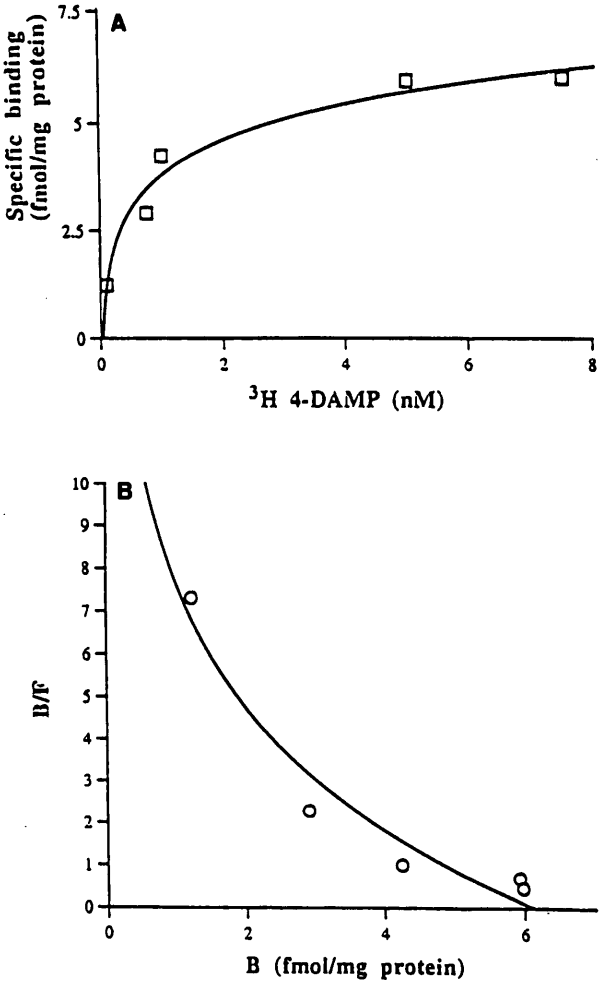

Fig. 5. An example to show the saturation curve (A) and Scatchard plot (B) for ^{3}H-labeled 4-DAMP binding assay.

around 9 (Fig. 4B). Similar results were observed in the trained group. The values of pA_2 for pirenzepine and 4-DAMP in this study were summarized in Table 2. A previous study has suggested that the pA_2 value of pirenzepine is 8 if the subtype of muscarinic receptors is M_1, and that the pA_2 value of 4-DAMP is 9 if the subtype of muscarinic receptors is M_3 (8). In our hands, the muscarinic receptors on rat aortic endothelial cells were identified as M_3 subtype. Therefore, we used ^{3}H-labeled 4-DAMP to perform receptor binding assays.

Table 3. Comparison of ^{3}H-Labeled 4-DAMP Binding Assay Results in Rat Thoracic Aortic Endothelial Cells between Control and Trained Groups

Parameters	Control	Trained
K_{dH} (nM)	0.3±0.1	0.3±0.1
B_{maxH} (fmol/mg protein)	2.1±0.4	1.5±0.3
K_{dL} (nM)	3.6±1.9	2.8±0.4
B_{maxL}(fmol/mg protein)	8.2±2.4	6.6±0.3

K_{dH} and K_{dDL} are high- and low-affinity sites dissociation constants, respectively.

B_{maxH} and B_{maxL} are the maximum binding numbers for high- and low-affinity sites, respectively.

Radioligand Binding Assay for Endothelial Muscarinic Receptors

Since the Scatchard plot of 4-DAMP binding assay was curvilinear (Fig. 5 demonstrated an example obtained from one experiment), there could be high-affinity and low-affinity binding sites. According to Rosenthal's method (17), we determined both B_{max} and K_d for high-affinity and low-affinity binding sites (Table 3). Our results showed insignificant differences in B_{max} and K_d between control and exercise trained groups.

Discussion

Our results indicated that in the thoracic aorta of Wistar rats, 1) ACh-induced vasorelaxation was mainly mediated by M_3 receptor; 2) chronic exercise enhanced ACh-evoked vasodilating responses. However, it was not caused by receptor upregulation, since maximal binding sites and affinity of M_3 receptor were not changed by chronic exercise.

M_3 receptor in other vessels of different animal species has been shown to be responsible for ACh-stimulated release of endothelium-derived relaxing factors (1, 6, 10, 12). The present study suggests that ACh-evoked vasorelaxation in rat thoracic aortae is also mediated by M_3 receptors. In addition, we found that chronic exercise could enhance ACh-evoked vasodilating responses in the thoracic aorta of male Wistar rats (Fig. 3). Previous reports have shown similar results in rabbit aortae, pulmonary arteries (4), thoracic aortae of Wistar-Kyoto rats and spontaneously hypertensive rats (2), canine epicardial coronary artery (23), porcine coronary resistance arteries (15), and rat abdominal aorta (7).

A previous study has reported that physical activity increases the density of muscarinic receptors on the hippocampus of rats (9). Our previous reports

also indicate that only receptor-mediated, but not nonreceptor-mediated, endothelium-dependent vasodilating responses are altered by chronic exercise (2, 4). It is, therefore, plausible to postulate that endothelial muscarinic receptors may be upregulated after chronic exercise. However, our results (Table 3) did not support this hypothesis. Since muscarinic receptors are G-protein coupled transmembrane protein, it is possible that the downstream signal transduction cascade, instead of the receptor itself, is altered by chronic exercise. For example, one can not rule out the possibility of an increase in nitric oxide synthase (NOS) gene expression after chronic exercise training. In fact, a 10-day running exercise has been reported to increase NOS mRNA expression in canine aortic endothelial cells (18). All of these possible mechanisms should be further verified. In this study, we found that N^{ω}-nitro-L-arginine (a NOS inhibitor), but not tetraethylammonium chloride (an EDHF inhibitor), could abolish ACh-induced vasorelaxation in the thoracic aorta of Wistar rats (data not shown). Therefore, NO, but not EDHF, contributes to the relaxation induced by ACh in Wistar rat thoracic aortae.

The curvilinear Scatchard plot for 4-DAMP suggests that there are heterogeneous binding sites of muscarinic receptors in rat aortic endothelial membranes or there is negative cooperativity. These binding characteristics are similar to the muscarinic receptor binding results from the rabbit aorta or from aortae of Wistar Kyoto rats and spontaneously hypertensive rats (19, 20).

In conclusion, 10 weeks of running exercise training in male Wistar rats can enhance M_3-mediated ACh-induced vasorelaxing response in thoracic aortae. This alteration is not cuased by endothelial M_3 receptor upregulation. Other possible mechanisms need to be further studied.

Acknowledgments

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