CALL FOR PAPERS | Exercise Training in Cardiovascular Disease: Mechanisms and Outcomes

Aerobic exercise training-induced changes in serum adropin level are associated with reduced arterial stiffness in middle-aged and older adults

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Fujie S, Hasegawa N, Sato K, Fujita S, Sanada K, Hamaoka T, Iemitsu M. Aerobic exercise training-induced changes in serum adropin level are associated with reduced arterial stiffness in middle-aged and older adults. Am J Physiol Heart Circ Physiol 309: H1642–H1647, 2015. First published September 14, 2015; doi:10.1152/ajpheart.00338.2015.—Aging-induced arterial stiffening is reduced by aerobic exercise training, and elevated production of nitric oxide (NO) participates in this effect. Adropin is a regulator of endothelial NO synthase and NO release, and circulating adropin level decreases with age. However, the effect of habitual aerobic exercise on circulating adropin levels in healthy middle-aged and older adults remains unclear. We sought to determine whether serum adropin level is associated with exercise training-induced changes in arterial stiffness. First, in a cross-sectional study, we investigated the association between serum adropin level and both arterial stiffness and cardiorespiratory fitness in 80 healthy middle-aged and older subjects (65.6 ± 0.9 yr). Second, in an intervention study, we examined the effects of 8-wk aerobic exercise training on serum adropin level and arterial stiffness in 40 healthy middle-aged and older subjects (67.3 ± 1.0 yr) divided into two groups: aerobic exercise training and sedentary controls. In the cross-sectional study, serum adropin level was negatively correlated with carotid β-stiffness (r = −0.437, P < 0.001) and positively correlated with plasma NOx level (r = 0.493, P < 0.001) and cardiorespiratory fitness (r = 0.457, P < 0.001). Serum adropin levels were elevated after the 8-wk aerobic exercise training intervention, and training-induced changes in serum adropin level were correlated with training-induced changes in carotid β-stiffness (r = −0.399, P < 0.05) and plasma NOx level (r = 0.623, P < 0.001). Thus the increase in adropin may participate in the exercise-induced reduction of arterial stiffness. Extra, age; adropin; carotid β-stiffness; exercise training; nitric oxide

NEW & NOTEWORTHY

Serum adropin level in middle-aged and older adults was elevated by aerobic exercise training. Additionally, serum adropin level was associated with exercise training-induced alteration of arterial stiffness and plasma NOx levels. Thus the increase in adropin may participate in the exercise-induced reduction of arterial stiffness, mediated by NO bioavailability.

Arterial stiffness increases with age (26) owing to declines in endothelial function and autonomic function and increases in arterial wall thickness and calcification (1, 3, 4, 11, 18, 24, 26, 31). This functional deterioration impairs the conduit and buffering functions of arteries, leading to several pathological conditions, including hypertension, atherosclerosis, and stroke (1, 4, 18, 26). A cross-sectional study revealed that arterial stiffness was lower in individuals with higher levels of cardiorespiratory fitness (23, 28). Furthermore, habitual aerobic exercise reduces arterial stiffness (10, 28).

Adropin consists of 76 amino acids and is encoded by a gene, Energy Homeostasis Associated (Enho) (16), and expressed in multiple tissues, including the brain, heart, kidney, liver, pancreas, skeletal muscle, and small intestine (2, 32). Circulating adropin level decreases with age (7). Recent work showed that adropin is expressed in vascular endothelial cells and promotes nitric oxide (NO) release by regulating endothelial nitric oxide synthase (eNOS) expression via vascular endothelial growth factor receptor 2 (VEGFR2), suggesting that adropin might be involved in endothelial function (20). Aerobic exercise training reduces arterial stiffness, concomitant with the elevation of plasma nitrite/nitrate (NOx) level, in middle-aged and older adults (10), and plasma NOx level is higher in athletes than in sedentary individuals with lower cardiorespiratory fitness (9). Moreover, aging-induced impairment of arterial eNOS protein and mRNA expression is increased by aerobic exercise training in aged rats (27). Thus adropin may participate in the mechanism underlying the reduction in arterial stiffness mediated by NO-derived vasodilation in response to aerobic exercise training in middle-aged and older adults. Moreover, circulating adropin level is positively correlated with plasma NOx level in patients with cardiovascular disease (8). However, the association between aerobic exercise training-induced changes in arterial stiffness and circulating adropin level in healthy middle-aged and older adults remains unclear.

In this study, we investigated whether serum adropin level is elevated by habitual aerobic exercise, as well as whether the change in serum adropin level is associated with exercise training-induced elevation of plasma NOx level and reduction of arterial stiffness in healthy middle-aged and older adults. In experiment 1, a cross-sectional study, we examined the correlation between serum adropin levels and both cardiorespiratory fitness and plasma NOx level in middle-aged and older adults. In experiment 2, an interventional study, we examined the effect of aerobic exercise training on serum adropin levels and the association between plasma NOx and serum adropin levels in middle-aged and older adults.

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METHODS

Subjects. In experiment 1, 80 healthy middle-aged and older adults (total: n = 80, 65.6 ± 0.9 yr; males: n = 37, 66.6 ± 1.4 yr; females: n = 43, 64.6 ± 1.1 yr) were enrolled in a cross-sectional study. The subjects were sedentary or moderately physically active (at least 60 min of physical activity per week), and did not participate in any other vigorous sports activity. In experiment 2, 40 healthy middle-aged and older sedentary subjects (total: n = 40, 67.3 ± 1.0 yr; males: n = 14, 70.8 ± 15 yr; females: n = 26, 65.4 ± 1.2 yr) volunteered to participate; these subjects were divided into two groups: the aerobic exercise training group [training group: n = 28 (11 males, 17 females), 66.9 ± 1.2 yr] and the sedentary control group [control group: n = 12 (3 males, 9 females), 68.2 ± 1.8 yr]. Subjects were recruited via advertisement from a local community health center and a community recreation center. All volunteers provided written informed consent before participating in the study, which was approved by the Ethics Committee of Ritsumeikan University and was conducted in accordance with the Declaration of Helsinki. Subjects with a history of stroke, diabetes, hypertension, hyperlipidemia, cardiac disease, chronic renal failure, or mental disorders, were excluded from the study. The subjects in this study hardly drank alcohol.

Experimental design. In experiment 1, we measured peak oxygen uptake (V\textsubscript{O\textsubscript{2}peak}), carotid \(\beta\)-stiffness, body weight, body fat, height, resting systolic blood pressure (SBP), resting diastolic blood pressure (DBP), resting heart rate (HR), resting plasma NOx concentration, resting serum adropin concentration, and serum concentrations of total cholesterol, HDL cholesterol, triglycerides, and fasting plasma glucose for all subjects. In experiment 2, 28 middle-aged and older subjects completed the aerobic exercise-training intervention. Carotid \(\beta\)-stiffness was examined as an index of arterial stiffness. Before subjects were tested, they sat quietly for 30 min. Resting brachial SBP, DBP, and HR were measured in duplicate in the supine position at rest, using a vascular testing device (OMRON COLIN, Tokyo, Japan). At the beginning and end of the study period, fasting blood samples were drawn following at least 48 h of rest after the last exercise-training session. All subjects were instructed not to eat or drink fluids other than water for at least 12 h prior to blood sampling. In addition, since NOx level can be affected by the diet, we checked to be sure that participants in both groups did not ingest any dietary sources of NOx over 24 h prior to testing. Thus we were able to avoid both acute effects from the most recent bout of exercise and oral sources of NOx other than NO. Serum and plasma samples were immediately centrifuged (1,500 g, 15 min, 4°C). Blood samples were stored at −80°C until use. Room temperature was maintained at 24°C throughout the experiment.

Exercise intervention. The aerobic exercise-training program consisted of cycling on a leg ergometer (828E Monark cycle ergometer, Stockholm, Sweden) for 55 min, 3 days/wk, for 8 wk. Each exercise session consisted of a 5-min warm-up period at 40% \(V\text{O}_2\text{peak}\), followed by 45 min of cycling at a resistance that elicited 60–70% \(V\text{O}_2\text{peak}\), and ended with a 5-min cool-down period at 40% \(V\text{O}_2\text{peak}\). Exercise compliance was carefully monitored by direct supervision. Additionally, the sedentary control subjects were encouraged to maintain the activities of daily living without significant changes during the 8-wk experiment. Subjects in both groups were encouraged to maintain their food intake as usual during the experiment.

Measurement of \(V\text{O}_2\text{peak}\). \(V\text{O}_2\text{peak}\) was measured during exercise by breath-by-breath assessment using an incremental cycle exercise test on a cycle ergometer (MINATO, AE-310SRD, Osaka, Japan). Incremental cycle exercise began at a work rate of 60 W (30–90 W) for men and 30 W (0–60 W) for women, and power output was increased by 15 W/min until the subjects could not maintain a fixed pedaling frequency of 60 rpm (10, 22). During the ergometer test, the subjects were encouraged to exercise at maximum intensity. Heart rate and rating of perceived exertion (RPE) were monitored minute by minute during the exercise. \(V\text{O}_2\text{peak}\) was obtained using the modified Borg scale. The highest 30-s averaged value of \(V\text{O}_2\) during the exercise test was designated as \(V\text{O}_2\text{peak}\) if three of four of the following criteria were met: 1) plateau in \(V\text{O}_2\) with an increase in external work, 2) maximal respiratory exchange ratio ≥ 1.1, 3) maximal heart rate ≥ 90% of the age-predicted maximum [208 – 0.7 × age; (29)], and 4) RPE ≥ 18.

Measurement of carotid \(\beta\)-stiffness index. Carotid \(\beta\)-stiffness was examined as an indicator of arterial stiffness. A combination of ultrasound imaging of the pulsatile common carotid artery and simultaneous application of tonometrically obtained arterial pressure from the contralateral carotid artery allowed noninvasive determination of arterial compliance (10, 15). The carotid artery diameter was measured from images obtained using an ultrasound system equipped with a high-resolution linear array transducer (15). A longitudinal image of the cephalic portion of the common carotid artery was acquired 1–2 cm proximal to the carotid bulb. All image analyses were performed by the same investigator.

Pressure waveforms and amplitudes were obtained from the common carotid artery using a pencil-shaped probe with a high-fidelity strain gauge transducer (SPT-301; Millar Instruments, Houston, TX [10, 15]). Because baseline blood pressure levels are dependent on hold-down pressure, the pressure signal obtained via tonometry was calibrated by equating the carotid mean arterial blood pressure and DBP to brachial artery values (10, 15). The carotid \(\beta\)-stiffness index was calculated using the equation \([\ln(P1/P0)]/[(D1 – D0)/D0]\), where D1 and D0 are the maximum (systolic) and minimum (diastolic) diameters, and P1 and P0 are the highest (systolic) and lowest (diastolic) blood pressures, respectively (14). The day-to-day coefficients of variation for the carotid \(\beta\)-stiffness were 3.2 ± 2.1%.

Measurement of plasma NOx concentrations. NOx concentration in the plasma was measured using the Total Nitric Oxide and Nitrate/Nitrite Parameter Assay Kit based on the Griess assay (R&D Systems, Minneapolis, MN). All samples were assayed in duplicate. Optical density at 540 nm was measured using an XMark microplate spectrophotometer (Bio-Rad Laboratories, Hercules, CA). Readings were converted to concentrations by a linear fitting to the log-log plot of the standard curve. The day-to-day coefficients of variation of plasma NOx concentrations were 5.3 ± 2.3%.

Measurement of serum adropin concentrations. Adropin concentration in the serum was measured using an enzyme-linked immunosorbent assay (ELISA; Phoenix Pharmaceuticals, Burlingame, CA). All samples were assayed in duplicate. Optical density at 450 nm was measured using an XMark microplate spectrophotometer. Readings were converted to concentrations by linear fitting to the log-log plot of the standard curve. The day-to-day coefficients of variation of serum adropin concentrations were 4.1 ± 3.3%.

Measurements of serum cholesterol and triglyceride levels. Fasting concentrations of total cholesterol, HDL cholesterol, and triglyceride levels were determined using standard enzymatic techniques.

Statistical analysis. Values are expressed as means ± SE. In experiment 1, correlations between serum adropin concentration and age, \(V\text{O}_2\text{peak}\), plasma NOx concentration, and carotid \(\beta\)-stiffness were determined using the Pearson correlation coefficient. The partial correlation coefficient was adjusted for age and/or sex. In experiment 2, differences between groups and time points were assessed by two-way repeated-measure ANCOVA adjusted for age and sex, followed by a Fisher’s post hoc test that was applied when a measurement was significantly different. Unpaired Student t-tests were used to compare differences in the change from baseline to 8 wk in carotid \(\beta\)-stiffness, plasma NOx concentration, and serum adropin concentration between the training and control groups. The relationships between serum adropin concentration, carotid \(\beta\)-stiffness, and plasma NOx concentration, and between serum adropin concentration and other parameters, were determined by using the Pearson correlation coefficient. The partial correlation coefficient was adjusted for age,
sex, body weight, heart rate, blood pressure, lipid profiles, and fasting plasma glucose. Multiple linear regression analysis was used to test the independent association between serum adropin concentration and age, sex, body weight, heart rate, blood pressure, lipid profiles, fasting plasma glucose, plasma NOx, VO$_2$peak, and carotid β-stiffness. $P < 0.05$ was defined as statistically significant. All statistical analyses were performed using StatView (5.0, SAS Institute, Tokyo, Japan).

RESULTS

Cross-sectional study (experiment 1). We investigated whether the serum adropin level was associated with arterial stiffness or cardiorespiratory fitness. Subject characteristics are listed in Table 1. A negative correlation was observed between serum adropin levels and carotid β-stiffness whether the comparison was unadjusted ($r = -0.437$, $P < 0.001$, Fig. 1A) or adjusted for age and sex ($\beta = -0.441$, $P < 0.001$). Serum adropin levels were positively correlated with plasma NOx levels (unadjusted for age and sex: $r = 0.493$, $P < 0.001$, Fig. 1B; adjusted: $\beta = 0.501$, $P < 0.001$). Plasma NOx levels were positively correlated with carotid β-stiffness (unadjusted for age and sex: $r = -0.405$, $P < 0.001$; adjusted: $\beta = -0.389$, $P < 0.001$). Serum adropin level was positively correlated with VO$_2$peak (unadjusted for age and sex: $r = 0.457$, $P < 0.001$, Fig. 2; adjusted: $\beta = 0.392$, $P < 0.001$). In addition, we found no significant correlations between age and serum adropin concentration, plasma NOx concentration, and carotid β-stiffness.

Intervention study (experiment 2). We next performed an interventional study to examine the effect of aerobic exercise-training on the association between serum adropin and arterial stiffness. There was no significant difference in VO$_2$peak between the training and control groups at baseline. However, there were significant effects of the interaction of group and time on VO$_2$peak (Table 2). In the training group, VO$_2$peak was significantly elevated after the exercise-training intervention (Table 2). Although there was no significant difference in carotid β-stiffness between the training and control groups before the exercise training, there were significant effects of the interaction of group and time on carotid β-stiffness (Fig. 3A, $P = 0.023$) and the carotid β-stiffness significantly decreased after aerobic exercise-training intervention (Fig. 3A). No significant differences between groups or times were noted for age, height, body weight, BMI, HR, SBP, DBP, total cholesterol level, HDL cholesterol level, triglyceride level, or blood glucose (Table 2).

Before exercise training, there were no significant differences in serum adropin or plasma NOx concentrations between the training and control groups. There were significant effects of the interaction of group and time on plasma NOx concentration (Table 2, $P < 0.001$). Namely, after exercise training, plasma NOx concentration was significantly elevated in the training group relative to the control group (Table 2). The interaction among groups and time points was significant for serum adropin concentration (Fig. 3B, $P < 0.01$). After exercise training, serum adropin concentration was significantly higher in the training group than in the control group (Fig. 3B).

In the training group, training-induced changes in serum adropin concentration were negatively correlated with training-induced changes in carotid β-stiffness, whether the comparison was unadjusted ($r = -0.399$, $P < 0.05$, Fig. 4A) or adjusted for age, sex, body weight, heart rate, blood pressure, lipid profiles, and fasting plasma glucose ($\beta = -0.452$, $P < 0.05$). Furthermore, the training-induced changes in serum adropin levels positively correlated with plasma NOx levels (unadjusted: $r = 0.623$, $P < 0.001$, Fig. 4B; adjusted: $\beta = 0.744$, $P < 0.0001$) or VO$_2$peak (unadjusted: $y = 241x + 0.847$, $r = 0.420$, $P < 0.05$; adjusted: $\beta = 0.493$, $P < 0.05$) but was not associated with the training-induced changes in plasma adropin levels and other parameters. Training-induced changes in plasma NOx levels were negatively correlated with training-induced changes in carotid β-stiffness (unad-

Table 1. Characteristics of subjects in cross-sectional study

<table>
<thead>
<tr>
<th>Variable</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, yr</td>
<td>65.6 ± 0.9</td>
</tr>
<tr>
<td>Height, cm</td>
<td>161.3 ± 1.1</td>
</tr>
<tr>
<td>Body weight, kg</td>
<td>60.3 ± 1.2</td>
</tr>
<tr>
<td>BMI, kg/m$^2$</td>
<td>23.2 ± 0.4</td>
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<tr>
<td>HR, beats/min</td>
<td>59.2 ± 1.0</td>
</tr>
<tr>
<td>SBP, mmHg</td>
<td>125.6 ± 1.8</td>
</tr>
<tr>
<td>DBP, mmHg</td>
<td>76.0 ± 1.3</td>
</tr>
<tr>
<td>Total cholesterol, mg/dl</td>
<td>219.6 ± 3.5</td>
</tr>
<tr>
<td>HDL cholesterol, mg/dl</td>
<td>68.0 ± 2.3</td>
</tr>
<tr>
<td>Triglycerides, mg/dl</td>
<td>119.7 ± 8.6</td>
</tr>
<tr>
<td>Blood glucose, mg/dl</td>
<td>102.2 ± 1.8</td>
</tr>
<tr>
<td>Plasma NOx, µmol/l</td>
<td>36.9 ± 2.1</td>
</tr>
<tr>
<td>VO$_2$peak, ml·kg$^{-1}$·min$^{-1}$</td>
<td>27.3 ± 0.8</td>
</tr>
</tbody>
</table>

Values are means ± SE. BMI, body mass index, SBP, systolic blood pressure, DBP, diastolic blood pressure, HDL, high-density lipoprotein, VO$_2$peak, peak oxygen uptake.
EXERCISE TRAINING INCREASES SERUM ADROPIN LEVEL

through VEGFR2, mediated via activation of phosphoinositide after the aerobic exercise-training intervention.

middle-aged and older adults, and this elevation in serum cardiorespiratory fitness. Therefore, these results suggest that the training-induced change in serum adropin level was positively correlated with cardiorespiratory fitness in middle-aged and older adults. Therefore, based on the previous study, our findings suggest that the association between arterial stiffness and circulating adropin level is mediated by exercise training-induced acceleration of NO production in middle-aged and older adults. Several studies have suggested that the increases in NO production and arterial eNOS expression resulting from endurance exercise training may play a causal role in reducing the risk of arterial stiffness, in both humans and animals (21, 27). Recently, we reported that NO production is elevated after aerobic exercise training in healthy middle-aged and older adults (10). This increased arterial NO bioavailability induced by adropin may influence arterial stiffness in middle-aged and older adults. Future studies should examine the molecular mechanism underlying the relationship between training-induced changes of serum adropin and plasma NOx using exercise-trained rats.

In this study, we showed that aerobic exercise training increases circulating adropin levels in middle-aged and older adults. However, the source of the exercise training-induced increase in serum adropin levels remains unclear. Adropin is expressed in vascular endothelial cells, as well as in brain, heart, kidney, liver, pancreas, skeletal muscle, and small intestine (2, 32). An in vitro study showed that adropin treatment promotes NO production from the human umbilical vein endothelial cells in a time-dependent manner, mediated via phosphorylation of eNOS protein (20). Tanabe et al. showed that aerobic swimming training elevated aortic eNOS protein and mRNA expression in older rats (27). Therefore, considering that elevated adropin induced by exercise training may participate in upregulation of arterial eNOS, mediated by autocrine and/or paracrine effects in endothelial cells, adropin expression in endothelial cells may contribute to circulating adropin levels. In this study, however, we did not investigate whether endothelial adropin gene expression was augmented by exercise training in middle-aged and older subjects. Future studies

Table 2. Comparison of characteristics in training and control groups

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Pre</th>
<th>Post</th>
<th>Pre</th>
<th>Post</th>
<th>Two-Way ANCOVA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, yr</td>
<td>68.2 ± 1.8</td>
<td>68.6 ± 1.8</td>
<td>66.9 ± 1.2</td>
<td>67.1 ± 1.2</td>
<td>0.941</td>
</tr>
<tr>
<td>Height, cm</td>
<td>157.0 ± 2.4</td>
<td>156.8 ± 2.4</td>
<td>159.7 ± 2.0</td>
<td>159.7 ± 1.9</td>
<td>0.975</td>
</tr>
<tr>
<td>Body weight, kg</td>
<td>54.7 ± 3.9</td>
<td>54.7 ± 3.9</td>
<td>61.3 ± 1.8</td>
<td>60.7 ± 1.9</td>
<td>0.920</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>22.0 ± 1.2</td>
<td>22.1 ± 1.2</td>
<td>24.1 ± 0.7</td>
<td>23.9 ± 0.7</td>
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<td>HR, beats/min</td>
<td>59.9 ± 2.2</td>
<td>57.3 ± 2.5</td>
<td>61.9 ± 1.6</td>
<td>59.7 ± 1.6</td>
<td>0.836</td>
</tr>
<tr>
<td>SBP, mmHg</td>
<td>130.5 ± 3.4</td>
<td>127.0 ± 4.5</td>
<td>129.4 ± 3.1</td>
<td>119.6 ± 2.7</td>
<td>0.405</td>
</tr>
<tr>
<td>DBP, mmHg</td>
<td>75.8 ± 1.7</td>
<td>73.5 ± 2.3</td>
<td>77.6 ± 2.4</td>
<td>73.3 ± 2.1</td>
<td>0.740</td>
</tr>
<tr>
<td>Total cholesterol, mg/dl</td>
<td>238.8 ± 8.1</td>
<td>227.6 ± 9.1</td>
<td>221.4 ± 6.3</td>
<td>215.8 ± 6.0</td>
<td>0.716</td>
</tr>
<tr>
<td>HDL cholesterol, mg/dl</td>
<td>84.2 ± 7.4</td>
<td>83.4 ± 8.1</td>
<td>62.9 ± 3.0</td>
<td>61.5 ± 2.8</td>
<td>0.875</td>
</tr>
<tr>
<td>Triglycerides, mg/dl</td>
<td>96.7 ± 16.7</td>
<td>93.4 ± 15.8</td>
<td>135.9 ± 15.6</td>
<td>128.9 ± 12.7</td>
<td>0.834</td>
</tr>
<tr>
<td>Blood glucose, mg/dl</td>
<td>96.8 ± 2.5</td>
<td>92.9 ± 2.7</td>
<td>102.0 ± 3.4</td>
<td>108.9 ± 3.5</td>
<td>0.283</td>
</tr>
<tr>
<td>Plasma NOx, μmol/l</td>
<td>22.1 ± 2.6</td>
<td>16.8 ± 1.8</td>
<td>29.3 ± 2.1</td>
<td>50.7 ± 3.8*</td>
<td>0.001</td>
</tr>
<tr>
<td>VO₂peak, ml·kg⁻¹·min⁻¹</td>
<td>24.0 ± 1.0</td>
<td>24.3 ± 1.1</td>
<td>22.8 ± 0.8</td>
<td>27.5 ± 1.0*</td>
<td>0.047</td>
</tr>
</tbody>
</table>

Values are means ± SE. *P < 0.05, after training vs. before training. Two-way ANCOVA was adjusted for sex and age.
Exercise training increases serum adropin level

In conclusion, in a cross-sectional study, we found that the serum adropin level was negatively correlated with arterial stiffness and positively correlated with cardiorespiratory fitness.
in middle-aged and older adults. Furthermore, a program of 8-wk aerobic exercise training in such subjects increased the levels of serum adropin and plasma NOx, and concomitantly reduced arterial stiffness. Furthermore, we observed negative correlations between the training-induced changes in serum adropin level and arterial stiffness. Thus the increased in serum adropin level in response to aerobic exercise training may be related to the reduction in arterial stiffness, mediated by NO bioavailability, in middle-aged and older adults.

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DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS
Author contributions: S. Fujie, N.H., K. Sato, S. Fujita, K. Sanada, T.H., and M.I. performed experiments; S. Fujie, N.H., S. Fujita, K. Sanada, and T.H. analyzed data; S. Fujie, S. Fujito, Sato, T.H., and M.I. interpreted results of experiments; S. Fujie prepared figures; S. Fujie, K. Sato, and M.I. drafted manuscript; S. Fujie, N.H., K. Sato, S. Fujita, K. Sanada, T.H., and M.I. approved final version of manuscript; M.I. conception and design of research.

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