Aerobic exercise training increases plasma Klotho levels and reduces arterial stiffness in postmenopausal women

Tomoko Matsubara,1,3 Asako Miyaki,1,3 Nobuhiko Akazawa,2 Youngju Choi,1,3 Song-Gyu Ra,1,3 Koichiro Tanahashi,1 Hiroshi Kumagai,1 Satoshi Oikawa,1 and Seiji Maeda2

1Division of Sports Medicine, Graduate School of Comprehensive Human Sciences, University of Tsukuba, Ibaraki, Japan; 2Division of Sports Medicine, Faculty of Health and Sport Sciences, University of Tsukuba, Ibaraki, Japan; and 3Research Fellow of the Japan Society for the Promotion of Science, Tokyo, Japan

Submitted 24 May 2013; accepted in final form 27 November 2013


The Klotho gene is a suppressor of the aging phenomena, and the secretion as well as the circulation of Klotho proteins decrease with aging. Although habitual exercise has antiaging effects (e.g., a decrease in arterial stiffness), the relationship between Klotho and habitual exercise remains unclear. In the present study, we investigated the effect of habitual exercise on Klotho, with a particular focus on arterial stiffness. First, we examined the correlation between plasma Klotho concentration and arterial stiffness (carotid artery compliance and β-stiffness index) or aerobic exercise capacity [oxygen uptake at ventilatory threshold (VT)] in 69 healthy, postmenopausal women (50–76 years old) by conducting a cross-sectional study. Second, we tested the effects of aerobic exercise training on plasma Klotho concentrations and arterial stiffness. A total of 19 healthy, postmenopausal women (50–76 years old) were divided into two groups: control group and exercise group. The exercise group completed 12 wk of moderate aerobic exercise training. In the cross-sectional study, plasma Klotho concentrations positively correlated with carotid artery compliance and VT and negatively correlated with the β-stiffness index. In the interventional study, aerobic exercise training increased plasma Klotho concentrations and carotid artery compliance and decreased the β-stiffness index. Moreover, the changes in plasma Klotho concentration and arterial stiffness were found to be correlated. These results suggest a possible role for secreted Klotho in the exercise-induced modulation of arterial stiffness.

Klotho; aerobic exercise; arterial stiffness

An increase in arterial stiffness is an independent risk factor for cardiovascular morbidity and mortality (22, 51). Arterial stiffness is known to increase with aging (39). Moreover, menopause augments the age-related increase in arterial stiffness (56), and older women have higher arterial stiffness than do men (52). Furthermore, habitual aerobic exercise decreases arterial stiffness in middle-aged and elderly individuals and postmenopausal women (45, 47). Several reports have suggested that the mechanisms by which aerobic exercise training decreases the arterial stiffness could be partly mediated by the enhancement of endothelial function, suppression of oxidative stress, and inflammation (24, 27, 46). However, the precise mechanism underlying the aerobic exercise-induced modulation of arterial stiffness remains unclear.

The Klotho gene was identified originally by insertional mutagenesis in mice that developed multiple aging phenotypes, such as ectopic calcification, osteoporosis, skin atrophy, infertility, and shortened lifespan (19). Klotho transcripts encode membrane-bound and secreted proteins (30), and the extracellular domain of membrane-bound Klotho is processed into the secreted form by ectodomain shedding (4). Klotho is expressed primarily in the kidney, although lower levels of expression have been detected in other organs. Secreted Klotho is detected in the blood, urine, and cerebrospinal fluid (15).

Secreted Klotho functions as a humoral factor and is involved in nitric oxide (NO) production in the endothelium (41), regulation of calcium channel activity (16), suppression of oxidative stress (3), transforming growth factor (TGF)-β1 signaling (9), insulin/IGF-I signaling (20). Plasma Klotho concentrations decrease with advancing age in healthy humans (55). Furthermore, it has been reported that the levels of circulating Klotho are lower in certain diseases, such as diabetes, acute kidney injury, and chronic kidney disease (CKD) (8, 13, 44). Particularly, in patients with CKD, the decrease in the levels of circulating Klotho is associated independently with the signs of vascular dysfunction, such as arterial stiffness (18). Therefore, the decrease in the levels of circulating Klotho may have important clinical significance in the pathophysiology of specific diseases.

The purpose of this study is to determine whether Klotho is associated with the reduction of arterial stiffness after aerobic exercise training. First, we measured plasma Klotho concentrations and examined their correlation with arterial stiffness as well as aerobic exercise capacity in postmenopausal women through a cross-sectional study. Second, we studied the effect of aerobic exercise training on plasma Klotho concentrations and arterial stiffness in postmenopausal women through an interventional study.

Materials and Methods

Subjects

For experiment 1, 69 healthy and postmenopausal women (aged 50–76 years old) were enrolled in a cross-sectional study. For experiment 2, a total of 19 healthy and postmenopausal women (aged 50–76 years old) participated in an exercise intervention study. Subjects were divided into two groups: control group (n = 8) and exercise group (n = 11). The exercise group was analyzed before and after the 12 wk of aerobic exercise training. All subjects were nonsmokers and free of cardiovascular disease, as indicated by their medical history. None of the subjects was taking dietary supplements and cardiovascular medications, including hormone replacement therapy. The subjects were instructed to maintain current eating behaviors.
for the duration of the intervention. The subjects were either sedentary or recreationally active. All subjects gave a written, informed consent to participate in this study. All procedures were reviewed and approved by the Ethical Committee of the University of Tsukuba.

Experimental Design

In experiment 1, ventilatory threshold (VT), carotid arterial compliance, β-stiffness index, resting arterial blood pressure, blood biochemistry, and plasma Klotho concentration were measured in the postmenopausal women. In experiment 2, the postmenopausal women completed aerobic exercise training. VT, carotid arterial compliance, β-stiffness index, resting arterial blood pressure, blood biochemistry, and plasma Klotho concentration were measured before and after the 12-wk aerobic exercise training. Age and height of the subjects were measured before the interventional study. Subjects were instructed not to alter their dietary habit throughout the intervention period. Before they were tested, subjects abstained from caffeine and fasted for at least 12 h. The subjects were tested 48 h after their last exercise training session to avoid the immediate (acute) effects. In experiment 2, the postmenopausal women were tested 48 h after their last exercise training, subjects abstained from caffeine and fasted for at least 12 h. The subjects were tested 48 h after their last exercise training session to avoid the immediate (acute) effects. In experiments 1 and 2, all measurements were performed at a constant room temperature (25°C).

Exercise Intervention

Subjects in the exercise group underwent aerobic exercise training for >3 days/wk (two to three supervised sessions and additional home-based training) for 12 wk. Initially, the training included cycling and walking for 30 min/day at a relatively low intensity (60% of their individually determined maximal heart rate). As their exercise tolerance improved, the intensity and duration of the aerobic exercise were increased to 40–60 min/day at an intensity of 70–80% of the maximal heart rate. Subjects in the control group were instructed not to change their level of physical activity.

Measurements

Arterial blood pressure. Resting blood pressure was measured from the brachial artery by using a semiautomated device (form pulse wave velocity (PWV)/ankle brachial index (ABI); Colin Medical Technology, Komaki, Japan) with the subjects in a supine position.

Carotid artery compliance. Dynamic arterial compliance was determined noninvasively using a combination of ultrasonography of the common carotid artery and simultaneous applanation tonometry of the contralateral carotid artery. Subjects were examined in the supine position under quiet resting conditions. The diameter of the common carotid artery was measured using the images from an ultrasound machine (EnVisor; Koninklijke Philips Electronics, Eindhoven, The Netherlands) equipped with a high-resolution (7.5 MHz) linear array transducer. Longitudinal images of the cephalic portion of the common carotid artery were obtained 1–2 cm proximal to the carotid bulb, with a transducer placed at a 90° angle to the vessel so that the proximal and distal wall boundaries were clearly discernible. Carotid arterial pressure waveforms were obtained with arterial applanation tonometry incorporating an array of 15 micropiezoresistive transducers (form PWV/ABI; Colin Medical Technology) (7) and were calibrated by equating the carotid mean arterial and diastolic blood pressure to the brachial mean arterial and diastolic blood pressure. These images were recorded on a computer for subsequent offline analysis and were analyzed using image analysis software. All image analyses were performed by the same investigator. Common carotid intima-media thickness (ccIMT) of the far wall was evaluated as the distance between the lumen-intima interface and the media-adventitia interface in 10 frozen basal diastolic frames. The ccIMT measurement was obtained from three continuous sites at 1-mm intervals in each frame, and an average of 30 measurements was used for the analysis. Time points that corresponded with maximum systolic expansion and the basal (minimum) diastolic relaxation of the carotid artery were selected to measure the vascular diameter. Subsequently, the distances (or diameter) between the distal boundaries of the vessel wall, corresponding to the boundary of the tunica adventitia and tunica media, were measured. To characterize the central arterial compliance comprehensively, two different measures, namely, arterial compliance (50) and β-stiffness index (11), were calculated. The β-stiffness index serves as an indicator of arterial compliance adjusted for distending pressure.

Plasma Klotho concentration and blood biochemistry. Blood samples were collected from the antecubital vein after overnight fasting. Serum concentrations of cholesterol and triglycerides were determined using the standard enzymatic techniques. Plasma Klotho concentrations were measured by ELISA using a soluble α-Klotho ELISA assay kit (Immuno-Biological Laboratories, Tokyo, Japan).

Physical activity. Daily step counts and physical activity levels were assessed using a pedometer with a uniaxial accelerometer (Life-Order; Kenz, Nagoya, Japan) and a daily physical activity diary. Participants wore the LifeOrder for 14 consecutive days, except while bathing. We calculated the physical activity, which could not be measured with the accelerometer, such as in bicycling, with an estimated value of: physical activity level (kcal) = 5 metabolic equivalent of tasks × time (h) × body mass (kg) × 1.05, as described in a previous study (1).

Oxygen consumption at VT. All subjects underwent an incremental cycle exercise test (after 2 min at 40 W, with 20-W increases/min) until they felt exhausted or reached 85% of the age-predicted maximal heart rate. Oxygen consumption (VO₂) was measured using a metabolic cart throughout the exercise test. Their individual VT was calculated using regression analysis of the slopes of carbon dioxide production, VO₂, and minute ventilation plot (28).

Statistical Analysis

In experiment 1, the correlation between plasma Klotho concentration and carotid arterial compliance, β-stiffness index, or VO₂ at VT was calculated by partial Pearson correlation analysis. The partial correlation coefficients were adjusted for age, body mass index (BMI), pulse pressure, and VO₂ at VT. In experiment 2, the effect of exercise intervention on all outcome measures was analyzed using repeated-measures ANOVA. When indicated by a significant main effect or interaction, specific mean comparisons were performed to identify significant differences within each intervention. When a significant F value was obtained, a post hoc test using the Bonferroni method was used to identify the significant differences among the mean values. Regression lines of plasma Klotho concentration and arterial stiffness parameters before and after exercise training were compared with respect to slope and intercept values by using analysis of covariance (ANCOVA). ANCOVA analyses were adjusted for age, BMI, and pulse pressure. All data are reported as means ± SE. Statistical significance was set a priori at P < 0.05 for all comparisons.

RESULTS

Experiment 1

To investigate the relationship between plasma Klotho concentration and arterial stiffness or aerobic exercise capacity, we performed a cross-sectional study on 69 healthy and postmenopausal women within the age range of 50–76 years. The characteristics of subjects are listed in Table 1. Table 2 shows the hemodynamics of the subjects. Plasma concentrations of Klotho ranged from 281 to 770 pg/ml. Plasma Klotho concentration and carotid arterial compliance were positively correlated after adjusting for age, pulse pressure, and BMI (β = 0.370, P < 0.01; Fig. 1A). A positive correlation was observed between plasma Klotho concentrations and β-stiffness indices.
after adjusting for age and BMI ($\beta = -0.301, P < 0.05$; Fig. 1B). No correlation was observed between plasma Klotho concentration and cIMT ($R = -0.130, P = 0.272$). Furthermore, plasma Klotho concentration positively correlated with $V_O2$ at VT after adjusting for age and BMI ($\beta = 0.329, P < 0.01$; Fig. 2). Plasma Klotho levels correlated significantly with arterial compliance ($\beta = 0.335, P < 0.01$) but not with $\beta$-stiffness index ($\beta = -0.230, P = 0.062$) after adjusting for $V_O2$ at VT.

**Experiment 2**

We conducted an interventional study to examine the effect of aerobic exercise training on the association between Klotho and arterial stiffness. The characteristics of the subjects are presented in Table 3. Before exercise intervention, we found no differences between the two groups with respect to age, height, body mass, BMI, total cholesterol, HDL cholesterol, LDL cholesterol, triglyceride, daily step counts, or physical activity level. After exercise intervention, body mass and BMI decreased significantly, whereas a mild decrease was observed. Total cholesterol, HDL cholesterol, and LDL cholesterol also decreased with the exercise intervention. Daily step counts, physical activity level, and $V_O2$ at VT increased significantly after the exercise intervention. Blood pressure and heart rate did not differ between the two groups before the intervention (Table 4). After the intervention, the heart rate decreased significantly with the exercise intervention.

Before the intervention, the plasma Klotho concentration was at similar levels in both the control and exercise groups (Fig. 3). However, plasma Klotho concentrations increased significantly in the exercise group after intervention ($414 \pm 28$ vs. $453 \pm 36$ pg/ml, $P < 0.05$). No statistical correlation was observed between the changes in $V_O2$ at VT and those in plasma Klotho concentration ($R = 0.386, P = 0.103$). Baseline carotid arterial compliance or $\beta$-stiffness index was similar in the two groups. The exercise group showed a significant increase in carotid arterial compliance ($0.084 \pm 0.007$ vs. $0.097 \pm 0.008$ mm$^2$/mmHg, $P < 0.05$) and a decrease in $\beta$-stiffness index ($8.16 \pm 0.80$ vs. $7.15 \pm 0.80$, $P < 0.05$) after the intervention (Fig. 4).

To analyze the relationship between Klotho concentration and arterial stiffness parameters, the linear regression lines, obtained before and after exercise training, were compared with respect to slope and intercept values by using two-way ANCOVA. No significant difference was observed in both the slopes ($P = 0.511$) and intercepts ($P = 0.254$) of the linear regression lines between plasma Klotho concentration and carotid arterial compliance after adjusting for age and BMI. However, a significant difference was observed in intercepts ($P < 0.05$) but not in slopes ($P = 0.651$) between plasma Klotho concentrations and $\beta$-stiffness index after adjusting for age, BMI, and pulse pressure.

Furthermore, the changes in the plasma Klotho concentration were positively correlated with the changes in carotid arterial compliance after adjusting for age, BMI, and pulse pressure ($\beta = 0.534, P < 0.05$). Changes in plasma Klotho concentration were negatively correlated with the changes in the $\beta$-stiffness index after adjusting for age and BMI ($\beta = 0.329, P < 0.05$).
Fig. 2. Correlation between plasma Klotho levels and oxygen uptake at the ventilatory threshold (VT). Each open circle represents an individual value of each subject. The line represents the regression slope. BW, body weight.

−0.673, \( P < 0.01 \)). No correlation was observed between the changes in the plasma Klotho concentration and those in cholesterol levels (total, HDL, and LDL).

**DISCUSSION**

The salient features of the present study are the correlation between plasma Klotho concentration and arterial stiffness as well as aerobic exercise in postmenopausal women. We found that aerobic exercise training induced an increase in plasma Klotho concentration and a decrease in arterial stiffness in postmenopausal women. Therefore, we propose that the increase in the plasma Klotho concentration, after aerobic exercise training, might be partly responsible for the decreased arterial stiffness.

Secreted Klotho diminishes cellular apoptosis and senescence, which impair endothelial function in the vascular endothelial cells (14). Inflammation and oxidative stress induce endothelial dysfunction (37); however, secreted Klotho suppresses inflammation in the endothelial cells (29) and oxidative stress in the smooth muscle cells (54). Supplementation of a secreted form of Klotho maintains endothelial integrity via regulating calcium ion entry to endothelial cells (21). Additionally, recent studies have suggested that the secreted Klotho has a protective effect against calcification by inhibiting vascular smooth muscle cell differentiation to osteoblast-like cells (34). Furthermore, secreted Klotho protects endothelial function through NO production, which modulates smooth muscle tones (42). In mice, kidney-secreted Klotho inhibits TGF-β1 signaling (9), which induces oxidative stress and remodeling (10). Thus, these findings indicate that Klotho acts as a humoral factor that promotes vascular protection. It has been reported that endothelial dysfunction, increase in inflammation, vascular oxidative stress, vascular calcification, and remodeling influence arterial stiffness (10, 31, 35, 38, 49).

In the present study, a correlation was observed between plasma Klotho levels and aerobic exercise capacity. Moreover, exercise improves aging-related arterial deterioration, i.e., vascular calcification (43). However, it was not known whether plasma Klotho levels are influenced by aerobic exercises. Habitual aerobic exercise improves aging-related arterial deterioration, i.e., vascular calcification (10) and reduction of NO availability (46).

In the present study, a correlation was observed between plasma Klotho levels and aerobic exercise capacity. Moreover, we also demonstrated that aerobic exercise training induced an increase in plasma Klotho levels with the modulation of arterial stiffness.

Table 3. **Characteristics of selected subjects before and after 12-wk exercise training**

<table>
<thead>
<tr>
<th>Variable</th>
<th>Control Before</th>
<th>Control After</th>
<th>Exercise Before</th>
<th>Exercise After</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>8</td>
<td>11</td>
<td>8</td>
<td>11</td>
</tr>
<tr>
<td>Age, yr</td>
<td>62 ± 3</td>
<td>62 ± 2</td>
<td>55.3 ± 1.4</td>
<td>54.2 ± 1.1*</td>
</tr>
<tr>
<td>Height, cm</td>
<td>155.0 ± 1.5</td>
<td>152.6 ± 1.8</td>
<td>23.8 ± 0.6</td>
<td>23.3 ± 0.5*</td>
</tr>
<tr>
<td>Body mass, kg</td>
<td>55.2 ± 3.6</td>
<td>55.1 ± 3.6</td>
<td>5.4 ± 0.3</td>
<td>5.7 ± 0.3*</td>
</tr>
<tr>
<td>Body mass index, kg/m²</td>
<td>23.0 ± 1.3</td>
<td>22.9 ± 1.3</td>
<td>54.2 ± 0.3</td>
<td>3.2 ± 0.3</td>
</tr>
<tr>
<td>Total cholesterol, mmol/l</td>
<td>1.6 ± 0.2</td>
<td>1.5 ± 0.1</td>
<td>1.6 ± 0.2</td>
<td>1.5 ± 0.1†</td>
</tr>
<tr>
<td>HDL cholesterol, mmol/l</td>
<td>3.4 ± 0.3</td>
<td>3.2 ± 0.3</td>
<td>3.8 ± 0.2</td>
<td>3.4 ± 0.3‡</td>
</tr>
<tr>
<td>Triglyceride, mmol/l</td>
<td>1.7 ± 0.1</td>
<td>1.6 ± 0.1</td>
<td>1.7 ± 0.1</td>
<td>1.7 ± 0.1</td>
</tr>
<tr>
<td>Daily step counts, steps/day</td>
<td>7,874 ± 1,465</td>
<td>6,713 ± 1,240</td>
<td>7,125 ± 672</td>
<td>12,560 ± 686†</td>
</tr>
<tr>
<td>Physical activity level, kcal/day</td>
<td>170 ± 29</td>
<td>139 ± 26</td>
<td>158 ± 19</td>
<td>426 ± 30†</td>
</tr>
<tr>
<td>Ventilatory threshold, ml · min⁻¹ · kg⁻¹</td>
<td>12.9 ± 0.8</td>
<td>13.3 ± 1.0</td>
<td>11.4 ± 0.4</td>
<td>14.2 ± 0.4‡</td>
</tr>
</tbody>
</table>

Values are means ± SE. *\( P < 0.05 \); †\( P < 0.01 \) vs. before intervention.
stiffness. However, in the interventional study, the changes in plasma Klotho concentration did not correlate significantly with the changes in aerobic exercise capacity. The reason for this discrepancy in the results is not clear at present. The two potential ways to increase secreted Klotho include upregulation of secreted Klotho itself by alternative splicing and activation of membrane Klotho shedding. The changes in circulating Klotho concentrations might depend on either or both of these pathways. Hence, it is possible that these pathways might interfere with the effect of aerobic exercise training on plasma Klotho levels.

In the present study, aerobic exercise training induced an increase in plasma Klotho concentrations with a decrease in arterial stiffness in postmenopausal women. Klotho influences the production of various factors. It has been reported that secreted Klotho inhibits TGF-\(\beta\)1 signaling in the kidney (9). Furthermore, silencing of brain Klotho upregulates endothelin-1 (ET-1) production (53). TGF-\(\beta\)1 and ET-1 affect arterial stiffness, and their levels have been shown to be altered by exercise training (9, 36). An exercise training-induced increase in Klotho may modulate the production of TGF-\(\beta\)1 and ET-1. Furthermore, several studies have suggested that the mechanisms underlying aerobic exercise training–induced modulation of arterial stiffness were mediated by the enhancement of endothelial function, suppression of oxidative stress, and inflammation (24, 27, 46). Therefore, an exercise training-induced increase in plasma Klotho levels might act on the endothelium to increase NO production, calcium channels in endothelial cells to regulate calcium influx, endothelial cells to suppress inflammation, and smooth muscle cells to suppress oxidative stress. Endogenous Klotho might thus partly facilitate the mechanism that regulates the decrease in arterial stiffness after aerobic exercise training.

Klotho is affected by physiological as well as pathological conditions, both at the transcriptional and post-transcriptional levels. Peroxisome proliferator-activated receptor-\(\gamma\) (PPAR\(\gamma\)), a transcription factor involved in adipogenesis, glucose homeostasis, and inflammation, has been shown to increase both Klotho messenger RNA (mRNA) and protein expression in the kidney (57). Angiotensin II downregulates both Klotho mRNA and protein expression levels in the kidney (32), and blockade of angiotensin II type I receptor (AT1R) increases circulating Klotho in human (25). Oxidative stress also downregulates Klotho mRNA and protein expression levels in mouse kidney cells (33), and a free radical scavenger upregulates Klotho mRNA and protein expression levels (40). Moreover, previous studies have suggested that exercise training increases PPAR\(\gamma\) (17) and decreases AT1R (6) and oxidative stress (2). Therefore, exercise training might also increase circulating Klotho through the increases in PPAR\(\gamma\) and decreases in AT1R and oxidative stress in the kidney. Further studies are needed to determine the mechanism underlying the exercise training-induced increase in Klotho.

Comparative analysis of pre-exercise training and postexercise training by ANCOVA indicated that both the slope and the intercept of the linear regression line of plasma Klotho concentration and carotid arterial compliance were similar. This observation suggested that the relationship between carotid arterial compliance and plasma Klotho concentration was not altered after exercise training, and therefore, the increase in plasma Klotho concentration after exercise training might be associated with the increase in carotid arterial compliance. However, for the \(\beta\)-stiffness index, the intercept but not the slope was different between pre-exercise training and postexercise training. This finding suggests that the relationship...
between the β-stiffness index and plasma Klotho concentration could be influenced by exercise training. These findings support the data, which indicate that plasma Klotho concentration correlated significantly with arterial compliance but not with the β-stiffness index after adjusting for VO₂ at VT. Elevation of blood pressure increases the arterial wall tension, i.e., functional stiffness. Carotid arterial compliance indicates structural and functional stiffness, and the β-stiffness index is more reflective of structural stiffness with minimal influence of blood pressure (23). Therefore, it is possible that there is a differential effect on the relationships between plasma Klotho concentration and carotid arterial compliance or the β-stiffness index due to the effect of blood pressure. However, we could not determine the factors that influence these relationships.

There are certain limitations in this study that should be considered while interpreting the findings. First, we measured only secreted Klotho in the plasma. Klotho is produced predominantly in the kidney, and secreted Klotho circulates in the blood (15). However, it is not known how exercise training enhances the secretion or ectodomain shedding of Klotho from the kidney. Furthermore, membrane Klotho-mediated FGFR3 signaling in the kidney has important roles in phosphate and vitamin D homeostasis, which affect vascular calcification (5, 12). Hence, further studies are needed to clarify the dynamics of Klotho production and secretion. Second, we did not investigate the mechanisms underlying the effects of aerobic exercise on Klotho and arterial stiffness. Further studies are required to establish the cause-and-effect relationship between secreted Klotho and arterial stiffness. Third, although a small sample size is a limitation of this study, power calculation indicated that the number of subjects in experiment 2 was sufficient to establish statistical significance. Finally, this study focused only on postmenopausal women; there is no gender difference in circulating Klotho levels (55). It has also been reported that aerobic exercise training reduced arterial stiffness in men and women, young and elderly (47, 48). However, the findings of this study cannot be generalized to include other populations.

In conclusion, our study demonstrated a correlation between plasma Klotho concentration and arterial stiffness, as well as aerobic exercise capacity. Furthermore, aerobic exercise training increased plasma Klotho concentration and decreased arterial stiffness. The decrease in the arterial stiffness was associated with an increase in plasma Klotho concentration. These results suggest that aerobic exercise training induced an increase in plasma Klotho concentration, which in turn, could contribute to a decrease in arterial stiffness.

ACKNOWLEDGMENTS

We thank Dr. Ryuichi Aijaka (University of Tsukuba) for his constructive advice and Mr. Ryota Higashino (University of Tsukuba) for his technical assistance.

GRANTS

Support for this work was provided by research fellowships from the Japan Society for the Promotion of Science for Young Scientists 251001.

DISCLOSURES

The authors have no conflicts of interest to disclose.

AUTHOR CONTRIBUTIONS

Author contributions: T.M. and S.M. conception and design of research; T.M., A.M., N.A., Y.C., S.G.R., K.T., H.K., and S.O. performed experiments; T.M. analyzed data; T.M. interpreted results of experiments; T.M. prepared figures; T.M. drafted manuscript; S.M. edited and revised manuscript; T.M. and S.M. approved final version of manuscript.

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