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ORIGINAL RESEARCH

Aerobic Exercise Restores Aging-Associated Reductions in Arterial Adropin Levels and Improves Adropin-Induced Nitric Oxide-Dependent Vasorelaxation

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BACKGROUND: Adropin is a peptide hormone that promotes nitric oxide (NO) production via activation of endothelial NO synthase (eNOS) in endothelial cells. Its circulating levels are reduced with aging and increased with aerobic exercise training (AT). Using a mouse model, we hypothesized that AT restores aging-associated reductions in arterial and circulating adropin and improves adropin-induced NO-dependent vasorelaxation. Further, we hypothesized these findings would be consistent with data obtained in elderly humans.

METHODS AND RESULTS: In the animal study, 50-week-old SAMP1 male mice that underwent 12 weeks of voluntary wheel running, or kept sedentary, were studied. A separate cohort of 25-week-old SAMP1 male mice were used as a mature adult sedentary group. In the human study, 14 healthy elderly subjects completed an 8-week AT program consisting of 45 minutes of cycling 3 days/week. In mice, we show that advanced age is associated with a decline in arterial and circulating levels of adropin along with deterioration of endothelial function, arterial NO production, and adropin-induced vasodilation. All these defects were restored by AT. Moreover, AT-induced increases in arterial adropin were correlated with increases in arterial eNOS phosphorylation and NO production. Consistently with these findings in mice, AT in elderly subjects enhanced circulating adropin levels and these effects were correlated with increases in circulating nitrite/nitrate (NOx) and endothelial function.

CONCLUSIONS: Changes in arterial adropin that occur with age or AT relate to alterations in endothelial function and NO production, supporting the notion that adropin should be considered a therapeutic target for vascular aging.

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Epidemiological data indicate that advanced age is a major risk factor for the development and progression of cardiovascular disease (CVD), the primary cause of overall morbidity and mortality worldwide.[1,2] Research further shows that a common underlying mechanism associated with CVD in the elderly is endothelial dysfunction,[3–6] which is characterized by a reduced bioavailability of nitric oxide (NO), a potent vasodilator also known for its role in maintaining vascular health.[7] Congruently, a main target for reducing the incidence of CVD is to improve endothelial function and NO bioavailability. In this regard, aerobic exercise training (AT) has been shown to be an effective therapeutic strategy. Indeed, cross-sectional and longitudinal

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CLINICAL PERSPECTIVE

What Is New?

- Using a mouse model, we show that advanced age is associated with a decline in arterial and circulating levels of adropin along with deterioration of endothelial function, arterial nitric oxide production and adropin-induced vasodilation.
- All these defects were restored by aerobic exercise training.
- In elderly human participants, we show that aerobic exercise training-induced increases in circulating adropin levels are correlated with improvements in endothelial function and increased circulating by-products of nitric oxide, thus validating the findings from the pre-clinical mouse model.

What Are the Clinical Implications?

- These findings suggest that the increases in adropin induced by aerobic exercise training may participate in improving arterial endothelial function in the elderly.
- Accordingly, aerobic exercise training should be considered an effective therapeutic strategy for increasing arterial adropin levels and endothelial function in the elderly.

Nonstandard Abbreviations and Acronyms

ACh	acetylcholine
Akt	protein kinase b
AT	aerobic exercise training
BAT	brown adipose tissue
cfPWV	carotid-femoral pulse wave velocity
CS	citrate synthase
DAPI	4,6-diamidino-2-phenylindole
DBP	diastolic blood pressure
eNOS	endothelial nitric oxide synthase
ERK1/2	extracellular signal-regulated kinases 1/2
FMD	flow-mediated dilation
HR	heart rate
L-NAME	Nω-nitro-L-arginine methyl ester
NO	nitric oxide
NOx	nitrite/nitrate
$\dot{V}O_{2peak}$	peak oxygen uptake
PGF2α	prostaglandin F2α
SAMP1	senescence-accelerated prone mouse 1
SBP	systolic blood pressure
TBARS	thiobarbituric acid reactive substance
VEGFR2	vascular endothelial growth factor receptor 2
WAT	white adipose tissue

studies in humans indicate that AT can prevent and reverse age-associated vascular endothelial dysfunction.[8–10] The role of exercise in mitigating endothelial dysfunction in aging is also supported by animal studies. For example, a number of studies in rodent models of aging have demonstrated that AT increases vascular endothelial NO synthase (eNOS) mRNA and protein expression, as well as NO-dependent vasodilation.[11,12] However, the specific mechanism(s) by which AT improves vascular NO bioavailability and endothelial function remain unclear.

Adropin is a 76-amino acid hormone encoded by the Energy Homeostasis Associated (i.e., Enho) gene.[13] It is expressed in multiple tissues, including brown adipose tissue (BAT), white adipose tissue (WAT), liver, aorta, small intestine, heart, kidney, skeletal muscle, brain, lung, and spleen.[13–15] Within the vasculature, adropin is expressed in endothelial cells,[14] where it modulates NO release by regulating eNOS expression and phosphorylation through activation of VEGFR2 (vascular endothelial growth factor receptor 2) and downstream Akt (protein kinase b) and ERK1/2 (extracellular signal-regulated kinases 1/2) signaling pathways.[14] Recently, we showed that AT increases circulating adropin as well as nitrite/nitrate (NOx) levels, while decreasing arterial stiffness in healthy and obese middle-aged adults as well as in older individuals.[16,17] This suggests that the changes in circulating adropin levels associated with aging or AT may play a role in altering endothelial function via changes in NO production, and that the levels of circulating adropin may be particularly associated with artery-derived production of the peptide hormone. Of note, it has been recently shown that aging-associated decreases in adropin protein expression in muscle feed arteries is accompanied by reductions in eNOS phosphorylation and endothelial dysfunction.[18] Furthermore, low circulating adropin levels are linked to endothelial dysfunction in patients with metabolic syndrome[19] or type 2 diabetes mellitus.[20]

Herein, using a mouse model, we hypothesized that AT restores aging-associated reductions in arterial levels of adropin and improves adropin-induced NO-dependent vasorelaxation. Furthermore, we hypothesized these findings would be consistent with data obtained in elderly human subjects.

METHODS

The data, analytic methods, and study materials will not be made available to other researchers for purposes of reproducing the results or replicating the procedure.

Animal Study

Animals and Protocol

The use of animals in this study was approved by the Committee on Animal Care at Ritsumeikan University. Male senescence-accelerated prone mouse 1 (SAMP1) mice were obtained from Japan SLC (Shizuoka, Japan) at 8 weeks of age and cared for according to the Guiding Principles for the Care and Use of Animals, based on the Declaration of Helsinki, and the National Research Council *Guide for the Care and Use of Laboratory Animals*. Only male mice were used to exclude the effect of female hormonal cycles. All mice were individually housed in cages under controlled conditions on a 12:12 hour light-dark cycle and were allowed access to standard laboratory diet (MF; Oriental Kobo, Tokyo, Japan) and water ad libitum for the duration of the study. The following three experimental groups were included in this study: (1) a mature adult sedentary control group (i.e., 25-week-old SAMP1 mice; Mature adult, n=7); (2) an aged sedentary control group (i.e., 50-week-old SAMP1 mice; Aged-Con, n=7); and (3) aged aerobic exercise trained group (i.e., 50-week-old SAMP1 mice) with access to running wheel during their last 12 weeks of life (Aged-AT, n=10).

Post-treatment experiments on Aged-AT mice were performed more than 48 hours after the last exercise session to avoid any acute effects of the exercise training intervention. Additionally, food was removed from all cages for 12 hours before measuring body weight and collecting blood samples from retro orbital vessels in mice under 3% isoflurane general anesthesia. Blood samples were collected in EDTA-coated tubes and immediately placed on ice. Plasma was isolated from whole blood by centrifugation (4000*g*, 15 minutes at 4°C) and stored at –80°C until further assays were performed. In addition, the quadriceps femoris muscle, tibialis anterior muscle, BAT, WAT, liver, small intestine, heart, kidney, brain, lung, spleen, and aorta were quickly resected, rinsed in ice-cold saline, frozen in liquid nitrogen, and stored at –80°C for further analysis. Before freezing, a portion of the thoracic aorta was excised and cut in 2-mm aortic rings to assess arterial function by wire myography.

Aerobic Exercise Training Protocol

AT in the Aged-AT group consisted of voluntary running on a wheel (diameter 15.5 cm, Med Associates, Inc. VT, USA) for 12 weeks beginning at 38 weeks of age.[21] Running distance was electronically monitored and recorded daily by a magnetic switch interfaced to a computer using Wheel Manager Data Acquisition Software (Med Associates Inc, VT, USA).

Functional Assessment of Isolated Aortic Rings

Aortic rings (2 mm) were mounted on wires connected to isometric force transducers for assessment of arterial function as previously described.[22–24] The mounted rings were submerged in 5 mL baths containing physiological Krebs–Henseleit solution containing 130 mmol/L NaCl, 4.7 mmol/L KCl, 1.16 mmol/L $CaCl_2$, 1.17 mmol/L $MgSO_4$, 1.18 mmol/L NaH_2PO_4, 5.5 mmol/L glucose, and 0.026 mmol/L EDTA. The solution was aerated with 95% O_2 to 5% CO_2 (pH 7.4) and maintained at 37°C for 1 hour to allow for equilibration during which the bath solution was changed every 20 minutes. Rings were stretched to an optimum preload force of 20 mN[25,26] and subsequently preconstricted with 30 nmol/L prostaglandin F2α (PGF2α U-46619, Cayman Chemical, Michigan, USA). Vasomotor function was assessed with cumulative concentration-response curves to the endothelium-dependent vasodilator, acetylcholine (ACh, 10^{-9} to 10^{-4} mol/L) and the endothelium-independent vasodilator, sodium nitroprusside (SNP, 10^{-9} to 10^{-4} mol/L), as previously described.[22] Adropin-induced vasodilation (0.5, 1, 5, 10, 25, 50, 100 ng/mL) was also assessed. Furthermore, in order to determine the role of NO in mediating adropin-induced vasorelaxation, a series of aortic rings were preincubated with the NOS inhibitor, Nω-nitro-L-arginine methyl ester (L-NAME; 300 μmol/L) for 30 minutes before exposure to increasing concentrations of adropin.[23] Additionally, to assess the upstream involvement of VEGFR2 and Akt in mediating adropin-induced NOS activation and vasorelaxation, aortic rings from matured adult mice were preincubated with the VEGFR2 inhibitor, SU1498 (500 nmol/L) or the Akt 1/2 kinase inhibitor (Akti-1/2, 500 nmol/L) for 30 minutes before adropin administration.[27] Relaxation at each concentration was measured and expressed as percent maximum relaxation, where 100% is equivalent to losing all tension developed in response to PGF2α.[24]

Real-Time RT-PCR

Total tissue RNA in the BAT, WAT, liver, aorta, small intestine, heart, kidney, tibialis anterior muscle, brain, lung and spleen samples was isolated using the ReliaPrep RNA Tissue Miniprep System (Promega, Madison, WI, USA) and the RNeasy mini kit (#74104, QIAGEN, Hilden, Germany) as previously described.[28] Single-stranded cDNA was synthesized from prepared RNA using OmniScript reverse transcriptase (QIAGEN). The mRNA expression of adropin was analyzed by real-time PCR with TaqMan Gene Expression assays (assay ID, Mn01223541_m1; Applied Biosystems). Real-time

PCR was performed on a Prism 7500 Fast Sequence Detection System 2.2 (Applied Biosystems), and cycle threshold values were calculated using the system's software. These mRNA expression levels were normalized against the expression levels of β-actin mRNA in the same sample (assay ID, Mm00607939_s1; Applied Biosystems).

Immunoblot Analysis

Western blot analysis was performed to detect arterial phospho-eNOS, total eNOS, phospho-Akt, total Akt, phospho-ERK1/2, total ERK1/2, VEGFR2 and GAPDH, as previously described.[29] We homogenized the descending thoracic aorta and separated nuclear and cytosolic fractions. Briefly, proteins from aorta homogenates including all fractions (10 μg of total protein) were separated by 10% SDS-PAGE, and transferred to PVDF membranes (Millipore, Billerica, MA, USA). The membranes were incubated for 1 hour in blocking buffer (2% skim milk in PBS with 0.1% Tween 20 (PBS-T)) and for 12 hours with antibodies against VEGFR2 (1:1000; #2479, Cell Signaling Technology, Tokyo, Japan), phosphorylated Akt Ser-473 (1:1000; #9271, Cell Signaling Technology, Tokyo, Japan), total Akt (1:1000; #9272, Cell Signaling Technology, Tokyo, Japan), phosphorylated ERK1/2 (1:1000; #9101, Cell Signaling Technology, Tokyo, Japan), total ERK1/2 (1:3000; #9102, Cell Signaling Technology, Tokyo, Japan), phosphorylated eNOS Ser-1177 (0.5 μg/mL; #612393, BD Biosciences, Tokyo, Japan), total eNOS (0.5 μg/mL; #610297, BD Biosciences, Tokyo, Japan), in blocking buffer at 4°C. GAPDH (1:1000; #AB2302, MilliporeSigma, MA, USA) was used as loading control. The membranes were washed thrice with PBS-T, and incubated for 1 hour at room temperature (24°C) with horseradish peroxidase (HRP)-conjugated secondary anti-rabbit or anti-mouse (1:3000) immunoglobulins in blocking buffer. After washing the membranes with PBS-T, phospho-eNOS, total eNOS, phospho-Akt, total Akt, phosphor-ERK1/2, total ERK1/2, VEGFR2 and GAPDH were detected using the Enhanced Chemiluminescence Plus system (GE Healthcare Biosciences) and visualized on an FUSION FX (Vilber Lourmat, Collégien, France).

Measurement of Plasma and Arterial NOx Concentrations

NOx concentrations in plasma and aortic tissues were measured using the Total Nitric Oxide and Nitrate/Nitrite Parameter Assay Kit based on the Griess assay (#KGE001, R&D Systems, Minneapolis, MN, USA). Arterial NOx levels were assayed using the cytosolic fractions obtained from the homogenized aorta. All samples were assayed in duplicate. Optical densities at 540 nm were measured using an xMark microplate spectrophotometer (Bio-Rad Laboratories, Hercules, CA, USA). Readings were converted to concentrations by a linear fitting to a log–log plot of the standard curve.

Measurement of Plasma and Arterial Adropin Concentrations

Adropin concentrations in plasma and aortic tissues were measured using an ELISA (#032–35, Phoenix Pharmaceuticals, Burlingame, CA, USA). All samples were assayed in duplicate. Optical densities at 450 nm were measured using an xMark microplate spectrophotometer (xMark microplate spectrophotometer; Bio-Rad Laboratories, Hercules, CA, USA). Readings were converted to concentrations by linear fitting to a log–log plot of the standard curve.

Measurement of Plasma Thiobarbituric Acid Reactive Substance Levels

Plasma thiobarbituric acid reactive substance (TBARS) levels were measured in duplicate using an colorimetric method (Cayman Chemical, Ann Arbor, Michigan, USA). Optical densities at 530 nm were measured using an xMark microplate reader (xMark microplate spectrophotometer; Bio-Rad Laboratories, Hercules, CA, USA). All colorimetric readings were converted into concentrations by linear fitting to the log–log plot of a standard curve.

Measurement of Citrate Synthase Activity

Quadriceps femoris muscle tissues (20 mg) were homogenized in 10 volumes of 250 mmol/L sucrose, 1 mmol/L Tris·HCl (pH 7.4), and 130 mmol/L NaCl on ice using a homogenizer. The homogenate was centrifuged at 9000*g* for 20 minutes at 0°C, and the pellet was resuspended in homogenate buffer and centrifuged at 600*g* for 10 minutes at 0°C. The resultant supernatant was centrifuged at 8000*g* for 15 minutes at 0°C, and the pellet was resuspended in 250 mmol/L sucrose. To determine citrate synthase (CS) activity, 8 μL of each sample was incubated for 2 minutes at 30°C in a 182-μL incubation mixture containing 100 mmol/L Tris·HCl (pH 8.0), 1 mmol/L 5,5-dithiobis [2-nitrobenzoic acid], and 10 mmol/L acetyl-CoA. The reaction was initiated by the addition of 10 μL of oxaloacetate (10 mmol/L) and measured spectrophotometrically at 412 nm for 3 minute as previously described.[29]

Immunohistochemical Analyses

For immunofluorescence detection of adropin, aortic arch samples were sliced into 7-μm-thick sections using a cryostat at –20°C.[28] Cryosections were

fixed in 3.7% paraformaldehyde followed by blocking for 1 hour in blocking buffer (1% bovine serum album in PBS), and then incubated overnight in adropin antibody (1:200, ab122800, Abcam, Cambridge, United Kingdom) at 4°C. The samples were then incubated with secondary antibodies (Alexa Fluor 594–conjugated anti-rabbit IgG antibody [3 μg/mL, A-11037, Thermo Fisher Scientific]) for 1 hour at room temperature. The nuclei were stained with 4,6-diamidino-2-phenylindole (DAPI; ProLong Antifade Reagents; Thermo Fisher Scientific, Waltham, MA, USA). Specimens were visualized at 320× magnification under a fluorescence microscope (BZ-9000; Keyence, Osaka, Japan).

Human Study

Subjects

In the human interventional study, 14 healthy elderly subjects (men: n=6, age: 68.7±2.3 years; women: n=8, age: 67.5±1.3 years) volunteered to participate. All subjects were recruited from local community health and recreation centers. Subjects were excluded if physician-diagnosed with hyperlipidemia, hypertension or hyperglycemia, and also if taking anti-hyperlipidemic, anti-hypertensive, or anti-hyperglycemic medication, or had a history of stroke, diabetes mellitus, hypertension, hyperlipidemia, cardiac disease, chronic renal failure, or mental disorders. None of the participants had a history of smoking for at least 12 months prior to the study. Women who had been postmenopausal for at least 5 years were not on hormone replacement therapy. All subjects were informed of the experimental procedures and risks, and provided written informed consent before participating in the study. The study was approved by the Ethics Committee of Ritsumeikan University and was conducted in accordance with the Declaration of Helsinki. This study was registered at the University Hospital Medical Information Network Clinical Trials Registry (UMIN-CTR, UMIN000035520).

Experimental Design

Measurements in this study were performed before and after AT and included height, body weight, percent body fat, peak oxygen uptake ($\dot{V}O_{2peak}$), resting heart rate (HR), systolic blood pressure (SBP), diastolic blood pressure (DBP), brachial artery flow-mediated dilation (FMD), plasma NOx levels, serum adropin levels and serum concentrations of total cholesterol, high-density lipoprotein cholesterol and triglycerides. All 14 elderly subjects completed an 8-week AT program. At the beginning and end of the study period, fasting blood samples were drawn following at least 48 hours after the last AT session to avoid the influence of acute effects of exercise. All subjects were instructed not to eat or drink fluids except for water for at least 12 hours before reporting to the laboratory. Serum and plasma samples were centrifuged (1500*g*, 15 minutes, 4°C) immediately after collection and stored at −80°C. All measurements were performed at a constant room temperature of 24°C. Percent body fat was assessed using dual-energy X-ray absorptiometry (Lunar Idxa; GE Healthcare UK, Buckinghamshire, UK).

Exercise Intervention

AT consisted of cycling on a cycle ergometer (828E Monark, Stockholm, Sweden) for 55 minutes, 3 days/week, for 8 weeks. Each 55-minute exercise bout included a 5-minute warm-up period at 40% $\dot{V}O_{2peak}$, followed by 45 minutes of cycling at 60–70% $\dot{V}O_{2peak}$, and a 5-minute cool-down period at 40% $\dot{V}O_{2peak}$. Exercise compliance was carefully monitored by direct supervision. We adopted this AT program in accordance with previous studies by our group demonstrating a favorable effect on arterial stiffness.[16,17] All subjects were instructed to maintain their dietary habits throughout the study.

Measurement of $\dot{V}O_{2peak}$

$\dot{V}O_{2peak}$ was measured during breath-by-breath oxygen consumption and carbon dioxide production using an incremental cycle exercise test on a cycle ergometer (MINATO, AE-310SRD, Osaka, Japan) as previously described.[16,17] The highest 30-second average value of $\dot{V}O_2$ during the exercise test was defined as $\dot{V}O_{2peak}$ if three out of four of the following criteria were met: (I) plateau in $\dot{V}O_2$ with an increase in external work, (II) maximal respiratory exchange ratio ≥1.1, (III) maximal heart rate ≥90% of the age-predicted maximum (208–0.7×age[30]), and (IV) rating of perceived exertion ≥18.

Measurement of Brachial Artery FMD

The participants remained supine throughout the measurement of FMD. Brachial artery FMD was assessed noninvasively with an ultrasound system (UNEXEF18G, Unex, Nagoya, Japan) as previously described.[31,32] In brief, high-resolution ultrasound with a 10 MHz linear array transducer was used to obtain a longitudinal image of the right brachial artery at baseline and then continuously for 2 minutes after deflation of a forearm cuff maintained at suprasystolic pressure (i.e., 50 mm Hg above SBP) for 5 minutes. The diameter within the same region of interest was monitored continuously after cuff deflation for determination of maximal dilation. FMD was calculated as the percentage change in peak vessel diameter from baseline,[31,32]

as follows: FMD=(maximal diameter–baseline diameter)×100/baseline diameter.

Measurement of Plasma NOx Levels

Plasma NOx levels were measured using the Total Nitric Oxide and Nitrate/Nitrite Parameter Assay Kit based on the Griess assay (R&D Systems, Minneapolis, MN, USA). All samples were assayed in duplicate. Optical densities at 540 nm were measured using an xMark microplate spectrophotometer (Bio-Rad Laboratories, Hercules, CA, USA). Readings were converted to concentrations by a linear fitting to the log–log plot of the standard curve. The day-to-day coefficient of variation of plasma NOx levels was 4.4±2.9%.

Measurement of Serum Adropin Levels

Serum adropin levels were measured in duplicate using an enzyme-linked immunosorbent assay (ELISA; Phoenix Pharmaceuticals, Burlingame, CA, USA). Optical densities at 450 nm were measured using an xMark microplate reader (xMark microplate spectrophotometer; Bio-Rad Laboratories, Hercules, CA, USA). All samples were converted into concentrations by linear fitting to the log–log plot of a standard curve. The day-to-day coefficient of variation of serum adropin levels was 2.8±0.7 %.

Measurements of Serum Cholesterol and Triglyceride Levels

Fasting serum concentrations of total cholesterol, high-density lipoprotein cholesterol, and triglycerides were measured by standard enzymatic techniques.

Statistical Analysis

In the animal study, values are expressed as means±standard error (SE). Statistical evaluations were performed using one-way analysis of variance (ANOVA). Bonferroni's post-hoc test was used to correct for multiple comparisons, when ANOVA analyses revealed significant differences. The relationships between arterial adropin concentrations and plasma adropin levels, arterial NOx concentrations or arterial eNOS phosphorylation, and the relationship between plasma adropin levels and plasma TBARS levels were determined using Pearson's correlation coefficient and simple linear regression model. $P<0.05$ was defined as statistically significant. All statistical analyses were performed using StatView (5.0, SAS Institute, Tokyo, Japan).

In the human study, values are expressed as means±SE. Differences before and after the AT intervention were compared using the paired Student's t-test. The relationships between AT-induced changes in the serum adropin levels and plasma NOx levels or FMD, and the relationship between AT-induced changes in plasma NOx levels and FMD were assessed using Pearson's correlation coefficients. $P<0.05$ was defined as statistically significant. All statistical analyses were performed using StatView (5.0, SAS Institute, Tokyo, Japan) after confirmation that data were normally distributed.

RESULTS

Animal Study

Animal Characteristics

No significant differences in body weight at the time of euthanasia were found between the three groups of mice, whereas adipose tissue mass (epididymal fat) was decreased in the Aged-AT group compared to the Aged-Con and Mature adult groups (Table 1). In addition, muscle CS activity in the Aged-Con group was significantly less compared to that in the Mature adult group. This aging effect on muscle CS was rescued by AT (Table 1).

ACh (Endothelium-Dependent)- and SNP (Endothelium-Independent)-Induced Vasorelaxation

ACh-induced aortic vasorelaxation was significantly reduced with aging (Figure 1A) and improved by AT (Figure 1A). No significant differences in SNP-induced vasorelaxation were observed between the three groups of mice (Figure 1B).

Adropin-Induced Vasorelaxation

Similar to vasomotor responses to ACh, aortic vasorelaxation in response to increasing concentrations of adropin was significantly reduced with aging

Table 1. Animal Characteristics

	Mature Adult	Aged	
		Con	AT
Body weight, g	35.3±1.1	37.5±1.4	34.6±1.3
Epididymal fat, g	1.65±0.12	1.71±0.15	1.04±0.19*†
Femoris CS activity, μmol/mg per min	13.16±0.77	11.11±0.66*	15.20±0.44*†
Average running distance, km/day	…	…	1.186±0.520

Mean value±SEM.

AT indicates aerobic exercise training; Con, control; CS, citrate synthase; and Femoris, quadriceps femoris muscle.

*$P<0.05$ vs Mature adult.

†$P<0.05$ vs Aged-Con.

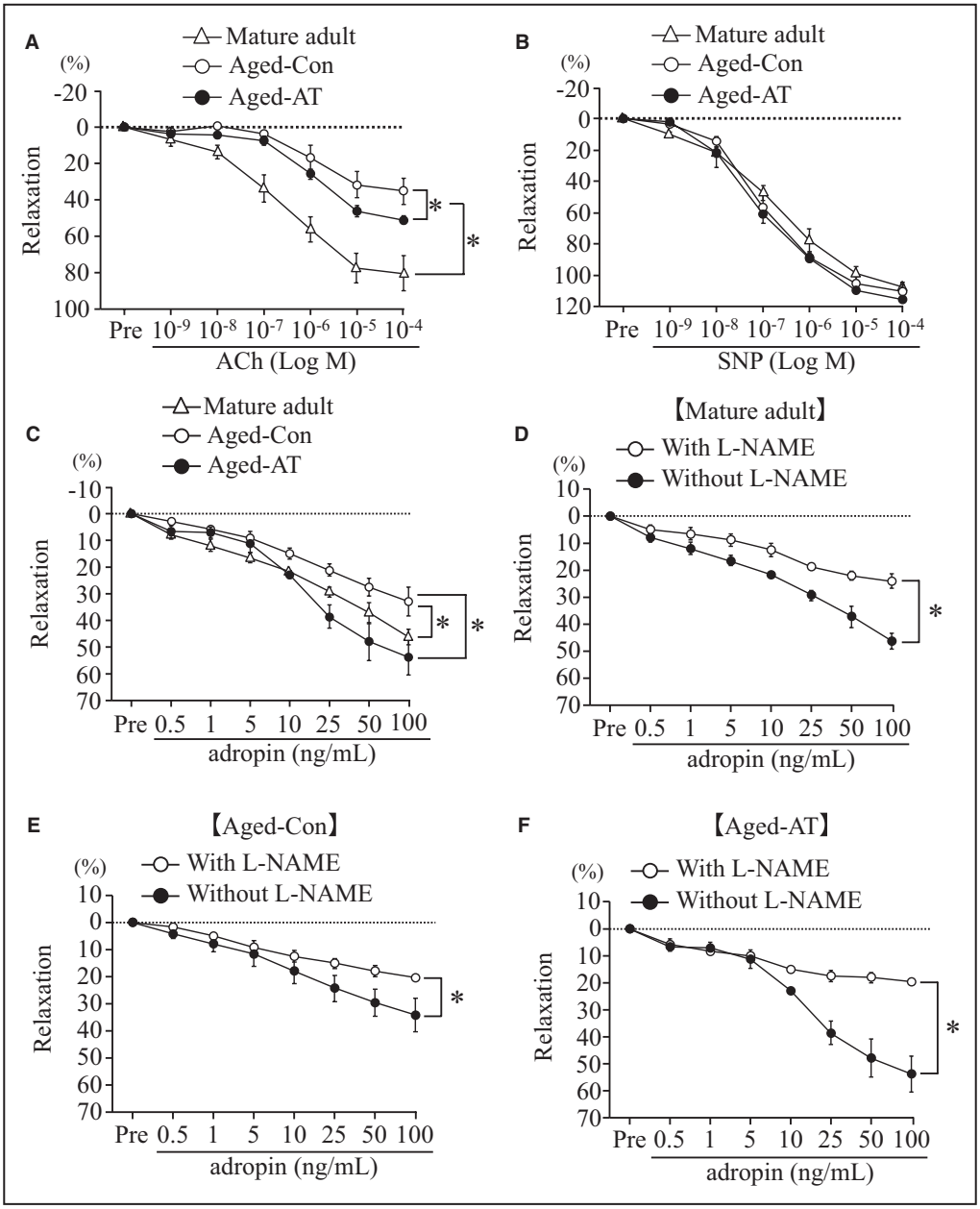


Figure 1. Aortic endothelium-dependent relaxation responses are diminished with age and increased by aerobic exercise training (AT).

Aortic rings were isolated from mature adult (25-week-old), aged control (50-week-old, Aged-Con), and aged with AT (50-week-old that underwent 12 weeks AT, Aged-AT) mice. Rings were hung in wire myography systems and exposed to increasing concentrations of the endothelium-dependent vasodilator acetylcholine (ACh, **A**) or the endothelium-independent vasodilator sodium nitroprusside (SNP, **B**). Adropin-induced aortic relaxation responses are nitric oxide synthase (NOS)-dependent, diminished with age and increased by aerobic exercise training (AT). Aortic rings were isolated from mature adult (25-week-old), aged control (50-week-old, Aged-Con), and aged with AT (50-week-old that underwent 12 weeks AT, Aged-AT) mice. Rings were hung in wire myography systems and exposed to increasing concentrations of adropin with or without the NOS inhibitor, Nω-nitro-L-arginine methyl ester (L-NAME). **C**, Comparison of adropin-induced relaxation between the Mature adult, Aged-Con and Aged-AT groups. **D**, Comparison of adropin-induced relaxation with and without L-NAME among the Mature adult group. **E**, Comparison of adropin-induced relaxation with and without L-NAME among the Aged-Con group. **F**, Comparison of adropin-induced relaxation with and without L-NAME among the Aged-AT group. Data are percent aortic relaxation responses from a preconstruction (Pre) induced with 30nM U-46619 and expressed as means±SEM, n=7 to 10/group, **P*<0.05.

(Figure 1C) and improved by AT (Figure 1C). As for the effect of NOS inhibition, adropin-induced vasorelaxation was significantly blunted by the presence of L-NAME in aortas from all groups (Figure 1D through F). Notably, the portion of vasorelaxation dependent on NO was greater in Aged-AT compared to the other two groups. Furthermore, we show that adropin-induced relaxation is blunted by VEGFR2 and Akt inhibition (Figure S1), suggesting that the NO-dependent vasodilatory effects of adropin are mediated through the VEGFR2-Akt axis.

Plasma and Arterial Adropin Concentrations

Arterial adropin concentrations were significantly lower in the Aged-Con group compared to the Mature adult group (Figure 2A). However, arterial adropin concentrations in the Aged-AT group were significantly greater than those in the Aged-Con group (Figure 2A). As with arterial adropin, plasma adropin concentrations were significantly lower in the Aged-Con group versus the Mature adult group, and greater in the Aged-AT group than in the Aged-Con group (Figure 2B).

Plasma TBARS Concentrations and Correlations Between Circulating Adropin Levels and TBARS Levels

Plasma TBARS concentrations were significantly greater in the Aged-Con group compared to the Mature adult group (Figure S2A). However, plasma TBARS concentrations in the Aged-AT group were significantly lesser than those in the Aged-Con group (Figure S2A). Plasma TBARS concentrations were negatively correlated with circulating adropin levels (r=−0.662, P=0.0004, Figure S2B).

Arterial VEGFR2 Presence, Akt, ERK1/2, eNOS Phosphorylation, and Plasma NOx

Arterial VEGFR2 protein expression was significantly lesser in the Aged-Con compared to the Mature adult group (Figure 2C), whereas it was greater in the Aged-AT versus the Aged-Con group (Figure 2C). As with VEGFR2 protein, arterial Akt Ser-473 phosphorylation was significantly reduced in the Aged-Con compared to the Mature adult group, and greater in the Aged-AT than in the Aged-Con group (Figure 2D). There were no significant differences in arterial ERK1/2 phosphorylation between the three groups of mice (Figure 2E). In contrast, arterial eNOS Ser-1177 phosphorylation was significantly lesser in the Aged-Con compared to the Mature adult group (Figure 2F), and greater in the Aged-AT versus the Aged-Con group (Figure 2F). Arterial NOx concentrations were also significantly reduced in the Aged-Con compared to the Mature adult group (Figure 2G) and significantly increased in the Aged-AT versus the Aged-Con group (Figure 2G). Furthermore, as with eNOS phosphorylation and arterial NO production, plasma NOx concentrations were significantly lesser in the Aged-Con group compared to the Mature adult group, and greater in the Aged-AT versus the Aged-Con group (Figure 2H).

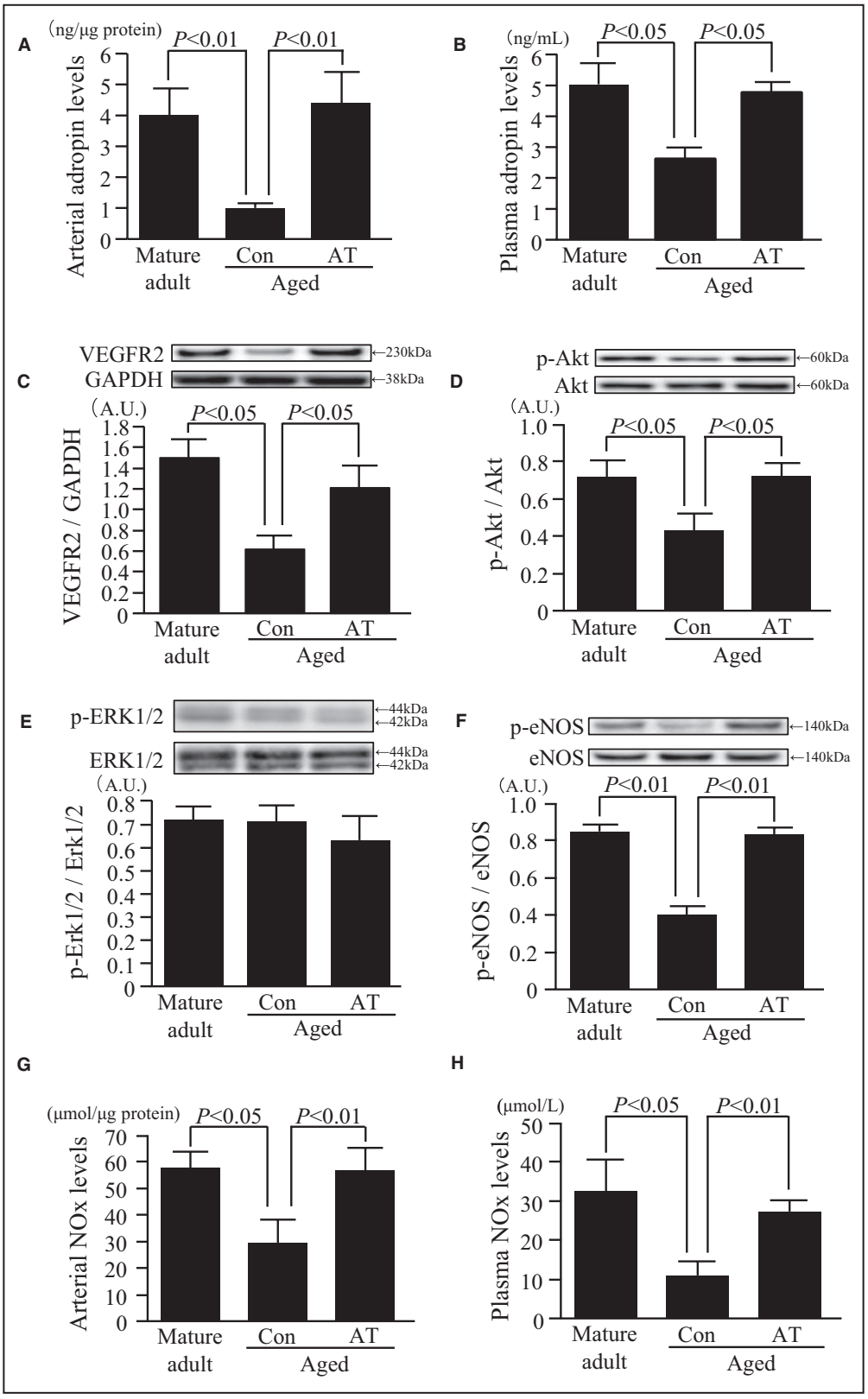
Adropin mRNA Expression in Various Tissues

To identify the potential source responsible for the changes in circulating adropin levels associated with aging and AT, we assessed adropin mRNA expression in various tissues. Of all tissues examined, only the aorta exhibited a reduction in adropin mRNA expression with aging which was restored by AT (Figure 3). There were no significant differences in adropin mRNA expression between the groups in BAT, WAT, liver, aorta, small intestine, heart, kidney, skeletal muscle, brain, lung, and spleen (Figure 3).

Correlations Between Arterial Adropin Concentrations and Circulating Adropin Levels, Arterial NOx Concentrations, and Arterial eNOS Phosphorylation

Arterial adropin concentrations were positively correlated with circulating adropin levels (r=0.613, P=0.0014,

Figure 2. Aortic and plasma adropin levels are diminished with age and increased by aerobic exercise training (AT). Adropin levels were measured using a commercial ELISA kit in Aortic tissue (**A**) and plasma (**B**) obtained from mature adult (25-week-old), aged control (50-week-old, Aged-Con), and aged with AT (50-week-old that underwent 12 weeks AT, Aged-AT) mice. Aortic endothelial growth factor receptor-2 (VEGFR2), phosphorylated Akt (p-Akt), phosphorylated endothelial nitric oxide synthase (p-eNOS) as well as aortic and plasma nitrate and nitrite (NOx) are diminished with age and increased by aerobic exercise training (AT). Aortic tissue and plasma were obtained from mature adult (25-week-old), aged control (50-week-old, Aged-Con) and aged with AT (50-week-old that underwent 12 weeks AT, Aged-AT) mice and subjected to Western blot analyses or NOx measurement. **C**, Representative blot and comparison of aortic VEGFR2 levels normalized to GAPDH between the three groups of mice. **D**, Representative blot and comparison of aortic p-Akt to total Akt ratios between the three groups of mice. **E**, Representative blot and comparison of aortic p-ERK1/2 to total ERK1/2 ratios between the three groups of mice. **F**, Representative blot and comparison of aortic p-eNOS to total eNOS ratios between the three groups of mice. **G**, Comparison of aortic NOx levels between the three groups of mice. **H**) Comparison of plasma NOx levels between the three groups of mice. Data are expressed as means±SEM, n=7 to 10/group.


A
(ng/μg protein)
P<0.01
P<0.01
Arterial adropin levels
Mature adult
Con
AT
Aged
B
(ng/mL)
P<0.05
P<0.05
Plasma adropin levels
C
VEGFR2
GAPDH
←230kDa
←38kDa
(A.U.)
P<0.05
P<0.05
VEGFR2 / GAPDH
D
p-Akt
Akt
←60kDa
←60kDa
p-Akt / Akt
E
p-ERK1/2
ERK1/2
←44kDa
←42kDa
p-Erk1/2 / Erk1/2
F
p-eNOS
eNOS
←140kDa
P<0.01
P<0.01
p-eNOS / eNOS
G
(μmol/μg protein)
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Arterial NOx levels
H
(μmol/L)
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Plasma NOx levels

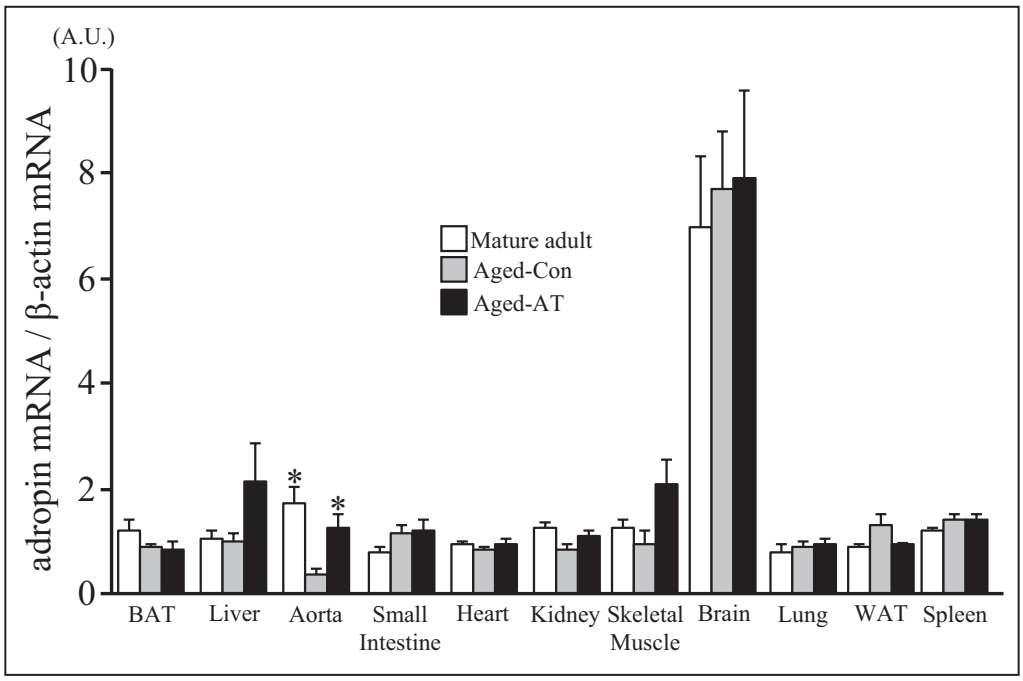

Figure 3. Aortic adropin mRNA is diminished with age and increased by aerobic exercise training (AT).
Adropin/β-actin mRNA expression was determined in brown adipose tissue (BAT), liver, aorta, small intestine, heart, kidney, skeletal muscle, brain, lung, white adipose tissue (WAT), and spleen and compared between samples obtained from mature adult (25-week-old), aged control (50-week-old, Aged-Con) and aged with AT (50-week-old that underwent 12 weeks AT, Aged-AT) mice. A.U., arbitrary units. Data are expressed as means±SEM, n=7 to 10, *P<0.05 vs Aged-Con.

Figure 4A), arterial eNOS phosphorylation (r=0.546, P=0.0057, Figure 4B) and arterial NOx concentrations (r=0.514, P=0.0102, Figure 4C).

Cellular Localization of Adropin

To determine the cellular localization of adropin, adropin protein content was vissualized via immunofluorescence in aortic samples. Adropin was detected in endothelial and vascular smooth muscle cells (Figure S3).

Human Study

To determine the translatability of the results obtained in the animal study, we examined the relationships between AT-induced increases in circulating adropin, plasma NOx levels, and vascular function in elderly human subjects.

Changes Before and After Aerobic Exercise Training

After the AT intervention, $\dot{V}O_{2peak}$ was significantly increased, whereas body weight, percent body fat, SBP, DBP, and total cholesterol levels were significantly decreased (Table 2). FMD and serum adropin levels were also significantly increased in the elderly after AT, as were their plasma NOx levels relative to those before AT (Table 2).

Correlations Between Serum Adropin Levels and Plasma NOx Levels or FMD, Plasma NOx Levels and FMD

AT-induced changes in the serum adropin levels were positively correlated with AT-induced changes in plasma NOx levels (r=0.732, P=0.0029, Figure 5A), and also with those in brachial artery FMD (r=0.578, P=0.0303, Figure 5B). Furthermore, AT-induced changes in plasma NOx levels were positively correlated with AT-induced changes in FMD (r=0.657, P=0.0107, Figure 5C).

DISCUSSION

Our main discoveries herein are that endothelial dysfunction in aged mice also includes reduced vasodilatory responses to increasing concentrations of adropin and that these age-associated vascular impairments are amerliorated by 12 weeks of AT. We further show that the endothelial dysfunction ocurring with age and rescued with AT is associated with reduced and restored levels of arterial adropin, VEGFR2, and Akt and eNOS phosphorylation,

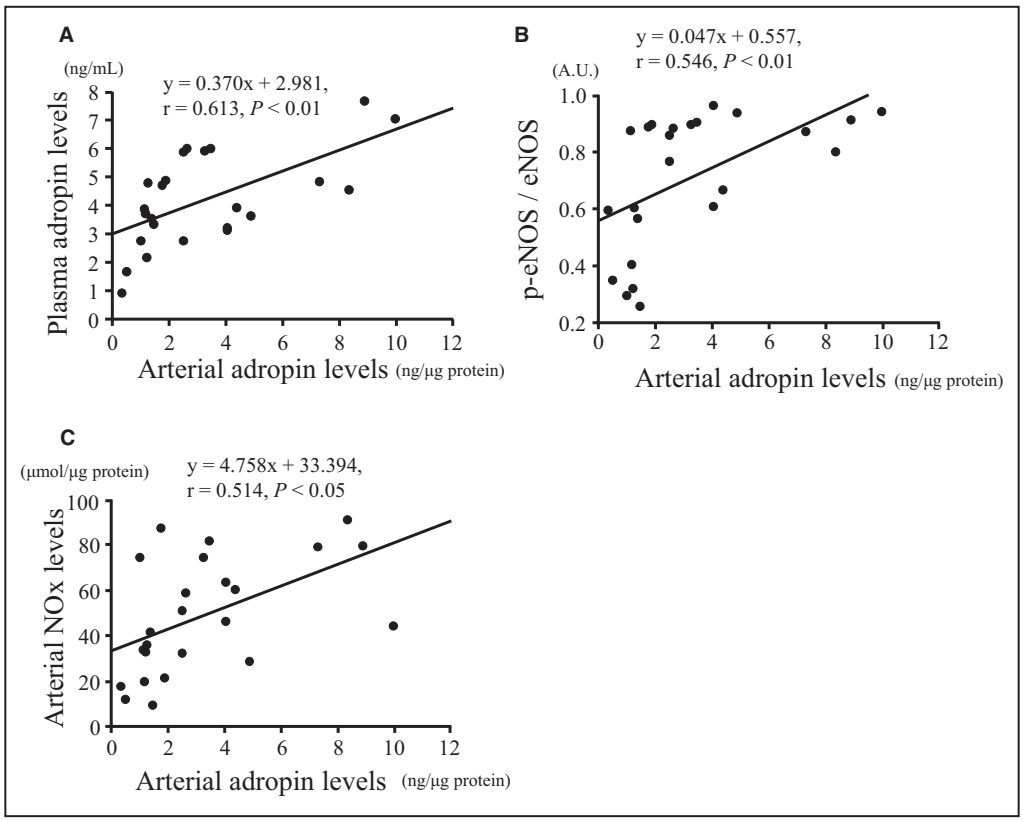

Figure 4. Aortic adropin levels are positively correlated with plasma adropin, phosphorylated endothelial nitric oxide synthase (p-eNOS) or aortic nitrate and nitrite (NOx).
A, Pearson's correlation coefficient and linear fit between arterial adropin and plasma adropin levels. **B**, Pearson's correlation coefficient and linear fit between arterial adropin and arterial p-eNOS/eNOS. **C**, Pearson's correlation coefficient and linear fit between arterial adropin and nitrite/nitrate (NOx) levels. Aortic tissues and plasma were obtained from mature adult (25-week-old), aged control (50-week-old, Aged-Con), and aged with AT (50-week-old that underwent 12 weeks AT, Aged-AT) mice. A.U., arbitrary units.

respectively. In addition, we demonstrate that our findings observed in the SAMP1 mouse model of aging are congruent with those observed in elderly human subjects, whom after an AT intervention had increased circulating adropin and NOx levels in conjunction with improved endothelial function, as assessed by FMD in the brachial artery.

Overall, our results in the SAMP1 mouse model support that aging is associated with endothelial dysfunction, characterized by reduced responses to the endothelium-dependent vasodilator, ACh, but not to the endothelium-independent vasodilator, SNP. We further show that aging dampens adropin-induced vasodilatory responses, and that these responses are mostly dependent on endothelial-derived NO production. The latter is based on the observation that the NOS inhibitor, L-NAME, consistently reduced adropin-induced vasodilation in all groups of mice. In addition, and consistent with previous reports,[33,34] we show that AT improves endothelial function in aged mice, as evidenced by increased vasodilatory responses to ACh and adropin. Notably, while aging was associated with decreased arterial adropin mRNA, decreased adropin circulating levels, lesser amounts of arterial and circulating NOx, and diminished phosphorylation of arterial eNOS, AT increased all these parameters and reversed endothelial dysfunction in aged mice. Moreover, the observation that arterial adropin levels were positively correlated with plasma adropin, arterial phospho-eNOS and arterial NOx suggests that the restoration of arterial adropin synthesis is related to the capacity of AT to reverse the endothelial dysfunction associated with aging. This is further supported by the fact that the amount of arterial VEGFR2 was reduced in aged mice and rescued by AT, which is consistent with the observation that AT restored the vasodilation responses to adropin by a greater margin than those induced by ACh. Our human data showing that AT increased circulating NOx and adropin levels as well as FMD in the elderly, and that circulating adropin levels were positively correlated with changes in circulating NOx levels and FMD demonstrate the translatability of our findings

Table 2. Elderly Human Subject Characteristics Before (Pre) and After (Post) Aerobic Exercise Training

	Aerobic Exercise Training		*P* Value
	Pre	Post	
Subjects (Male/Female), n	14 (6/8)		
Age, y	68.0±1.2		
Male, y	68.7±2.3		
Female, y	67.5±1.3		
Body weight, kg	58.5±1.8	57.5±1.9*	0.0084
Height, cm	160.7±2.6	160.7±2.5	0.7846
Body fat, %	28.8±2.3	28.0±2.3*	0.0006
HR, beats/min	64.4±2.9	60.4±2.4	0.0840
SBP, mm Hg	130.7±4.4	120.5±4.5*	0.0161
DBP, mm Hg	77.5±3.3	71.9±3.0*	0.0133
FMD, %	6.8±0.3	8.1±0.3*	0.0001
Total cholesterol, mg/dL	228.5±9.3	213.5±7.3*	0.0215
HDL cholesterol, mg/dL	63.5±4.3	60.3±4.2	0.2478
Triglycerides, mg/dL	118.6±18.3	121.5±17.4	0.8322
Plasma NOx, μmol/L	33.7±3.6	47.4±5.9*	0.0003
Serum adropin, ng/mL	2.66±0.23	3.5±0.31*	0.0001
$\dot{V}O_{2peak}$, mL/kg per min	23.3±1.3	27.0±1.4*	0.0001

Values are means and SEM.

DBP indicates diastolic blood pressure; FMD, flow-mediated dilation; HDL, high-density lipoprotein; HR, heart rate; NOx, nitrite/nitrate; SBP, systolic blood pressure; and $\dot{V}O_{2peak}$, peak oxygen uptake.

*$P<0.05$, vs Pre.

from the pre-clinical mouse model. Thus, our results from animal and human studies collectively show, for the first time, that AT can restore aging-induced endothelial dysfunction, likely through a process associated with increases in arterial adropin mRNA expression and production, as well as arterial eNOS phosphorylation and NO synthesis.

Increasing evidence indicates that adropin is modulated by exercise and involved in the regulation of vascular function. Indeed, as shown in the present and in our previous reports, AT increases circulating adropin levels in human subjects, including healthy middle-aged and older adults,[16] as well as obese elderly.[17] Adropin is highly expressed in brain[13,15] and in many other tissues, including the vascular endothelium, albeit at lower levels.[13–15] However, until now, which tissue(s) may be responsible for the changes in circulating adropin associated with aging and AT remained unidentified. Herein we show that from all the tissues we tested, which included brain, BAT, WAT, liver, aorta, small intestine, heart, kidney, skeletal muscle, lung, and spleen, only adropin mRNA in aorta followed the same pattern of changes seen in circulating adropin levels associated with aging and AT. Thus, it is plausible that reduced adropin synthesis in arterial tissue may be responsible for the reduction in circulating adropin associated with aging and that AT can restore this aging-induced decrease in arterial adropin production. In this regard, and in alignment with findings from others,[35] we show that adropin is expressed in endothelial and smooth muscle cells. Importantly, further studies are needed to determine the molecular mechanisms by which arterial adropin is regulated and how aerobic exercise intersects with these pathways.

Among the multiple mechanisms that are involved in endothelial dysfunction, reduced NO bioavailability plays a central role, as it has been implicated in the reductions of endothelium-dependent vasodilation associated with aging and many pathologies such as atherosclerosis, diabetes mellitus, essential and pulmonary hypertension, and heart failure.[36,37] Notably, in the animal study presented here, the changes in arterial adropin concentrations associated with aging and AT were positively correlated with changes in arterial eNOS phosphorylation and NOx levels. In addition, exposure of isolated arteries to exogenous adropin caused vasodilatory responses that were dependent on NO synthesis. Thus, our results indicating that circulating adropin levels were correlated with those of plasma NOx suggest that the changes in arterial and circulating adropin associated with aging and AT may be in part responsible for the concomitant changes in NO bioavailability under those conditions. It has been previously shown that aging is associated with eNOS uncoupling and that exercise training restores this uncoupling.[38] Although this may have occurred in our study, we did not assess eNOS uncoupling by aging or the effects of AT on these paramters. Nevertheless, our findings suggest that arterial and circulating adropin may be a novel target for the prevention or treatment of CVD associated with impairments in eNOS-derived NO synthesis.

Adropin regulates arterial NO release by regulating eNOS phosphorylation through the activation of VEGFR2 and its downstream signaling pathways including Akt and ERK1/2.[14] Indeed, we show that adropin-induced vasorelaxation is blunted with inhibition of VEGFR2, Akt, or NOS. In this regard, previous studies showed that Akt plays a central role in the regulation of NO production and endothelial function.[39–44] Of note, in alignment with earlier reports,[29,45] we show that AT increases arterial adropin levels, arterial VEGFR2 protein expression, and arterial Akt Ser-473 and eNOS Ser-1177 phosphorylation. However, in contrast with a previous study indicating that AT decreases ERK1/2 phosphorylation in porcine aortic endothelium,[46] AT did not change ERK1/2 phosphorylation in our mouse study. Also, oxidative stress decreases vascular NO bioavailability and causes endothelial dysfunction.[47] Prior studies demonstrate that aging results in increased circulating levels of TBARS, an index of oxidative stress, and that this induction is dampened

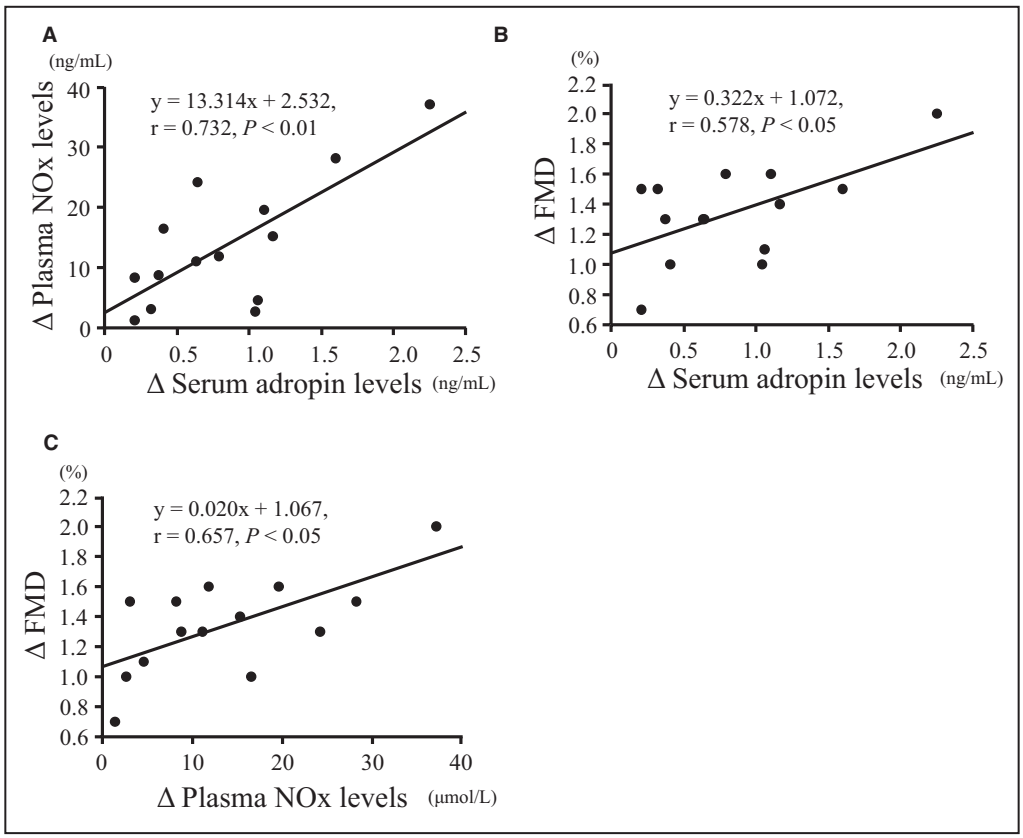

Figure 5. Serum adropin levels are positively correlated with plasma nitrate and nitrite (NOx) or brachial artery flow-mediated dilation (FMD), as plasma NOx is also correlated with brachial FMD in human elderly subjects.

A, Pearson's correlation coefficient and linear fit between serum adropin and plasma NOx levels. **B**, Pearson's correlation coefficient and linear fit between serum adropin and brachial artery FMD. C, Pearson's correlation coefficient and linear fit between plasma NOx levels and brachial artery FMD. Measurements were made before and after an 8-week aerobic exercise training intervention.

with aerobic exercise training.[48,49] In agreement with results from this prior work, we show that aerobic exercise training decreased aging-induced increases in circulating TBARS levels. It is possible that these exercise effects are in part driven by adropin, as adropin has anti-oxidant effects.[50] All this, in conjunction with our observation that NOS inhibition reduced adropin-induced vasodilation by 35% to 65%, suggest that adropin activation of the Akt-eNOS signaling pathway may contribute to AT-related systemic improvements in vasodilatory function. Adropin-induced vasodilation, however, appears to also include pathways other than NO synthesis, which remain to be elucidated.

The results from the present human study, despite the small sample size, revealed that AT increased serum adropin levels, which were positively associated with increases in brachial artery FMD. In addition, aerobic exercise training increased circulating adropin levels along with decreasing blood pressure. Given previous reports that circulating adropin is inversely correlated with blood pressure,[51,52] which is an important determinant of endothelial function, it is plausible that the exercise-induced improvement in endothelial function is in part secondary to a reduction in blood pressure and/or amelioration of other cardiovascular risk factors. Notably, low circulating adropin levels have been independently associated with reduced FMD in patients with metabolic syndrome[19] or type 2 diabetes mellitus.[20] Accordingly, adropin has the potential to be considered a novel biomarker for endothelial dysfunction.

There are some limitations in this study that should be considered while interpreting the findings. First, only 14 participants underwent the exercise training intervention. While the effects of exercise on FMD, adropin and NOx levels were statistically significant, the statistical power remained low. Thus, additional clinical trials involving larger cohorts are needed to confirm the present results. Second, this study showed a deterioration of endothelial function in aged SAMP1 mice. Previous studies show that other phenotypes associated with cardiovascular risk such as hypertension, hyperlipidemia, and hyperglycemia also develop in these mice.[53,54] Future studies using this mouse model

of aging should provide additional information on the relationship of its cardiovascular and metabolic characteristics with our current findings.

CONCLUSIONS

In conclusion, we show that advanced age is associated with a decline in arterial adropin mRNA expression and plasma concentrations along with deterioration of endothelial function, arterial NO production and adropin-induced vasodilation. Notably, a 12-week AT program in aged mice increased arterial adropin mRNA expression as well as its tissue and plasma concentrations along with improving endothelial function, arterial NO production and adropin-induced vasodilation. Furthermore, the AT-induced increases in arterial adropin concentrations were correlated with increases in arterial eNOS phosphorylation and NO production. As validation of these results in mice, we show that an 8-week AT program in elderly human subjects enhanced circulating adropin levels and that these effects were also correlated with increases in circulating NOx and FMD. These results suggest that the changes in arterial adropin levels that occur with age or AT are related to vascular endothelial function, NO production, thus providing the basis to consider adropin as a therapeutic target to reduce age-associated CVD.

ARTICLE INFORMATION

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Disclosures

None.

Supplementary Material

Figures S1–S3

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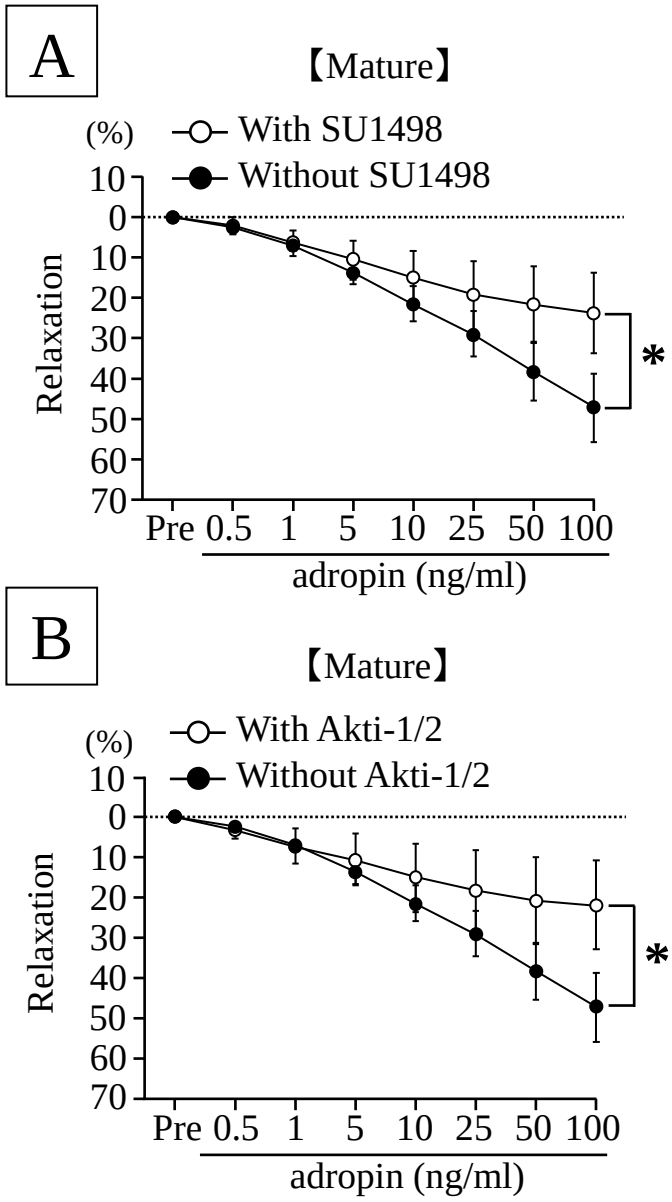
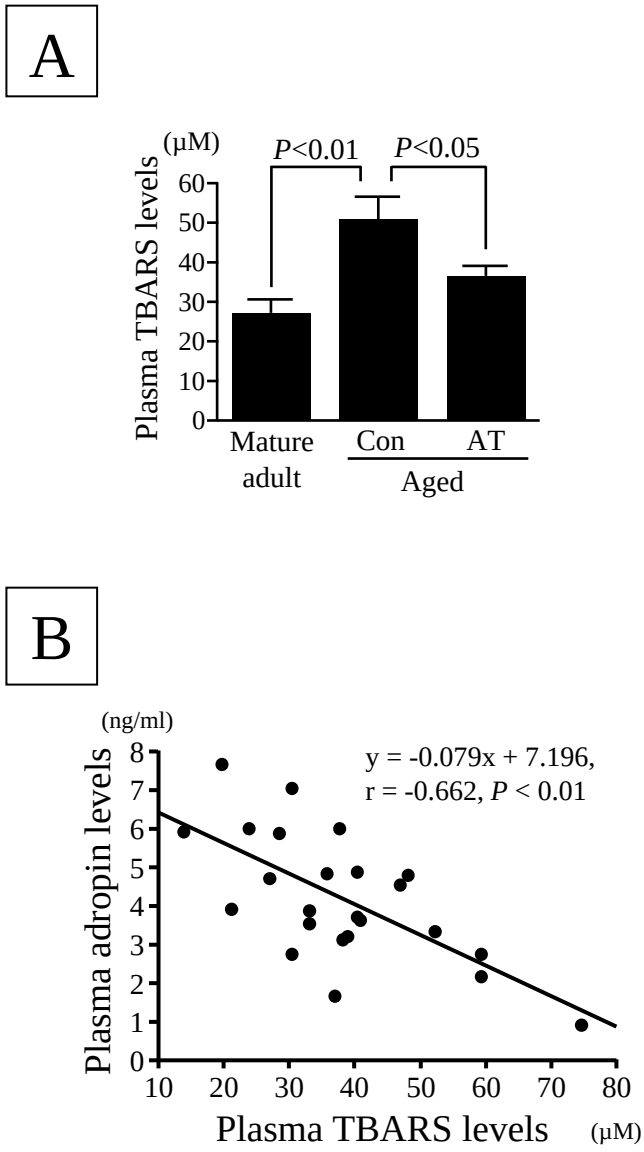
SUPPLEMENTAL MATERIAL

Figure S1. Aortic rings from matured adult mice were hung in wire myography systems and exposed to increasing concentrations of adropin with or without the VEGFR2 inhibitor, SU1498, and with or without the Akt 1/2 kinase inhibitor, Akti-1/2.



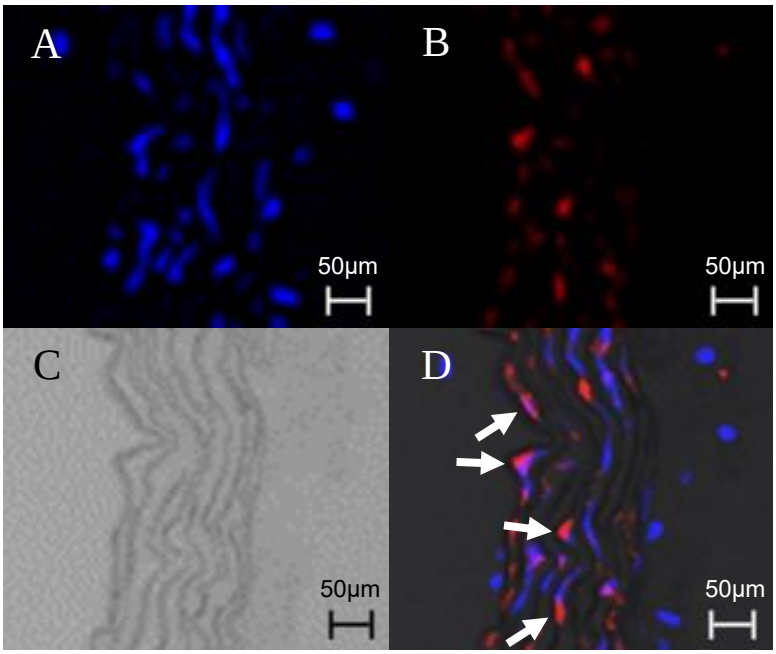
A) Comparison of adropin-induced relaxation with and without SU1498 (500nmol/L) .
B) Comparison of adropin-induced relaxation with and without Akti-1/2 (500nmol/L) .
Data are percent aortic relaxation responses from a preconstruction (Pre) induced with 30nM U-46619 and expressed as means ± SEM, n=7-10/group, *P<0.05.

Figure S2. Comparison of plasma TBARS levels among mature adult, aged-con and aged-AT groups, and correlation of plasma TBARS levels and adropin levels.



Plasma TBARS levels are increased with age and decreased by aerobic exercise training (AT) (A). TBARS levels were measured using a commercial ELISA kit in plasma obtained from mature adult (25-week-old), aged control (50-week-old, Aged-Con) and aged with AT (50-week-old that underwent 12 weeks AT, Aged-AT) mice. Plasma TBARS levels are negatively correlated with plasma adropin levels (B). Data are expressed as means ± SEM, n=7-10/group.

Figure S3. Representative immunofluorescence image of adropin in the aortic tissue section of a matured adult mice.



Images of 4,6-diamidino-2-phenylindole (DAPI)-staining of nuclei (blue, A panel), adropin-staining (red, B panel), brightfield transmitted light (C panel), and merged fluorescence images (D panel). All images were taken from the same sample. Representative immunofluorescence images are shown. Adropin: red. Scale bar: = 50 μm.