Online supplemental material

Aerobic exercise prevents rarefaction of pial collaterals and increased stroke severity that occur with aging

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Detailed materials and methods

Animals. Thirty-six male and thirty-six female C57BL/6J (B6) mice were purchased from the National Institute of Aging at one year of age and acclimated to the vivarium for one week. Animals were then randomly assigned to running and sedentary groups, body weight and composition by MRI were measured (EchoMRI-100, Echo Medical Systems, Houston, TX), and placed in individually-housed telemetry cages (Phenomaster/Labmaster, TSE SYSTEMS, Chesterfield, MO) for 48 hours to measure O_2 consumption and CO_2 production, respiratory exchange rate, energy expenditure, and food and water consumption. 3 months-old mice of both sexes served as sedentary controls (n=28). Endothelial cell-specific eNOS transgenic mice ($eNOS^{TG}$; B6 background, a gift of Dr. Rini de Crom[1]) were bred and maintained sedentary in the same vivarium as above until 3 or 26 months-age (n=4, n=10). Comparing wildtype and $eNOS^{TG}$ mice (both 3 months-old), $eNOS^{TG}$ evidenced: 2-fold increase in nitric oxide production; 30% decrease in each of MAP, systemic vascular resistance and mean pulmonary artery pressure; no change in body weight, heart weight and heart rate; 20% decrease in serum cholesterol.[1] **Figure I** provides the experimental design and allocation of subjects to the measurements obtained.

Regular aerobic exercise (RAEx). Animals in the running group were individually housed with access to running wheels (Lafayette Instruments, Lafayette, IN, model 80850L). Voluntary running was recorded electronically at 1-min intervals until 25-26.5 months-age (~26 months on average). Sedentary mice were single-housed with access to standard lab-animal enrichment, but with no access to running-wheels. The following parameters were calculated daily: distance (total revolutions), time spent running (cumulative 1-min intervals in which at least 1 revolution was recorded), average speed (total revolutions/time spent running), and maximum speed (highest number of revolutions in any 1-min interval within a 24-hr period).[2] Animals in both groups were removed from their cages for 48 hours of telemetry-assessment at six months and again at one year. Body-weight and composition were measured by MRI every 60 days. Animals were monitored daily, and all procedures were approved by and conducted in accordance with the guidelines set forth by the Institutional Animal Care and Use Committee at the University of North Carolina at Chapel Hill. Detailed data for wheel running (distance, duration, speed) and changes in body mass, body composition and metabolic traits can be found in a separate paper.[3] The following were extracted from these data and are included here to provide an indication of the amount of exercise per day, 7 days per week, that the mice experienced: Kilometers run per day (derived from number of wheel-revolutions recorded per day) on approximately day-14, day-246 and day-352 after wheel-running began in the 12 months-old mice were: ~3.3, ~2.8 and ~3.3 for males and ~7.2, ~6.1 and ~3.9 for females, respectively. Hours spent running per day (derived from cumulative 1-min intervals in which at least 1 revolution was recorded) on approximately day-14, day-246 and day-352 after wheel-running began were: ~4.7, ~3.3, and ~4.2 for males and ~8.3, ~6.2, and ~4.7 for females, respectively.

Permanent middle cerebral artery occlusion and infarct volume. At 26 months-age mice were anesthetized with isoflurane+oxygen and received permanent MCA occlusion. A 4 mm incision was made caudal to the right eye, and the temporal muscle was retracted. A 2 mm^2 craniotomy exposed the main trunk of the MCA, and the dura was incised with a 27 gauge needle. The MCA was cauterized (18010-00, FST, modified), the temporal muscle reseated, and the incision closed. Mice were euthanized 72 h later. Brains were removed and sliced into 1 mm coronal sections that were incubated in 2% 2,3,5-triphenyltetrazolium chloride in PBS at 37°C. Total forebrain and infarct areas were imaged with a stereomicroscope and analyzed with ImageJ (NIH). Percent infarct volume was normalized to forebrain volume.

Morphometry of pial collaterals, PCom arteries and primary cerebral arteries. As detailed elsewhere,[4] 5 minutes after heparinization, mice were euthanized. The thoracic aorta was cannulated with PE50 tubing and perfused retrograde with sodium nitroprusside (30 μg/ml) to dilate the vasculature. Following craniotomy to expose the cortical pial circulation, yellow Microfil™ (FlowTech Inc, Carver, MA), with viscosity adjusted to fill the pre-capillary vasculature and pial collaterals but with minimal capillary transit, was infused while imaging the pial circulation under a stereomicroscope. 4% PFA was applied topically to the cortex and brains were removed and fixed overnight in 4% PFA. Thereafter, specimens were imaged with a stereomicroscope and analyzed with

ImageJ. All pial collaterals between the anterior cerebral artery (ACA) and middle cerebral artery (MCA) trees of both hemispheres were counted. Their lumen diameter (measured at midpoint) and length were assessed at 50X magnification and averaged for each mouse. Pial collateral length was measured for each collateral that crossed the watershed zone between the MCA and ACA trees and averaged for each mouse. The beginning- and end-points of the collateral were defined as their points of departure from the ends of their parent distal-most arteriole of the MCA and ACA tree when the collateral's characteristic increased tortuosity becomes apparent (Fig 1A).[5] Relative resistance of the MCA-to-ACA collateral network of the left hemisphere was calculated for each mouse's average of the following measurements [collateral length/(collateral number x radius^4)].[5] Lumen diameter of the PCom collateral arteries and primary cerebral arteries were obtained at 100X magnification and averaged for each mouse. Measurements were made 250 μm from their bifurcations or at midpoint for the ICAs, PCAs and PCom collaterals (Figure 2A).

Immunohistochemistry and exercise training effects. The above methods to obtain morphometry of pial collaterals and intracranial arteries and infarct volume precluded use of the brains of these mice for immunohistochemistry of cerebral arteries (that requires immediate fixing, subsequent sucrose protection, and freezing of tissue) (see also Figure I). We thus had to perform immunohistochemistry on the hindlimb's proximal caudal femoral artery (PCFA); see comments in Methods of the paper for additional justifications. Hindlimb muscle was also required to determine morphometric changes in the a peripheral artery (PCFA, **Figure II**) as a measure of the exercise training effects, along with the following other measures typically reported for such: muscle fiber type switching (**Figure III**) and body weight and heart:body weight ratio (**Figure IV**). The exercise protocol used herein has not been studied previously. We thus quantified these endpoints that are commonly used to document exercise-training effects. Peripheral muscular arteries are known to experience large increases in blood flow during running that promote outward remodeling. The PCFA measurements were also obtained to compare with the novel effects we saw in the primary intracranial and PCom arteries (Figure 2). The hindlimb adductor of the thigh was embedded in paraffin, and 6 μm thick sections were deparaffinized, followed by antigen retrieval (Biocare Medical Decloaking Chamber). Sections were activated with H2O2 in PBS for 10 min, washed with PBS and incubated with 10% goat serum in 0.3% triton-PBS for 1h. Then, depending on the target, we incubated sections with: 1:50 anti-eNOS rabbit IgG (sc-654, Santa Cruz Biotechnology); 1:100 anti-phospho eNOS rabbit IgG (ab75639, Abcam); 1:200 anti-HO-1 rabbit IgG (ab13243, Abcam); 1:100 anti-SOD2 rabbit IgG (ab13533, Abcam); 1:250 anti-p16^{INK4a} rabbit IgG (ab189034, Abcam); 1:250 anti-NFkB rabbit IgG (ab16502, Abcam); 1:500 anti-8OHdG rabbit IgG in 10% goat serum in 0.3% PBS-triton, at 4°C overnight. After washing in PBS, sections were incubated 30 min in 1:200 biotinylated, goat, anti-rabbit Ig secondary antibody in 10% goat serum in 0.3% triton-PBS. Sections were then washed with PBS and incubated with R.T.U Vectastain ABC Reagent for 30 min. After washing with PBS, sections were incubated in substrate solution for 5-10 min depending on specific target, washed with water, and cover-slipped with DPX mounting media. Slides were stained in groups of six, three from each experimental group, and incubated for equal time. RAEx and sedentary samples were processed at the same time.

Morphometric analysis was performed with a light/fluorescent microscope at 630X magnification. Images were taken at similar illumination (small adjustments were made to better visualize each sample), with the same fluorescent exposure time. All targets were analyzed in the vascular wall of the PCFA. Results were quantified using Image J. The background was removed with manual tracing, leaving vascular wall, endothelial layer or both, depending on the particular antibody tested. Stained pixel area was then obtained after setting hue at 0 to 100 and brightness between 0 and the value at which the brown-stained pixel area was highlighted (usually 0 to 170, with small adjustments depending on brightness of the background). Each slide contained 8 sections from which 3-6 were included in the analysis based on the following criteria: presence of an artery, quality of staining (equal distribution), and assessment of appropriateness of staining, ie, negative and positive controls for absence and presence of signal in a given cell type (endothelial or smooth muscle cell or leukocyte) or interstitium. Finally pixel area was averaged for each animal (3-6 animals per group). As detailed elsewhere for eNOS and phospho-eNOS,[4] we measured the endothelial-stained pixel area adjusted to lumen circumference; for SOD2, intima+media-stained pixel area was normalized to

intima+media thickness; for HO-1, NFkB and $p16^{INK4a}$, media-stained pixel area was normalized to media thickness; for 8OHdG, stained cell number was normalized to media thickness. Vascular wall cells and leukocytes served as targets and positive controls, respectively: eNOS is expressed only in endothelium.[4] NFkB, $p16^{INK4a}$, 8OHdG, SOD2 and HO1 are expressed in both endothelium and leukocytes.[6-10] Given that aging causes increased leukocyte adhesion to the vascular wall[11] and that it was not possible to distinguish activated endothelial cells from adhering leukocytes with certainty, with the exception of SOD2, we quantified these targets as described above to take this into account. The above targets are also expressed in vascular smooth muscle cells, therefore we measured their expression in the media.[10,12-14] Additionally, expression in leukocytes served as a positive control.

As detailed elsewhere for muscle fiber-type,[4] adductor sections were incubated with MOM Mouse IgG Blocking Reagent (BMK-2202, Vector Laboratories, Inc.) for 1 h and MOM Diluent Protein Concentrate. Depending on the target, sections were then incubated in: 1:100 anti-Fast Myosin Skeletal Heavy chain (ab 51263, Abcam) or 1:400 anti-Slow Myosin Skeletal Heavy chain (ab11083, Abcam) for 30 min. After washing with PBS, sections were incubated with 1:250 MOM Biotinylated Anti-Mouse IgG Reagent. Specimens were then incubated with 1:300 streptavidin-Alexa fluor 568 for 15 min, washed with PBS and covered with fluoro-gel. Morphometric analysis was performed under a fluorescent microscope in the DSR red channel. After delineating the anterior and posterior gracilis and tibialis anterior muscles, results were quantified as the proportion of slow-twitch and fast-twitch fibers using Image J.

Experimental design and statistical analysis. Experiments were performed in accordance with the University of North Carolina IACUC and NIH guidelines, the ARRIVE guidelines, and the following suggested STAIR criteria:[15] n-sizes were specified based on our previous studies that have had sufficient power to test the endpoints measured herein;[eg 4,5] ~equal number of males and females were included; mice were randomized to treatment groups; controls and treatment groups were singly housed in the same conditions excepting presence of running wheels; investigators were blinded during data analysis; pMCAO was used to permanently recruit pial collaterals; no data points were determined to be outlier by statistical criterion and excluded; negative results were reported; and the literature in support and in disagreement with the results was reviewed. Experimental design, n-sizes, allocation of subjects, and reasons for loss of animals or endpoints are shown in Figure I.

Statistical analysis was performed using SPSS (PASW Statistics 18) and Graphpad Prism. Data are mean ± SEM for the n-sizes given in the figures (number of animals), with significance defined as $p<0.05$ determined by ANOVA, Bonferroni protected *t*-, Student's *t*- and X^2 tests. For all figures unless stated otherwise in the legend: data were subjected to the above tests with significance set at 2-tailed p values; each 3-bar group (3 months-old sedentary (Sed), 26 months-old Sed, 26 months-old RAEx or $eNOS^{TG}$) was tested, if significant by ANOVA, for 3 post-hoc comparisons using Bonferroni-protected 2-tailed *t*-tests, where *,**,*** = $p<0.05$, <0.01, <0.001 versus 3 months-old Sed and where #, ##, ### denotes $p<0.05$, <0.01, <0.001 versus 26 months-old Sed mice. If a comparison was not $p<0.05$ by the above test, the 2-tailed Bonferroni test was constrained to comparison of 2 groups/bars among the following groups: 3 months-old Sed, 26 months-old Sed, 26 months-old RAEx or 26 months-old $eNOS^{TG}$, where †, ††, ††† denotes $p<0.025$, <0.005, <0.0005 versus 3 months-old Sed and where ‡, ‡‡, ‡‡‡ denotes $p<0.025$, <0.005, <0.0005 versus 26 months-old Sed. Constraining was justified since the above 2-bar comparisons were pre-specified in the experimental design based on previous results;[16-27] nevertheless, the p values were halved, as above, to maintain protection against a type-II error in the 2-group comparison. See figure legends and Supplement for additional details. The following explains the justification for use of 1-tailed *t*-tests to analyze the below data.

Figure 4. Data were tested with 1-tailed *t*-tests, since: 1) We reported previously that aging was accompanied by a decrease in phospho-eNOS and its downstream target, phospo-VASP, and an increase in protein nitrotyrosine (a marker of oxidative stress) in the wall of arteries and collaterals in the hindlimb of aged mice.[5,28] 2) With aging, endothelial cells evidence decreased NO and SOD[23] and increased oxidative stress, inflammatory signals and DNA damage.[16,18-22,29] RAEx decreases the age-associated decline in eNOS-NO and endothelial dysfunction in peripheral arteries.[20] We thus predicted that the immunohistochemical assays would find either no change or an increase in the signals measured.

Figure III. Data were tested with 1-tailed *t*-tests, since it is well known that RAEx induces either no change or an increase in the percentage of slow-twitch oxidative fiber types present.[4,30]

Figure V. Data were tested with 1-tailed *t*-tests, since the 2-fold increase in eNOS activity in this $eNOS^{TG}$ mouse[1] would be predicted to have either no effect on the measured parameters or to promote lumen enlargement/outward remodeling of collaterals and arteries, since it is well known that: NO induces dilation, prolonged dilation promotes remodeling, and shear stress stimulates eNOS-NO that activates downstream signaling which drives remodeling.[19-22,23,24,26]

The following may be obtained from the senior author: original data, unstained slide sections and tissue samples where available. Antibodies were obtained from commercial sources identified above.

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Supplemental Figure Legends

Figure I. Experimental design, allocation of subjects to the measurements obtained, and reasons for loss of animals or endpoints.

Figure II. Exercise-training tended to inhibit the increase in the ratio of wall thickness-to-lumen diameter in the proximal caudal femoral artery that occurs with aging. A, Representative images of cyano-Massons-elastin staining of proximal caudal femoral artery supplying adductor muscle group. B-F, Values are mean ± SEM; number of animals is given at base of the columns; percentage values are relative to data to immediate left; ANOVAs were not significant. B, Lumen diameter (calculated from mean lumen circumference of several cross-sections for each animal) tended to decrease with aging and exercise-training tended to prevent this ($p=0.045$ for 2-tailed *t*-test; p-value doubled to protect for constraining to 2 pre-planned comparisons). C, IMT, intima-plus-media thickness. D, IMT-to-lumen diameter ratio. E, Adventitia thickness. F, The tendency of the ratio of wall thickness (WT, WT=IMT+adventitia thickness) and lumen diameter to increase with aging ($p=0.048$ for 2-tailed *t*-test, doubled to protect for constraining to 2 pre-planned comparisons) was inhibited by exercise-training. The above trends for lumen diameter and wall-to-lumen ratio in the RAEx mice are similar to those reported in humans for exercise-training, alone, without concomitant aging: Exercise-training induced outward remodeling of human arteries, most strongly in the muscles involved in the exercise. Dinenno et al reported a 7-9% increase in lumen diameter, ~15-20% decrease in IMT and IMT/lumen-ratio in femoral artery with exercise-training.[31] Wall thickness (WT) also decreased in carotid, brachial and femoral arteries of aerobic-trained athletes and in brachial and carotid arteries of wheelchair athletes.[32] The trends in the above figure for the effects of aging on a small resistance artery contrast with findings for large elastic conduit arteries: Lumen diameter of peripheral conduit arteries increased with aging in humans,[21] eg, by ~5-9% for carotid during 45-to-64 years-age and by ~17% for femoral artery during 25-to-67 years-age, and intima-medial thickness (IMT) increased in both vessels.[21,27] Likewise, lumen diameter of carotid arteries increased ~17% in 6 versus 30 months-old rats, with concomitant increase in IMT.[33,34]

Figure III. Exercise-training induced muscle fiber-type switching. A, Representative images of immunofluorescence staining for fast-twitch and slow-twitch fiber types (red signal) within the adductor muscle. B, As expected, exercise-trained animals had a higher proportion of slow-twitch fibers in the anterior gracilis and anterior tibialis muscles, with no difference in the posterior gracilis muscle. Values are mean ± SEM; number of animals is given at base of column. ** $p<0.01$ versus 3 months-old Sed by 1-tailed *t*-tests. The above changes are similar to a previous report in B6 mice after 4 weeks of voluntary wheel-running.[30]

Figure IV. Exercise-training lessened age-associated increase in body weight and increased heart-to-body weight ratio. Values are mean ± SEM; number of animals is given at base of column; percentage values shown are relative to 3 months-old Sed. A, ANOVA $p<0.001$; groups were then tested for all (10) possible comparisons using Bonferroni-protected 2-tailed *t*-tests, where *,*** = $p<0.05$, <0.001 versus 3 months-old Sed and where # $p<0.05$ versus 26 months-old Sed. $, $p<0.05$ for 26 months-old $eNOS^{TG}$ versus 26 months-old RAEx by 2-tailed Bonferroni test for 3 comparisons among last 3 bars (4th versus 5th bar are not significantly different). B, ANOVA $p<0.01$; groups were tested for 3 comparisons using a Bonferroni-protected 2-tailed *t*-test, where *,** = $p<0.05$, <0.01 versus 3 months-old Sed. C, ANOVA $p=0.05$. A, The body weight increase in the 26 months-old Sed groups reflects additional growth to the young adulthood when the rate of body weight increase declines (Mouse Physiome Database, Jackson Laboratories) plus accumulation of adipose tissue that occurs with aging to 26 months;[3] RAEx reduced the latter as expected.[3] B, Heart weight increased in both aged groups, as expected for the 26 months-old Sed group for the reason given above. C, After normalizing to body weight, exercise hypertrophy was evident in the RAEx group as expected given the well-known effects of RAEx to cause physiological hypertrophy. Previously reported findings are relevant to the above data: Heart weight increased 15% after 4 weeks of voluntary wheel-running in young mice,[30] and, aging and exercise-training each tended to increase heart weight although not significantly[35]—findings which may reflect the younger-aged mice than studied herein. Since aging and RAEx affect cardiac myocyte size and cardiac fat, collagen and other matrix components

differently,[36] it is possible that opposing changes in these parameters had a nulling effect in our aged mice, resulting in their similar increases in heart weight.

Figure V. Comparisons of pial, PCom and intracranial artery morphometrics between sedentary 3 months-old C57BL/6 wildtype and eNOS^{TG} mice. Values are mean ± SEM; number of animals is given at base of column. *,**,*** $p<0.05$, 0.01, 0.001 versus 3 months-old sedentary C57BL/6 mice, by 1-tailed *t*-tests (ICA, p=0.05).