Aging Causes Collateral Rarefaction and Increased Severity of Ischemic Injury in Multiple Tissues

James E. Faber, Hua Zhang, Roberta M. Lassance-Soares, Pranay Prabhakar, Amir H. Najafi, Mary Susan Burnett, Stephen E. Epstein

Objective—Aging is a major risk factor for increased ischemic tissue injury. Whether collateral rarefaction and impaired remodeling contribute to this is unknown. We quantified the number and diameter of native collaterals and their remodeling in 3-, 16-, 24-, and 31-month-old mice.

Methods and Results—Aging caused an "age-dose-dependent" greater drop in perfusion immediately after femoral artery ligation, followed by a diminished recovery of flow and increase in tissue injury. These effects were associated with a decline in collateral number, diameter, and remodeling. Angiogenesis was also impaired. Mechanistically, these changes were not accompanied by reduced recruitment of T cells or macrophages to remodeling collaterals. However, endothelial nitric oxide synthase signaling was dysfunctional, as indicated by increased protein nitrosylation and less phosphorylated endothelial nitric oxide synthase and vasodilator-stimulated phosphoprotein in collateral wall cells. The cerebral circulation exhibited a similar age-dose-dependent loss of collateral number and diameter and increased tortuosity, resulting in an increase in collateral resistance and infarct volume (eg, 6- and 3-fold, respectively, in 24-month-old mice) after artery occlusion. This was not associated with rarefaction of similar sized arterioles. Collateral remodeling was also reduced.

Conclusion—Our findings demonstrate that aging causes rarefaction and insufficiency of the collateral circulation in multiple tissues, resulting in more severe ischemic tissue injury. (Arterioscler Thromb Vasc Biol. 2011;31:1748-1756.)

Key Words: aging ■ angiogenesis ■ collateral circulation ■ ischemia ■ vascular biology

The occurrence of coronary and peripheral artery diseases increases with age, even in a population without other major risk factors. The prevalence of peripheral arterial disease is 5% to 10%, whereas 15% to 20% of individuals older than 70 years of age are affected. The risk of stroke and transient ischemic attack also increases with age. Besides prevalence, mortality due to ischemic cardiovascular disease is higher in elderly individuals. Thus, the severity of tissue injury following acute and chronic ischemia is increased by aging.

Mechanisms of age-associated decline in vascular function are complicated. Changes occur in cell signaling and matrix that predispose toward initiation and progression of cardiovascular diseases. For example, endothelial dysfunction is a prominent feature of the aged vascular wall. Many of the complications associated with cardiovascular risk factors and aging are initially attributable, at least in part, to attenuated endothelial function. Subsequent structural changes proceed, eg, intima-medial thickening and changes in extracellular matrix, as occur during atherosclerosis, aging, and hypertension. Additionally, evidence suggests that collateral remodeling is impaired, in part, by dysfunctional endothelial nitric oxide synthase (eNOS) signaling and oxidative stress.

 Recent studies in mice suggest that the extent of the native collateral circulation (ie, preexisting collateral number and diameter) is a primary determinant of the severity of tissue injury following acute arterial obstruction. Native collateral extent defines not only the initial conductance of the network after obstruction but also the number of collaterals available for remodeling—a process that takes days to weeks. However, no studies have determined whether aging reduces the extent of the collateral circulation and results in greater ischemic injury.

Here we report that aging is accompanied by a progressive loss of collateral number and diameter and increased tortuosity—changes that significantly increase resistance of the collateral circulation. These alterations, which are associated with impaired eNOS signaling, are compounded by impaired collateral remodeling after vascular occlusion, resulting in worse ischemic injury. The findings may explain, in part, the inconclusive results of previous therapeutic trials aimed at...
enhancing collateral function in older individuals with ischemic cardiovascular disease.

**Methods**

An expanded Materials and Methods section is available online at http://atvb.ahajournals.org.

Male C57BL/6 mice received right femoral artery ligation (FAL) distal to the lateral caudal femoral artery. Plantar foot (index of overall leg perfusion) of each leg was measured before and immediately, 3 days, and 7 days after FAL. Right hindlimb appearance and use scores (indexes of muscle ischemia and function) were obtained. Seven days later, histology was performed on the centermost region of the adductor musculature of the chronically ligated right and acutely ligated left legs to obtain lumen diameter at the approximate midpoint of the anterior and posterior gracilis muscle collaterals and to determine the number of CD3 and CD11b cells and phospho-eNOS- and phospho-vasodilator-stimulated phosphoprotein (phospho-VASP) levels in their pericolateral region. In adjacent sections, the number of smooth muscle actin–positive vessels (primarily collaterals) crossing the midpoint of the main adductor muscle (semimembranosis) was counted.

Capillary density, fiber size, and fiber number were obtained in the gastrocnemius of both legs. Collaterals connecting the middle cerebral artery (MCA) and anterior cerebral artery (ACA) trees of both hemispheres were examined for number, lumen diameter (D) at their midpoint, length, tortuosity (vector length/axial length [l]) and collateral circuit resistance (l/D). In 31-month-old mice, the number and diameter of the distal-most type I or type II arterioles (distal-most arteriole [DMA]) (not connecting or connecting to a collateral, respectively) of the MCA tree were also quantified, as was cerebral cortical area and territories of the MCA, ACA, and posterior cerebral artery trees. The number of penetrating arterioles branching from pial collaterals was quantified. These measures were made in 31-month-old mice that showed the greatest rarefaction.

In a separate group of 24-month-old mice, infarct volume and collateral remodeling were determined 3 days after permanent right MCA occlusion (MCAO). The 31-month-old mice were not examined because of their reduced chance of surviving MCAO. Circulating blood cell differential analysis was performed. Western blotting was conducted on mesenteric artery for nitrotyrosine, because the minute size of native collaterals prevents such analysis.

Data were subjected to ANOVA followed by Dunn-Bonferroni corrected t tests for preplanned comparisons or the Student t test.

**Results**

Aging Decreases Collateral Number and Diameter and Impairs Collateral Remodeling, Angiogenesis, and Recovery of Perfusion in Ischemic Hindlimb

We first examined whether aging causes collateral rarefaction using mouse hindlimb. Approximately 5 minutes after FAL (time required for Doppler scanning), perfusion was lower in 16-, 24-, and 31-month-old mice compared with young (3-month-old) mice (Figure 1a to 1c). Baseline perfusion before ligation did not differ between right and left legs or with aging (1236±38, 1214±22, 1190±30, and 1119±62 perfusion units for 3-, 16-, 24-, and 31-month-old mice, respectively; P=0.11). Aging is well known to favor an
increase in arterial pressure, thus indicating that lower perfusion immediately after acute FAL in the aged groups cannot be attributed to lower arterial pressure. These data therefore suggest that aging causes a loss of number, diameter, or both number and diameter of native collaterals. Aged mice also had reduced recovery of perfusion (Figure 1b), suggesting impaired collateral remodeling (arteriogenesis). Later time points were not obtained because our main goal was to assess the effect of aging on the native collateral circulation and because others have demonstrated lower recovery of perfusion after FAL with aging, although mechanisms vis-à-vis rarefaction and collateral remodeling were not studied.15–17

The number of α-smooth muscle actin–positive vessels at the midpoint (ie, collateral zone) of the semimembranosus muscle was determined in the nonligated limb, as an index of native collaterals.11,14 The number of 10- to 41-μm-diameter vessels declined with aging (Figure 1d). Because venules of these diameters lack smooth muscle and native collaterals in the adductor muscles average 22 μm diameter,11,14 these data suggest loss of collaterals with aging. This conclusion, which is consistent with the greater drop in perfusion immediately after ligation, is also supported by the higher appearance and use scores for the ligated limb, indicating worse ischemia (Figure 2a and 2b).

These findings were confirmed for the anatomically distinct collateral present in each of the anterior and posterior gracilis muscles. Lumen diameter trended smaller (eg, 13% lower in the nonligated limb of 31-month-old mice, P=0.09) (Figure 2c). Remodeling of these collaterals in the ligated limb (75% increase 7 days after ligation in young mice) was less with aging (54% in 31-month-old mice, P=0.02) (Figure 2c).

In 24-month-old mice, baseline capillary density in the nonligated left leg was not different from that of young mice (Figure 2d). This, together with the above evidence for fewer collaterals of smaller diameter in aged mice, suggests that the greater drop in perfusion immediately after ligation (Figure 1b and 1c) is not due to reduction of capillary density. Aged mice had impaired angiogenesis (Figure 2c), which could contribute modestly to their impaired recovery of perfusion (Figure 1b). Gastrocnemius fiber size was smaller in aged mice, reflecting known age-associated muscle atrophy (Figure 2e). Aged mice did not evidence greater atrophy after FAL, likely because atrophy was already evident, a model of moderate rather than severe ischemia was used, and C57BL/6 mice have abundant collaterals.11,14 The latter 2 considerations also explain the modest angiogenesis observed. The data in Figure 2 for the 3-month-old mice agree with our previous studies in C57BL/6 mice of the same age.11,14

Collateral remodeling is initiated by increased shear stress after arterial obstruction, followed by recruitment of hematopoietic cells to the pericollateral region, where they release cytokines and growth factors that direct remodeling. However, we did not detect a difference in abundance of pericollateral T lymphocytes (CD3+) and macrophages (CD11b+) in young and 24-month-old mice (Supplemental Figure I; for immunohistochemical controls, see Supplemental Figures II and III). Also, the number of circulating lymphocytes and monocytes did not differ (Supplemental Tables I and II). These data suggest that
Figure 3. Aging causes rarefaction of native cerebral collateral diameter and number. a, Image of dilated, fixed, and filled pial cortical circulation. b, Higher magnification image shows 2 collaterals (dotted lines) and penetrating arterioles (asterisks) branching from type II (black arrows) and type I (white arrows) DMAs that either are or are not cross-connected by collaterals, respectively. c through h, All collaterals interconnecting MCA and ACA trees in both cerebral hemispheres were quantified. e to h, Increased collateral tortuosity and resistance of the native collateral circulation with aging. e and f, Collateral length (l) indicates axial length of pial collateral; collateral span (L), scalar length connecting both ends of collateral. h, Relative resistance of the pial collateral circulation, calculated as collateral length/(Collateral number×Diameter⁴), was 6- and 10-fold higher in 24- and 31-month-old groups than in the 3-month-old group.
impaired collateral remodeling with aging (Figure 2c) is not due to reduced leukocyte recruitment.

Aging Decreases the Number and Diameter of Native Collaterals and Impairs Collateral Remodeling in the Brain, Resulting in Increased Infarct Volume

Similar to skeletal muscle, native pial collateral number and diameter also declined (Figure 3c and 3d), and collateral tortuosity doubled by 16 months of age (Figure 3e to 3g). Collateral span increased slightly, reflecting tortuosity acquired by the ends of the DMAs connecting to collaterals. The ≈40% decline in tortuosity in the 31-month-old mice (relative to the increase in 16- versus 3-month-old mice) is significantly larger than the 5% shrinkage of the cortex (see below) in this advanced age group. Thus, collateral tortuosity increases and then subsides with aging. The decrease in collateral number and diameter, plus increase in length, increased relative resistance of the collateral circulation by as much as 10-fold (Figure 3h).

This collateral insufficiency, which resulted in a 6-fold increase in collateral resistance (Figure 3h), was associated with 3-fold larger infarct volume 3 days after permanent MCAO in 24-month-old mice (Figure 4a to 4c). This cannot be attributed to increased MCA territory, because territory decreased by 5% (discussed below). Pial collateral remodeling was also examined 3 days after MCAO, when it had reached maximum in C57BL/6 mice. Aged mice had smaller baseline collateral diameters (Figure 3d), confirming the trend we detected in skeletal muscle (Figure 2). Collateral remodeling was 44% less in old mice (P<0.05) (Figure 4d, inset graph). The above cerebral and hindlimb data suggest that aging is associated in multiple tissues with a loss of native collateral number and diameter, plus impaired collateral remodeling.

Mechanisms of Aging-Associated Collateral Circulatory Insufficiency

The MCA tree was 5% smaller in 31- versus 3-month-old mice (Figure 5a) and thus showed a similar 6% reduction in the number of DMAs (Figure 3b) within the crown of the MCA tree (Figure 5b). These changes agree with the 5% smaller cortex in this aged group (Supplemental Figure IV). They are also consistent with absence of correlation of differences in collateral number and MCA tree size among 15 inbred mouse strains.13

Although the 6% decrease in DMAs was smaller than the 22% decrease in collateral number (Figure 5b), the absolute decreases in collaterals and DMAs were comparable (Figure 5b). This suggests that aging causes loss of native collaterals by a pruning process that involves or is preceded by a decline in diameter (Figures 2c, 3d, and 4d)—a process unique to collaterals (see below)—that extends retrograde to include the DMA from which the collateral arises. Consistent with this, the diameters of DMAs that connect to the extant collaterals (type II DMAs, Figure 3b) in aged mice were smaller than those in young mice, whereas the diameters of type I DMAs (which do not connect to collaterals) were not different (Figure 5c). The larger diameter of type II DMAs in both young and old mice would be expected, given the greater flow carried by these arterioles to the collaterals which, in turn, supply penetrating arterioles that branch from the collaterals into the cortex (Figure 3b). The number of penetrating arterioles branching from collaterals did not differ among aged mice (Figure 5d), suggesting that collaterals are not lost with aging because of loss of penetrating arterioles branching from them. Also, as with hindlimb capillary density, DMAs and penetrating arterioles did not decline in aged mice. These findings suggest that the collateral circulation may be especially susceptible to rarefaction with aging.

eNOS activation contributes to shear-stress-induced collateral remodeling,11 and aging is accompanied by reduced eNOS/NO activity/bioavailability.7–9,18–20 Moreover, native collateral number is reduced in eNOS-deficient mice, suggesting that NO is a maintenance factor for the collateral circulation.11 We therefore examined gracilis collaterals for phospho-eNOS (necessary for eNOS activation) and phospho-VASP (which undergoes phosphorylation when NO is increased). Before ligation, baseline phospho-eNOS and phospho-VASP were lower in aged mice (Figure 6a and 6b). Nitrotyrosine was increased in mesenteric arterioles (Figure 6c). These and previous findings11 suggest a role for impaired...
Discussion

The extent (density and diameter) of the native collateral circulation in healthy tissues varies widely as a function of differences in genetic background.\textsuperscript{13,14} Environmental factors may also affect collateral extent. The present study examined the hypothesis that 1 omnipresent environmental factor—the natural aging process—adversely affects the collateral circulation. Indeed, nitric oxide (NO) deficiency results in loss of collaterals during natural growth to adulthood,\textsuperscript{11} and endothelial/NO dysfunction accompanies aging.\textsuperscript{7–9,19,20} We found that aging caused a decline in the number and diameter of collaterals in skeletal muscle, resulting in larger decreases in blood flow and greater tissue injury following occlusion.

Similar collateral insufficiency also occurred in brain, resulting in a 6-fold increase in collateral resistance and a 3-fold increase in severity of infarct volume after MCAO in 24-versus 3-month-old-mice. Rarefaction was specific to the collateral circulation and was associated with impaired eNOS/NO signaling in the collateral wall—a finding congruent with evidence that eNOS-deficient mice lose collaterals in these same tissues by 3 months of age.\textsuperscript{11} Although multiple mechanisms are likely involved, these data suggest that aging-induced collateral insufficiency in humans could contribute significantly to the increase in severity of ischemic tissue injury in the later decades of life.

Our previous studies have shown that strain-specific differences in the extent of the native collateral circulation in the cerebral cortex mirror similar changes in other tissues.\textsuperscript{14,21} However, unlike in other tissues, the arterial trees and interconnecting collaterals are restricted to the pial cerebral circulation, permitting robust morphometry.\textsuperscript{11} These considerations allowed us to examine whether the magnitude of decline in collateral density and diameter with aging simply reflected arteriolar rarefaction in the general arteriovenous circulation. However, these declines were not observed for similarly sized DMAs and penetrating arterioles. The diameter of DMAs in the MCA tree that end by descending into the cortex was the same in 3- and 24-month-old mice. However, those DMAs that continued as collaterals had
smaller diameters in the aged mice, in accordance with the smaller diameter of their cognate collaterals.

Neither did aging cause a generalized loss of DMAs (losses of DMAs and collaterals were similar) or penetrating arterioles branching from the collaterals. Others have reported fewer collaterals in the cerebral circulation of aged rats and cats.22,23 In patients with acute myocardial infarction or stable coronary artery disease, the presence of Rentrop-defined coronary collaterals is inversely related to age.33,34 However, whether this results from a decline in native collateral extent before or from reduced collateral remodeling after onset of coronary atherosclerosis, and thus less detection, cannot be distinguished in such studies.

We speculate that collaterals are progressively pruned with aging and that this is accompanied by pruning of the DMAs to which they connect, rather than visa versa. Potential reasons why collaterals might be especially susceptible to age-induced pruning are discussed below. Similar to the absence of a generalized rarefaction of pial arterioles, capillarity in skeletal muscle did not decline with aging even though, as expected, skeletal muscle fibers underwent atrophy. Decline in capillary density in aged rats is controversial and may be tissue specific.24–34

Aging also inhibited remodeling of skeletal muscle and cerebral collaterals. This provides direct evidence that impaired remodeling likely contributed to reduced recovery of hindlimb flow after FAL reported in aged animals,16,18,35 wherein impaired increases in Hif1α, vascular endothelial growth factor (VEGF), angiopoietins, SDF1, and other cytokines in ischemic skeletal muscle were also observed. A probable mechanism for these observations is that shear-mediated increase in eNOS expression is reduced by aging, as are Hif1α and VEGF,8,16 each normally contributing significantly to collateral remodeling.11,21 Our current findings that phospho-eNOS (serine 1177) and a downstream marker/target of normal eNOS signaling, phospho-VASP, are decreased in the wall of collateral vessels in old mice provide evidence that aging-induced impairment of eNOS signaling contributes to the reduced collateral remodeling seen with aging.

Interestingly, exogenous VEGF35 or restoration of Hif1α expression16 ameliorated the decline in recovery of limb blood flow in aged mice, although the VEGF-induced improvement was not confirmed in humans with peripheral artery disease.37 Ligation-induced remodeling of ileal arteries upstream of a collateral circuit within the intestine was reduced in aged rats and in a rat strain with accelerated aging.38

The complexity of the eNOS system is illustrated by the fact that other studies demonstrated that phospho-eNOS (serine 1177) in endothelial cells from brachial arteries and antecubital veins of 61- versus 21-year-old individuals was higher and that total eNOS was unchanged.19 Aside from the heterogeneity of human samples, increased phospho-eNOS does not provide definitive evidence of intact eNOS signaling. Downstream signaling triggered by phosphorylation of eNOS is dysfunctional when eNOS is uncoupled by aging and other conditions associated with cardiovascular risk. Thus, Akt-mediated eNOS phosphorylation, which normally enhances production of NO, increased eNOS-derived superoxide rather than NO when eNOS was uncoupled.39 We also found that aged mice exhibited increased protein nitrosylation (a marker of oxidative stress) in mesenteric arteries. This finding and the finding of decreased formation of phospho-
eNOS and phospho-VASP in the collateral wall of aging mice are consistent with the concept that aging-associated disturbance in eNOS signaling occurs in collateral vessels, as it does in the general circulation, resulting in impaired collateral remodeling in occlusive arterial disease.

Tortuosity, a signature characteristic of collaterals, increases as collaterals remodel during chronic increases in shear stress. We found that tortuosity of native pial collaterals more than doubled between the ages of 3 and 16 months (the latter equivalent to a 55-year-old human). Tortuosity also increases, though much more modestly, in arteries and arterioles of healthy aged rats and humans.40

In the present study, aging caused an “age-dose-dependent” rarefaction of collaterals. This was associated with a progressively more severe ischemic injury of hindlimb tissue, decrease in conductance of the cerebral collateral network, and worse ischemic stroke. Many homeostatic systems decline with aging; thus, direct proof for cause and effect is difficult to establish. Future work studying eNOS-transgenic or VEGF-hypermorphic aged mice could help address this, because eNOS/NO and VEGF signaling, which decline with aging, also act as collateral “maintenance factors” and oppose rarefaction.10,21 One could also conduct logit regression analysis of collateral extent and stroke severity across a wide range of ages in CD1 mice, given the large variation in collateral extent in this genetically varying outbred strain.21 Nevertheless, a central role played by collateral extent per se in ischemic tissue injury was strongly suggested in a study of 15 strains of 3-month-old mice, where variation in stroke volume tightly correlated with variation in collateral number and diameter,13 and in a subsequent study of a smaller number of strains subjected to hindlimb ischemia.14

Although identification of the mechanisms responsible for the loss of collateral density and diameter require additional study, we offer 2 hypotheses. One is based on the unique hemodynamic stress that characterizes the normal collateral environment. The absence of a net pressure drop across collaterals in healthy tissues sets the prevailing environment as one of low and disturbed (oscillatory) shear stress and high circumferential wall stress.41 These conditions could predispose the collateral circulation to “accelerated aging,” compared with the general circulation, resulting in collateral rarefaction. A second possible cause relates to eNOS/NO signaling as a collateral maintenance factor.11 Thus, we have found that endothelial cells derived from aged mice exhibit an increased propensity to undergo apoptosis in association with impaired eNOS/NO activity (unpublished results). This predisposition is “rescued” by exposure to an NO donor, a finding that is compatible with the concept that endothelial cell dropout may, perhaps in conjunction with the first speculation above, underlie collateral rarefaction. Consistent with this, genetic eNOS deficiency, which has many of the features of the aged general cardiovascular system, causes accelerated loss of collaterals in young mice.11 Future investigation is needed to identify the underlying mechanisms and promote new therapeutic strategies to prevent or reverse collateral rarefaction in the aging vasculature.

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Disclosures

None.

References


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Aging causes collateral rarefaction and increased severity of ischemic injury in multiple tissues

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Supplemental Materials

Expanded Materials and Methods

Supplemental References

Supplemental table I
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Supplemental figure IV
Expanded Materials and Methods

**Animals.** Male, 3-, 16-, 24- and 31-months-old C57BL/6 mice were obtained from Jackson Laboratory (Bar Harbor, Maine) or the National Institute of Aging. Experiments were conducted according to IACUC approvals and NIH guidelines.

**Hindlimb ischemia.** Femoral artery ligation (FAL) was performed as described. Mice were anesthetized with 1.25% isoflurane/O₂ and the hindlimbs depilated. Rectal temperature was maintained at 37.0±0.5°C. The right femoral artery was exposed through a 2mm incision without retraction and with minimal tissue disturbance. A 7-0 ligature was placed distal to the origin of the lateral caudal femoral and superficial epigastric arteries (the latter was also ligated) and proximal to the genu artery. The femoral artery was transected between the sutures and separated 1-2 mm. The wound was irrigated with saline, closed and cefazolin (50mg/kg, im), furazolidone (topical) and pentazocine (10mg/kg, im) were administered.

**Laser Doppler perfusion imaging.** As detailed, under 1.125% isoflurane/O₂ anesthesia and 37±0.5°C, perfusion imaging (Moor Instruments, Wilmington, DE) of the plantar foot (index of the overall leg perfusion) and adductor thigh region (index of perfusion of superficial collaterals in the thigh) were performed before, immediately after, and 3 and 7 days after FAL on the ligated and non-ligated leg. Regions of interest were drawn to anatomical landmarks. To account for variation in ambient light and temperature and arterial pressure, perfusion was expressed as a ratio of ligated to non-ligated leg.

**Muscle function and ischemia.** At 3 and 7 after FAL, animals were evaluated for right hindlimb appearance score (index of ischemia): 0, normal; 1–5, cyanosis or loss of nail(s), where the score is dependent on the number of nails affected; 6–10, partial or complete atrophy of digit(s), where the score reflects number of digits affected; 11, partial atrophy of forefoot. Hindlimb use scores (index of muscle function) were obtained: 0, normal; 1, no toe flexion; 2, no plantar flexion; 3, dragging foot.

**Hindlimb histology and morphometry.** Seven days after surgery, the center-most regions of the adductor and gastrocnemius muscles of the ligated and non-ligated legs were excized after heparinization and pressure-perfused exsanguination (100 mmHg), maximal dilation and paraformaldehyde fixation. Paraffin sections (5 microns thick) of the adductor were stained with modified cyano-Massons elastin stain to obtain lumen diameter at the ~midpoint of the anterior and posterior gracilis muscle collaterals. Adjacent sections were immuno-stained for smooth muscle α-actin to obtain the number of vessels (primarily collaterals) crossing the midpoint of the adductor musculature. Adjacent sections were also immuno-stained for CD3⁺ and CD11b⁺ cells in the peri-collateral region, and for phospho-eNOS and phospho-VASP to quantify levels within gracilis collaterals. Skeletal muscle capillary density, fiber size and number were obtained in the gastrocnemius muscle from sections stained with lectin. Morphometry of digital microscopic images was used to obtain the above parameters.

**Postmortem cerebral artery micro-angiography.** As described before, 7 days after FAL, the abdominal aorta was cannulated retrograde and the circulation was heparinized, perfusion-cleared at 100 mmHg with phosphate-buffered saline (PBS, pH 7.4) containing adenosine (10 mg/ml) and papaverine (4 mg/ml), followed by fixation with 4% paraformaldehyde (PFA). The dorsal calvarium and dura mater were removed to expose the pial circulation. A second catheter was placed retrograde into the thoracic aorta, and a polyurethane solution with a viscosity sufficient to minimize capillary transit (1:1 resin-to-methylethyl ketone, PU4ii, Vasqtec, Zurich, Switzerland) was infused with the aid of a stereomicroscope to insure filling of all
collaterals. 4% PFA was applied topically, and the polyurethane was allowed to cure. After post-fixation overnight in 4% PFA, the pial circulation was imaged and digitized (Leica MZ16FA, Leica Microsystems, Bannockburn, IL). As described previously, all collaterals connecting the middle and anterior cerebral artery (MCA, ACA) trees of both hemispheres were analyzed (ImageJ, NIH) for number, lumen diameter (D) at the midpoint, length and tortuosity (vector length/axial length (l)), and collateral circuit resistance (l/n•D^4) was calculated. Number and diameter of the distal-most Type I or Type II arterioles (not connecting or connecting to a collateral, respectively) of the MCA were also quantified. Total cortex area and territories of the MCA, ACA and posterior cerebral artery (PCA) were determined from dorsal images. The number of penetrating arterioles branching from pial collaterals into the cortex was determined.

**Mouse middle cerebral artery occlusion and infarct volume.** The right middle cerebral artery trunk was exposed at its position midway between the zygomatic arch and the pinna of the ear and permanently occluded by micro-cautery. 3 days later, the mouse received an overdose of ketamine (100 mg/kg ip) and xylazine (15 mg/kg ip). The brain was removed, sliced into 1 mm coronal sections, and incubated in a PBS solution containing 2% 2,3,5 triphenyltetrazolium chloride (TTC) at 37°C for 20 min. Sections were washed in PBS, fixed in 10% formalin and imaged (MZ16FA, Leica). Infarct volume was calculated as the sum of the cortical volume devoid of TTC in each section, and expressed as a percent of total right cortex volume. Collateral remodeling was measured by determining diameter of the ACA-to-MCA collaterals on the ligated and non-ligated cerebral hemispheres.

**Immunohistochemistry and circulating blood differential analysis.** Tissue preparation and histological procedures have been detailed previously. Vessel density was determined by smooth muscle actin (SMA) staining in the adductor (semimembranosus) muscle. The staining was performed using M.O.M Kit (detecting mouse primary antibodies in mouse tissue - Vector). Briefly, the slides were incubated with 3% of H2O2 for 5 minutes and washed 2 times with PBS. Sections were incubated with blocking serum for one hour and with monoclonal mouse anti-human smooth muscle actin (1:200 - Dako) for 30 minutes. Slides were washed in PBS and incubated for 10 minutes with secondary anti-mouse IgG M.O.M reagent. DAB vector peroxidase substrate kit was applied as the detection system. SMA-positive vessels in the mid-zone of the semimembranosus muscle were counted as an index of collateral number. CBC analysis was performed by the Pathology Core of the University of North Carolina.

**Western blot.** 3-months-old and 24-months-old mice were sacrificed one day after FAL and the mesenteric artery was collected. The mesenteric artery was homogenized in lysis buffer (50mM HEPES,150mM NaCl,10% glycerol,1.5mM MgCl2,1mM EDTA,1%NP40). Total protein was measured by BCA kit (Pierce). 80ug protein was resolved using a 4-20% Tris-Glycine gel and was blotted on nitrocellulose membrane. The membrane was blocked with 5% milk and then incubated with primary antibody against nitrotyrosine (1:500, Cell Biolabs – Nitrotyrosine Immunoblot Kit) and actin (1:500, Sigma) at 4C overnight and with corresponding secondary antibody for 1 hour at room temperature. The signal was detected using an enhanced chemiluminescence substrate (Pierce).

**Statistics.** Data (means ± SEM) were subjected to ANOVA followed by Dunn-Bonferroni corrected t-tests, or Student’s t-tests, as indicated in the figures.
Supplemental References


### Supplemental Table I. **Granulocytes lower 7 days after MCA occlusion. Aging increases platelets (PLT).**

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<td>4.84</td>
<td>↓ 0.41#</td>
<td>0.76</td>
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<tr>
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<th>RBC 10^6/ul</th>
<th>HGB g/dl</th>
<th>MCH pg</th>
<th>MCHC g/dl</th>
<th>RDW %</th>
<th>MPV fl</th>
<th>PLT 10^9/ul</th>
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<tr>
<td>Mean</td>
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<td>8.32</td>
<td>13.41</td>
<td>16.14</td>
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3-months-old, n=8; 24-months-old, n=8. SE, standard error of mean; MCV, mean corpuscular volume; HGB, hemoglobin; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; MPV, mean platelet volume. Blood samples were taken 3 days after permanent MCA occlusion. *, p < 0.05 vs 3-months-old group; #, p <0.05 vs no-MCA occlusion control mice (Table 2). Granulocyte decrease may reflect accumulation in infarcted cerebral tissue, since same decrease observed in 3- and 24-months-old groups (dotted box = trend) and no decrease seen in 6-months-old control group shown in Supplemental Table 2. These data show that there are no differences in circulating leukocytes in 24-months-old control group, consistent with lack of a difference in T-cell and macrophage accumulation around remodeling collaterals (Suppl Fig I), to account for the observed decrease in collateral remodeling. Thrombocytosis, a marker of inflammation consistent with decreased eNOS activity and increased nitrotyrosine (Fig 6), supports reduced NO and increased oxidative stress known to characterize the aged vasculature.
Supplemental Table II. CBC blood analysis in 5-6 month old control mice that did not receive MCA occlusion.

<table>
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<th>LYMF $10^3/\mu$l</th>
<th>GRAN $10^3/\mu$l</th>
<th>MONO $10^3/\mu$l</th>
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<th>GRAN %</th>
<th>MONO %</th>
<th>HCT %</th>
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<tr>
<td></td>
<td>MCV fl</td>
<td>RBC $10^6/\mu$l</td>
<td>HGB g/dl</td>
<td>MCH pg</td>
<td>MCHC g/dl</td>
<td>RDW %</td>
<td>MPV fl</td>
<td>PLT $10^3/\mu$l</td>
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<tr>
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C57BL/6J, n=10. SE, standard error of the mean; MCV, mean corpuscular volume; HGB, hemoglobin; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; MPV, mean platelet volume.
Supplemental figure I. Aging does not impair peri-collateral leukocyte accumulation.
No change in T cell (CD3+) or macrophage (CD11b) recruitment to remodeling collaterals, detected by immunohistochemistry of gracilis collaterals at day-7 after ligation. Arrow indicates positives cells. Magnification same for both sections.
Supplemental figure II. Positive control for CD3 immunohistochemistry (pan-T cell marker), showing CD3+ T cells in lymph node.
Supplemental figure III. Positive control for CD11b immunohistochemistry, showing CD11b+ macrophages in lymph node.
Supplemental figure IV. 5% decrease in cortex surface area is consistent with 5% decrease in MCA tree territory (Figure 5), indicating a much smaller decrease in brain size with advanced aging than the 22% decline in collateral number and 30% decrease in diameter (Figure 3). That is, the latter decreases cannot be ascribed to reduced brain size with aging. Body weight data show no significant loss of body weight after FAL surgery, indicating good recovery from surgery and the mild stress caused by Doppler and use/appearance score determinations on day-3 and day-7. 31-months-old group shows expected decline in baseline body weight (before FAL) with advanced age.