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Cardiovascular risk factors cause premature rarefaction of the collateral circulation and greater ischemic tissue injury

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Abstract

Rationale—Collaterals lessen tissue injury in occlusive disease. However, aging causes progressive decline in their number and smaller diameters in those that remain (collateral rarefaction), beginning at 16 months-age in mice (ie, middle age), and worse ischemic injury—effects that are accelerated in even 3 months-old eNOS$^{-/-}$ mice. These findings have found indirect support in recent human studies.

Objective—We sought to determine if other cardiovascular risk factors (CVRFs) associated with endothelial dysfunction cause collateral rarefaction, investigate possible mechanisms, and test strategies for prevention.

Methods and Results—Mice with nine different models of CVRFs of 4–12 months-age were assessed for number and diameter of native collaterals in skeletal muscle and brain, and for collateral-dependent perfusion and ischemic injury after arterial occlusion. Hypertension caused collateral rarefaction whose severity increased with duration and level of hypertension, accompanied by greater hindlimb ischemia and cerebral infarct volume. Chronic treatment of wildtype mice with L-N^G-nitro-arginine methylester caused similar rarefaction and worse ischemic injury that were not prevented by lowering arterial pressure with hydralazine. Metabolic

syndrome, hypercholesterolemia, diabetes mellitus, and obesity also caused collateral rarefaction. Neither chronic statin treatment nor exercise training lessened hypertension-induced rarefaction.

Conclusion—Chronic CVRF presence caused collateral rarefaction and worse ischemic injury, even at relatively young ages. Rarefaction was associated with increased proliferation rate of collateral endothelial cells, effects that may promote accelerated endothelial cell senescence.

Keywords

collateral circulation; cardiovascular risk factors; endothelial dysfunction; hypertension; diabetes mellitus; metabolic syndrome; hyperlipidemia; obesity; cerebral circulation; ischemic stroke; peripheral vascular disease

Occlusive arterial disease is the most common cause of death in developed countries. The health care cost for cardiovascular disease and stroke, recently estimated at \$312 billion, is more than any other diagnostic group.[1] The presence of cardiovascular risk factors (CVRFs) increases the incidence of acute coronary syndrome, stroke, peripheral artery disease, and other occlusive vascular diseases. In data collected from 2007 to 2010, 35 percent of US adults were classified as obese, 33 percent had hypertension, and 38 percent met criteria for pre-diabetes[1]. In addition to increasing the risk of ischemic vascular disease, CVRF presence also predicts poorer outcomes in acute myocardial infarction,[2] stroke,[3] and peripheral artery disease.[4] Current treatment for ischemic disease is limited to risk factor reduction to slow disease progression and revascularization strategies that include percutaneous intervention and open surgical bypass. Despite these efforts, a large fraction of patients are not adequately revascularized by traditional therapies; for example, only one third with coronary artery disease (CAD) achieves adequate revascularization.[5] Furthermore, success with therapies to promote new blood vessel growth has thus far been limited.[6]

Most tissues in healthy individuals have a native (pre-existing) collateral circulation that provides protection against ischemic injury. Collaterals are arteriole anastomoses that cross-connect a small fraction of the outer branches of adjacent arterial trees. They thus provide an alternative source of perfusion, whose magnitude depends on their number and diameter (collateral extent), in the event of arterial occlusion of one of the trees.[7–9] If the obstruction is sustained or slowly developing, the collaterals enlarge their anatomic lumen diameter over days-to-weeks, which increases the collateral-dependent blood flow. A number of studies have found that aging, hypertension, diabetes and other CVRFs impair this collateral remodeling process.[8,12–14] However, little is known about whether CVFRs affect the extent of the native collateral circulation before obstruction ensues.

Collateral extent measured in animals[7] and estimates of collateral-dependent blood flow in humans[15–17] indicate that wide variability exists in the abundance of these vessels among healthy individuals prior to occlusive disease. Studies in mice have found that differences in genetic background are a major contributor to this variation.[7] Environmental factors also affect collateral extent; loss of collateral number and/or diameter (collateral rarefaction) occurs in brain and hindlimb of 16 months-old mice (~50 years in human), compared to 3 months-old young adults.[8] Moreover, rarefaction worsens with further aging, resulting in more severe injury in both tissues following arterial occlusion. This age-associated collateral

rarefaction, which was not seen in similarly sized end-arterioles in the surrounding trees, was associated with reduced eNOS activity and other markers of endothelial dysfunction in cells of the collateral wall.[8] In support of a role for nitric oxide (NO) deficiency, collateral rarefaction in brain and hindlimb was greatly accelerated in eNOS$^{-/-}$ mice, occurring at even 3 months-age, becoming worse by 6 months, and resulting in more severe ischemic tissue injury.[9] These results led us to suggest that eNOS-derived NO, through its anti-proliferative, -inflammatory and –oxidative stress properties, serves as a "maintenance factor" for preserving collateral extent during aging.[8]

Reduced eNOS activity and NO bioavailability are central features of endothelial dysfunction that accompanies the presence of CVRFs. However, aside from the above-mentioned studies of aging and targeted eNOS deficiency[8,9] and studies of diabetic rodents,[12,13] none has examined whether the presence of other CVRFs also cause collateral rarefaction. The major aim of the present study was to examine this possibility. We studied nine mouse models of hypertension, diabetes mellitus, obesity, hyperlipidemia, metabolic syndrome, combinations of these risk factors, and chronic exposure to cigarette smoke. In addition, since both chronic exercise training and statin treatment lessen endothelial dysfunction and risk factors in several or more of the above disturbances,[18–21] we also examined whether these therapies could inhibit collateral rarefaction in one of the most common disease/risk factor —hypertension.

Materials and Methods

Details are available in the Online supplement.

Models of chronic cardiovascular risk factor presence

Unless indicated otherwise, each group had approximately equal male and female C57BL/6 mice aged to ~eight months before studying, some while receiving certain treatments, to provide the following models of chronic CVRF presence. Hypertension: RTGMK renin transgenic mice (RTG$^{+/-}$, RTG$^{+/+}$) were generously provided by Dr Kathleen Caron; littermate controls (wildtype, wt) were used. Endothelial dysfunction: daily L-N^G-nitro-arginine methylester (L-NAME) from 1.5 to 8 months-old ± hydralazine, the latter to control for L-NAME-induced hypertension. Obesity: *Lep$^{ob/ob}$*. Hyperlipidemia and mild obesity: *Ldlr$^{-/-}$* fed a high-fat western diet starting at 1.5 months-age. Mild hyperlipemia: *Apoe$^{-/-}$* fed normal chow. Mild hypertension and mild hyperlipidemia: *Pparg$^{L/+}$;Apoe$^{-/-}$* fed normal chow. Metabolic syndrome/Type 2 diabetes: *Lep$^{ob/ob}$;Ldlr$^{-/-}$* (2KO, "double knock out") studied at 6 months-age. Type 1 diabetes: *Ins2^{Akita}* (Akita, male). Additional ages besides the above were examined for duration of cardiovascular risk factor exposure: Moderately hypertensive (RTG$^{+/-}$) and hypertensive mice (RTG$^{+/+}$): 1.5, 4, 8 and 12 months-age; *Ldlr$^{-/-}$* at 12 months-age; Akita at 10–11 months-age. Six week-old male mice were exposed to smoke from 6 cigarettes, 5 days per week, for 6 months. Control mice and those with risk factors, and/or their tissue sets, were coded and studied by blinded observer where possible.

Hindlimb ischemia and cerebral stroke

After femoral artery ligation (FAL), perfusion and deficits ischemic appearance and leg use were measured.[7–9] Infarct volume was determined 72 and 24 hours after permanent right middle cerebral artery (MCA) occlusion in the RTG and smoke-exposed mice, respectively.[7,8]

Angiography of cerebral and skeletal muscle collaterals, collateral remodeling

Angiography of pial and abdominal wall vasculature, after maximal dilation and fixation, was accomplished by infusion of Microfil with viscosity adjusted to fill the arterial and collateral circulations.[7–9] For each mouse, all collaterals between the anterior cerebral artery (ACA) and MCA trees of both hemispheres were counted; their diameter at midpoint and tortuosity were obtained and an average value calculated for each mouse (similar methods were used in the abdominal wall). Remodeling of collaterals (anatomic increase in lumen diameter) in the right hemisphere was determined 72h and 24h after permanent MCA occlusion in the RTG and smoke-exposed mice, respectively.

Chronic daily statin treatment, chronic exercise training

Hypertensive RTG^{+/−} mice and normotensive littermates received pitavastatin from 1.5 through 8 months-age. In separate groups of RTG^{+/−} and littermates, mice were swim-trained from 2-to-8 months-age for 1h/day, 5 days/wk,[22] or sham treated.

Proliferation of collateral wall cells, immunohistochemistry, immunoblotting

RTG^{+/−} mice and age-matched wildtype littermates were infused with 5-ethynyl-2-deoxyuridine (EdU) for 28 days by minipump, then processed for whole-mount immunohistochemistry. Immunoblot analysis was conducted on descending thoracic aorta. Fiber type composition of the tibialis anterior was determined using immunohistochemistry for myosin heavy chain isoforms.

Statistics

Data are mean ± SEM and were tested for significance ($p < 0.05$) by t-tests or ANOVA followed by Bonferroni tests for comparison of more than two groups.

Results

Chronic hypertension results in more severe ischemia after acute femoral artery ligation

Mice were bred with different durations and severity of renin-dependent hypertension to determine if rarefaction of the native collateral circulation occurs. Mean arterial pressures of 4, 8 and 12 months-old RTG^{+/−} mice were 31, 22 and 31mmHg higher, respectively, than their normotensive age-matched littermates (Figure 1A, Online Table 1). Four months-old RTG^{+/+} were more hypertensive than heterozygotes (153 ± 3 vs 136 ± 3, $P < 0.001$). Heart rate did not differ among the groups (Figure 1B). These findings agree with the original characterization of this model.[21] Plantar and adductor perfusion dropped to lower values immediately after FAL in 8 months-old RTG^{+/−} ("Post-op", Figure 2A and 2B **insets**). Perfusion of the plantar region after ligation, as measured herein, correlates with collateral-

dependent blood flow to the hindlimb determined with microspheres.[24] Plantar perfusion was lower in the hypertensives over the first 7 days (Figure 2A, $P<0.05$). A similar trend was seen in the adductor region where lower perfusion, compared to the control group, reflects lower collateral-dependent perfusion.[9,24] These findings suggest hypertensive mice have fewer and/or smaller-diameter native collaterals and may also experience a delay in collateral remodeling after FAL (both findings confirmed below). Baseline perfusion ("Pre-op") in plantar and adductor regions did not differ between the right and left hindlimb (data not shown) or between hypertensive and wildtype mice (left plantar: 1375 vs 1397, left adductor: 296 vs 336 perfusion units; $P>0.9$). We used distal FAL to produce moderate hindlimb ischemia thus minimal tissue damage. This consisted of cyanosis in one or more toes, with occasional nail loss in these B6 and B6;129SvEv strains that have abundant native collaterals.[7,8] Hypertensive mice had worse scores for ischemic appearance and limb use one day after FAL (Figure 2C), a finding consistent with collateral rarefaction.

Progressive collateral rarefaction occurs with increasing duration and severity of hypertension and leads to more severe stroke

The above data suggest that renin-dependent hypertension causes lower collateral conductance, possibly due to collateral rarefaction. This is supported by the fact that higher arterial pressure in the hypertensive groups would be expected to increase rather than decrease collateral-dependent perfusion immediately after FAL if native collateral conductance was unaffected. However, the possibility of greater resistance above or below the collateral network in the RTG mice (eg, from increased angiotensin-dependent smooth muscle contraction) complicates this interpretation. Thus, to determine if hypertension causes rarefaction of native collaterals we measured their number and average diameter (at maximal dilation) in a different tissue. Because the complex three-dimensional orientation of the vascular trees in the hindlimb requires use of x-ray or CT angiography which have limited resolution and ability to distinguish collaterals from arteries and arterioles in terms of number and diameter,[9] we developed an angiography method for use in skeletal muscle of the abdominal wall where the vascular trees are arranged in near-2-dimension. We first confirmed the reliability of the method in BALB/cByJ and B6 strains, which have wide differences in native collateral extent in multiple tissues (eg, brain, hindlimb and intestine)[7] (Online figure 1). We then used it in 8 months-old RTG^{+/−} mice, finding reduced collateral number and diameter (Figure 3A, B). Similar results were obtained in brain (Figure 3), consistent with our previous studies showing that native collateral extent in one tissue reflects extent in other tissues of the same individual.[7–9] Analysis at additional ages showed that loss of collateral number began between 4 and 8 months-age and was slightly greater by 12 months-age (Figure 4A), whereas loss of diameter occurred earlier (16% decrease at 4 months-age; Figure 4B). Thus, exposure to even 4 months of relatively moderate hypertension caused collateral rarefaction. Aging-induced loss of collateral diameter also preceded loss of collateral number.[8]

We next examined homozygous RTG mice to determine if more severe hypertension causes greater rarefaction. Compared to 4 months-old heterozygotes, 4 months-old RTG^{+/+} had greater reduction in collateral number and diameter (Figure 4B). To confirm that hypertension-induced rarefaction has a functional effect, we examined the severity of infarct

volume 72h after MCA occlusion. Eight months-old RTG$^{+/-}$ had a 1.8-fold increase in infarct volume (Figure 5A). Furthermore, collateral remodeling was reduced in the hypertensive mice by 34% when measured 72h after MCA occlusion (Figure 5B)—the time required for maximal remodeling of pial collaterals in B6 mice.[7]

Genetic-dependent differences in collateral extent in the brain are shared in the hindlimb of the same mouse.[7,9] Therefore, to determine if the above pial collateral rarefaction of 4 months-old RTG$^{+/+}$ extends to hindlimb, we performed acute FAL as a functional test of collateral extent, since we found, above, that collateral extent in skeletal muscle of the abdominal wall correlated with flow immediately after hindlimb ligation in the above 8 months-old mice. The reduction in perfusion immediately after FAL was greater in the RTG$^{+/+}$ compared to RTG$^{+/-}$ mice (Figure 4C).

Chronic L-NAME causes collateral rarefaction independent of increased arterial pressure

A previous study found that collateral rarefaction occurred in brain and hindlimb of 3 months-old B6 mice with genetic deletion of eNOS, while collaterogenesis in the embryo/ neonate was unaffected, leading to the suggestion that eNOS-derived NO is a "maintenance factor" for the native collateral circulation.[9] However, whether rarefaction was caused by the mild hypertension also evident in eNOS$^{-/-}$ mice or from other consequences of eNOS deficiency was not evaluated. To answer this question and also determine if combined deficiency of eNOS, nNOS and iNOS causes greater collateral rarefaction (ie, if other NOS isoforms contribute to maintenance of native collaterals), B6 mice were treated with L-NAME ± hydralazine (3.0 and 0.2 mg/ml, respectively, in drinking water) starting at 6 weeks-age. Previous studies using these doses have shown that L-NAME causes a ~20 mmHg increase in arterial pressure that is reduced by hydralazine[25]—findings that were confirmed herein (Online Table 1). At 8 months-age, plantar and adductor perfusion both fell to lower values immediately after FAL in the L-NAME group compared to control mice, and limb use and ischemic appearance were worse when measured the next day (Figure 2D–F). Collateral number and diameter were reduced in brain and abdominal wall (Figure 3C, D). Recovery of plantar and adductor perfusion and hindlimb use were strongly impaired in L-NAME treated mice. The above changes are similar in magnitude to those reported for 3 months-old eNOS$^{-/-}$ mice,[9] suggesting that the presence or absence of nNOS and iNOS does not influence native collateral extent or remodeling. Co-administration of hydralazine reduced the hypertension but had no significant effect on the above measurements, except for lessening ischemic appearance score. These data strengthen previous evidence that eNOS-derived NO is required to maintain native collaterals in tissues and permit full collateral remodeling after arterial obstruction.[9] They also do not support a role for nNOS and iNOS in these processes. In addition, they demonstrate that deficiency in eNOS causes collateral rarefaction in brain and skeletal muscle that is independent of the accompanying hypertension.

Increased cell proliferation and tortuosity in collaterals of hypertensive mice

How might deficient eNOS-NO/endothelial dysfunction cause collateral rarefaction? Collaterals form late in gestation and have no tortuosity at birth.[32] However, this hallmark of collaterals in adult tissues rapidly develops over the next 3 weeks, followed by a slower

increase through 3 months-age that peaks at 16 months.[8, 32] Beginning at 16 months, collaterals evidence a loss of diameter that is not seen in distal-most arterioles.[8] This is accompanied by a progressive decline in tortuosity at 24 and 31 months. Since the distance between the crowns of adjacent arterial trees (ie, the width of the collateral zone) does not decrease between birth and advanced aging,[8, 32] this biphasic pattern of change in collateral diameter and tortuosity prompted speculation[8] that collateral endothelial cells have a higher proliferation rate than arterioles of similar diameter, hence their progressively increased tortuosity from birth through middle age (16 months). And furthermore, that this increase in proliferation favors proliferative senescence of collateral endothelial cells with additional aging, leading to loss of collaterals and a decline in diameter and tortuosity in those that are still present at advanced age.[8]

Given that RTG mice evidence rarefaction at a much younger age (Figures 3,4), we sought to determine if this is accompanied by increased tortuosity since this is suggested by the above findings to be a surrogate for endothelial proliferation. Compared to wildtype mice, collateral tortuosity increased 24% more in 4 months-old, 56% more in 8 months-old, and 24% more in 12 months-old RTG^{+/−} mice (Figure 6A). This biphasic pattern recapitulates, at a much earlier age, the pattern seen in mice with advanced age,[8] suggesting that collateral rarefaction in RTG and aged mice may share common mechanisms and that elevated angiotensin and/or hypertension accelerates the process. To determine directly if the increase in tortuosity in hypertensive mice is accompanied by increased proliferation, EdU was infused by minipump for 28 days before sacrifice. Increased proliferation of cells in the collateral wall with location and nuclear orientation characteristic of endothelial cells was observed in 8 months-old RTG^{+/−} mice, with a similar trend seen at 4 months (Figure 6B). These and the tortuosity findings suggest that collateral rarefaction, evident in relatively young mice with renin-dependent hypertension, may be caused by an increase in the already-accelerated proliferative senescence of collateral endothelial cells that accompanies natural aging. The small number of proliferating cells observed in non-vascular cells of the pia mater (Figure 7), which could include fibroblasts and/or immune cells of peripheral or central origin, did not differ between wild-type and hypertensive RTG^{+/−} mice (0.24 ± 0.03 vs 0.20 ± 0.05 cells/100 μm^2, $p = 0.47$, $n = 6$ of each strain, randomly selected fields at or within the vicinity of the collateral zone). Hazama et al[33] reported that young spontaneously hypertensive rats (which are more hypertensive than the mice studied herein, had, compared to normotensive controls, increased proliferating cells in the watershed zones [where collaterals reside] that they described as endothelial, smooth muscle and adventitial cells within the walls of pial and intracerebral arterioles, as well as glial and arachnoid cells in the surrounding pia. However, the authors did not make clear whether the pial arterioles were collaterals, nor was it evident which cell types had significantly increased proliferation.

Obesity, hyperlipidemia, diabetes mellitus and metabolic syndrome also cause collateral rarefaction

We examined other cardiovascular risk factors in addition to hypertension. All mouse models were on the B6 background and 8 months-age except metabolic syndrome (6-months age) (Online Table 1). Responses to FAL in obese mice (*Lep^{ob/ob}*) were equivalent to wildtype, as were native pial collateral number; however collateral diameter was reduced

(Figure 7A). Mice with hyperlipidemia and moderate obesity (*Ldlr*$^{-/-}$ fed a western diet) tended to have lower perfusion immediately after FAL (*P*=0.05), consistent with lower collateral extent, and lower recovery of perfusion thereafter (*P*=0.06), and fewer native pial collaterals (*P*=0.06) (Figure 7B). Mildly hyperlipidemic mice (*Apoe*$^{-/-}$ fed a normal diet) had lower perfusion immediately after FAL (Online figure 2). Mice with mild hypertension (~10 mmHg increase) and hyperlipidemia (*Pparg*$^{L/+}$;*Apoe*$^{-/-}$ fed a normal diet) had lower perfusion immediately after FAL and fewer pial collaterals (Online figure 2). Responses to FAL in mice with Type 1 diabetes (*Ins2*^{Akita}) were consistent with rarefaction of native collaterals in hindlimb; however, only pial collateral number (not diameter) trended lower (*P*=0.07) (Figure 7C). Since diabetes is well-known to induce endothelial dysfunction and impaired flow-mediated dilation,[34] the latter could also contribute to the hindlimb findings. Consistent with this, we found a near-complete loss of NO-dependent flow-mediated dilation of the feed arteries that supply the prominent collaterals in the gracilis muscles (Online figure 3). Mice with metabolic syndrome (*Lep*$^{ob/ob}$;*Ldlr*$^{-/-}$) that have Type 2 diabetes, hypertension, moderate obesity, and hyperlipidemia (Online Table 1) had pronounced reduction in perfusion following FAL both acutely and over 21-days, and reduced number and diameter of pial collaterals (Figure 7D). Although the trends toward fewer pial collaterals in 8 months-old Akita mice did not become significant at 11–12 months-age, they did when both ages were combined and also almost did-so in the *Ldlr*$^{-/-}$ mice (Online figure 4). Measurements of hindlimb use and ischemic appearance support the above findings for these models of CVRF presence (Online figure 5).

Neither pitavastatin nor exercise training prevented collateral rarefaction in hypertension

The chronic presence of the above cardiovascular risk factors in humans and animal models, including the above, are known to associate with deficiencies in endothelial function and eNOS-NO[29]. Statins reduce endothelial dysfunction and increase eNOS-NO activity/bioavailability, among other actions.[35] Since the above and our previous data in aging and eNOS$^{-/-}$ mice implicate eNOS deficiency/endothelial dysfunction in rarefaction of collaterals,[8,9] we asked whether chronic statin treatment from 1.5 through 8 months-age could prevent rarefaction in RTG$^{+/-}$ hypertensive mice. However, neither the deficits following FAL nor pial collateral rarefaction were affected by pitavastatin (Online figure 6). Chronic statin treatment did not reduce arterial pressure in the RTG$^{+/-}$ mice but did in the normotensive vehicle-treated controls (Online figure 7).

Exercise training is well known to lessen endothelial dysfunction and impaired eNOS-NO activity/bioavailability in hypertension and aging.[36] We therefore examined whether chronic daily swimming from 2 through 8 months-age would prevent collateral rarefaction in RTG$^{+/-}$ mice. However, like pitavastatin treatment, exercise did not prevent the deficits in perfusion following FAL nor rarefaction of pial collaterals (Online figure 8). This was despite a training regimen sufficient to cause modest resting bradycardia, reduction in arterial pressure, and skeletal muscle fiber type switching (Online figures 9, 10).

Although rarefaction of native collaterals in brain and indices of it in hindlimb (flow immediately after FAL) were not lessened by chronic statin treatment or exercise training, hindlimb use and ischemic appearance were improved by both treatments (Online figure 11).

This was true for both wildtype and hypertensive mice. Consistent with this, recovery of hindlimb flow trended higher with pitavastatin treatment (however this was not the case with exercise training) (Online figures 5,7). This may result—in the case of exercise training—from its effect to increase capillary density and improve oxygen utilization (eg, increased mitochondrial volume/fiber length),[37] and—in the case of statin treatment—to increase Sirt-1 and eNOS/NO thus reduce inflammation and leukocyte/platelet adhesion.[18] These effects favor increased capillary flow and capillary surface area receiving flow, which can both improve tissue oxygenation without a significant change in overall blood flow to a limb or organ.

No significant differences were observed in male and female mice in any of the above experiments (~equal numbers were studied, except for Type 1 diabetic mice that were all male).

Trend toward collateral rarefaction and increased infarct volume with exposure to cigarette smoke

Six months of exposure to cigarette smoke caused a trend toward loss of collaterals and increased infarct volume after middle cerebral artery occlusion (Online figure 12). Absence of a significant effect may reflect the need for a larger sample size and/or that humans generally smoke for a longer time than 6 months before encountering smoking-related health problems, although 6 months amounts to 17–20% of the C57BL/6 mouse lifetime of 30–36 months.

Discussion

In the present study, hypertension caused rarefaction whose severity increased with the level and duration of hypertension over 4-to-12 months-age. This resulted in more severe stroke and hindlimb injury after acute arterial occlusion. Hypertensive rarefaction occurred more rapidly than with aging alone, ie, diameter and number had declined significantly by 4 and 8 months-age in moderately hypertensive RTG$^{+/-}$ mice, respectively, whereas significant loss of diameter and number were not seen until 16 and 24 months-age, respectively, with aging alone.[8] These latter ages in B6 mice are ~equivalent to 50 and 67 years of age in humans. This "premature" rarefaction that occurred with even moderate hypertension and duration (8 months-old RTG$^{+/-}$ mice) was accompanied by—in the collaterals that had not been lost—a decrease in expression of markers of eNOS-derived NO activity/bioavailability, an increase in rate of endothelial cell proliferation, and a loss of diameter and tortuosity (length) which is a hallmark of collaterals.[8, 32] Chronically increased proliferation rate, when accompanied by a decline in diameter and length, suggest the possibility of accelerated senescence thus progressive loss of collateral wall cells. These findings support a previously proposed hypothesis[8,9] that endothelial dysfunction caused by natural aging, hypertension and other CVRFs results in reduced NO-mediated inhibition of proliferation, oxidative stress, inflammation, and thus increased aging of collateral endothelial cells leading to apoptosis and loss of length, diameter and ultimately number. Consistent with this hypothesis and our conclusion reached in a previous study of eNOS$^{-/-}$ mice that eNOS-NO is required to maintain collateral number and diameter during aging,[9] chronic treatment with L-NAME

from 1.5-to-8 months-age caused collateral rarefaction and greater cerebral infarct volume and hindlimb ischemia. It is also noteworthy that the severity of rarefaction and hindlimb ischemia, as well as deficit in collateral remodeling and recovery of perfusion caused by L-NAME treatment, were similar to that reported in eNOS$^{-/-}$ mice.[9] This suggests that other NOS isoforms do not participate in maintaining native collaterals or in their remodeling in occlusive disease, nor do these isoforms compensate in these activities when eNOS is deficient.

Comparable levels of hypertension (20–22 mmHg increase) in 8 months-old RTG$^{+/-}$ and L-NAME-treated mice were accompanied by similar levels of collateral rarefaction. L-NAME-induced rarefaction was not reduced when hypertension was prevented by simultaneous administration of hydralazine (arterial pressure remained 7 mmHg above baseline; non-significant). We thus postulate that reduced NO but not the mild hypertension in this model, causes collateral rarefaction. This is supported by our finding that well-characterized models of metabolic syndrome, hypercholesterolemia, diabetes mellitus, and to a lesser degree, obesity, ie, CVRFs that have in common reduced eNOS-NO but not hypertension, were also accompanied by collateral rarefaction. It is also noteworthy that inhibition of NO production, either genetically or by L-NAME, causes upregulation of the renin-angiotensin system (RAS).[38, 39] This plus evidence that chronic increase in angiotensin causes endothelial dysfunction independent of angiotensin's direct vasoconstrictor and hypertensive effects[40] may explain the above results in the RTG and L-NAME±hydralazine experiments. However, since a greater level of hypertension and thus, potentially, collateral wall stress could itself cause rarefaction, it remains to be determined if a normal-renin model of hypertension also causes collateral rarefaction. In support of this, endothelial dysfunction accompanies essential hypertension in humans.[41] And indirect evidence consistent with reduced collateral extent has been reported in the brain and heart of individuals with essential hypertension (discussed below).[42, 43] Interestingly, reduced capillary density in skin and brain has been reported in human essential hypertension,[44, 45] as well as in skeletal muscle,[46] intestinal mesentery,[47] skin of spontaneously hypertensive rats,[48] and brain of renal hypertensive rats.[49] Reduction in arteriole density was also observed in the rat studies, although collateral vessels were not assessed. Spontaneously hypertensive rats have larger infarctions following MCA occlusion and less collateral-dependent blood flow than their normotensive counterparts;[50] however, dysregulated vascular tone and limited dilatory reserve were theorized as the responsible mechanism in these early studies. Interestingly, both pressure-dependent and independent effects of angiotensin have been linked to microvascular rarefaction[51, 52] that is associated with increased endothelial cell apoptosis and oxidative stress.[53]

In 8 week-old eNOS$^{-/-}$ mice whose arterial pressure under pentobarbital anesthesia was not significantly elevated, capillary and arteriole densities in cardiac and skeletal muscles were not different.[54] By 12 months-age, arteriolar densities were 15% lower, an effect prevented by hydralazine. These findings suggest that hypertension caused by eNOS deficiency results in arteriolar rarefaction. However, collateral vessels, which have many properties unique from arterioles,[55] were not examined in the above study. The purpose of our study did not include studying the effect and mechanisms by which certain CVRFs reduce collateral remodeling after arterial ligation, because many reports have addressed this topic.[12–14] We

used the hindlimb ischemia model to determine blood flow immediately after ligation, as a functional index of CVRF-induced rarefaction of the pre-existing collateral circulation in the hindlimb. Our purpose was to determine if the anatomic rarefaction that we measured directly in the brain and skeletal muscle of the abdominal wall might also occur in hindlimb. These flow values immediately after ligation, which are important to the purpose of the paper, are shown in the bar graphs of Figure 1A, B, D, E and Figure 7A–D, as well as several of the online figures. We measured hindlimb flow over 21 days because recovery of flow is determined by both collateral extent before ligation and collateral remodeling thereafter. The data in these figures thus add functional significance to our findings of CVRF-induced collateral rarefaction in brain and abdominal wall. Parenthetically, the time-course data also provide confirmation of impaired recovery of flow in the presence of certain CVRFs reported by others, and provide additional data about recovery in CVRF models not reported elsewhere. It was previously shown[56] in eNOS$^{-/-}$ mice that hindlimb flow after FAL was only decreased until day-7, suggesting delayed collateral growth after FAL. However, histology found no differences in collateral diameter in eNOS$^{-/-}$, wildtype and eNOS-transgenic mice at 1 and 3 weeks. Administration of an NO donor induced vasodilation in eNOS$^{-/-}$ but not wildtype mice, suggesting impaired dilation of collaterals and/or the vascular beds upstream or downstream of the collateral network, since collateral vasoactivity was not directly measured. This study demonstrated that eNOS activity is crucial for NO-mediated vasodilation of peripheral vessels in the setting of arterial occlusion.

Recent studies in humans without obstructive disease, ie, wherein the confounding effects of differences in collateral remodeling on estimates of native collateral extent are avoided, have reported findings consistent with our results. In individuals lacking identifiable CAD, the presence of hypertension associated with (p<0.006) low collateral flow index (CFI) on multivariate analysis, while no significant association was found for other classic CVRFs including diabetes and obesity (p=0.07 for age).[42] Since collateral extent can only be determined postmortem in humans, CFI remains the best indicator, albeit indirect, of conductance of the collateral network and thus collateral extent. Likewise, a similar index— collateral score measured as the extent of retrograde filling of the occluded tree on neuroimaging of patients with acute ischemic stroke—was associated when low with age, hypertension, metabolic syndrome, and higher plasma glucose, D-dimer and uric acid, but not with other classic CVRFs.[43] On multivariate analysis, only age, metabolic syndrome and hyperuricemia remained independent predictors of poor pial collateral score. While studies in mice benefit from the ability to hold genetic and environmental factors constant, larger patient samples will be required to address whether congruence exists between humans and our findings in mice.

Statin therapy is associated with improvements in endothelial function, and newer agents such as pitavastatin have shown promise in promoting eNOS activity/signaling,[18, 57] anti-senescence factors,[18, 58] and improved collateral-dependent perfusion in CAD[59] and acute ischemic stroke.[60] Because our current and previous results[8,9] implicate decreased eNOS activity/vascular senescence in CVRF-associated collateral rarefaction, we tested pitavastatin administration in hypertensive mice from 1.5-to-8 months-age. However, no inhibition of rarefaction was observed in brain or hindlimb. Likewise, no inhibition was seen

with chronic exercise training over the same duration. This was despite changes indicative of training-induced cardiovascular conditioning in hypertensive and normotensive mice (resting bradycardia and muscle fiber-type switching) that have been reported in similar swim-training programs.[61] These negative results were unexpected, since exercise training increases eNOS activity in arteries and arterioles[20, 62] and decreased senescence of vascular wall cells.[21] In addition, 2.5 months of swim-training reduced arterial pressure and heart rate, caused fiber-type switching, restored eNOS activity and prevented capillary rarefaction in spontaneously hypertensive rats.[61] Since the training effects we observed were small, it remains possible that a more vigorous training program, or its testing in the presence of renin-independent hypertension or a different CVRF, would find that exercise training lessens or prevents collateral rarefaction.

We were also surprised that recovery of flow after FAL, which is dependent on both native collateral extent prior to obstruction and subsequent remodeling of collateral diameter, failed to improve in normotensive and hypertensive groups following exercise-training, since better recovery has been reported in several studies. Yang et al. found in rats that femoral artery ligation, followed by 4 weeks of treadmill training, increased collateral-dependent flow more than with bFGF alone.[63] They also found that L-NAME abolished the improvement in recovery with exercise. However, microvascular density was not affected, suggesting that increasing NO with exercise training may predominantly occur in larger vessels thus improve eNOS-dependent dilation.[64] Interestingly, collateral-dependent perfusion is partly dependent on flow-mediated dilation upstream of the collateral network.[9] Furthermore, increased NO-dependent collateral conductance following acute arterial obstruction was seen whether the exercise regimen was begun before or after the occlusive event.[65] It is important to note, however, that previous studies have not quantified native collateral extent in exercised animals in the presence or absence of CVRFs. Endurance training augmented coronary CFI in a healthy individual[66] and in normal coronary artery trees of patients with CAD elsewhere in the heart.[67] However, the short 3–6 month duration of training suggests these findings may result from improved outward remodeling of existing collaterals rather than inhibition of age- or CVRF-induced rarefaction. Prior exercise training is associated with improved outcome and reduced infarct size in the setting of stroke.[68] While the coronary effects might reflect improved remodeling in collaterals already recruited by CAD, whether the latter results in stroke reflect improved remodeling, less rarefaction, better autoregulation of blood flow within trees adjacent to the occluded territory or enhanced neuroprotective mechanisms were not addressed. Our findings suggest that the beneficial effects of exercise in the above studies may reflect greater outward remodeling of collaterals and angiogenesis in the downstream tissues, rather than from preservation of native collaterals, although metabolic changes could also contribute. A stronger exercise regime remains to be tested for inhibition of collateral rarefaction.

While the focus in the present study was on rarefaction of the native collateral circulation, we also examined recovery of perfusion after femoral artery ligation, which is dependent on both native extent and collateral remodeling. Diabetes, hypertension, metabolic syndrome and L-NAME reduced recovery, whereas obesity and hyperlipidemia did not. Although we only measured remodeling in the hypertensive model (where it was reduced, Figure 5B), impaired recovery after arterial obstruction has been well-documented in animal models of

diabetes mellitus,[69] hypertension,[70] obesity,[71] metabolic syndrome,[72] and hyperlipidemia.[73] Similarly, human studies have also reported reduced collateral score/status in patients with diabetes mellitus,[42, 43, 66] hypertension,[43, 74, 75] and obesity/metabolic syndrome/ hyperlipidemia.[43, 76] As with the exercise studies discussed above, whether these observations reflect rarefaction of native collaterals, impaired remodeling or other mechanisms is not known. However, our finding of reduced remodeling in hypertensive mice is in agreement with two recent studies.[77, 78]

Hyperlipidemic and obese mice did not show any significant deficit in recovery of perfusion. In contrast, van Weel et al. found that hyperlipidemic mice with the Apo3*Leiden mutation and altered diet showed strong impairment in recovery after FAL, accompanied by reduced collateral remodeling.[79] This disagreement might be explained by a predominance of low-density lipoprotein in $Ldlr^{-/-}$ versus greater very low-density lipoprotein in the Apo3*Leiden mice. Interestingly, mice with metabolic syndrome, which was produced by a combined deletion of $Ldlr$ and $Leptin$ (ie, 2KO) showed collateral rarefaction and strong impairment in recovery from hindlimb ischemia. In addition to obesity and hyperlipidemia, the 2KO mouse is characterized by high fasting blood glucose and insulin resistance.[80] Yan et al. recently reported that Type 2 diabetic/insulin resistant mice have more pronounced impairment in recovery from hindlimb ischemia than Type 1 diabetic mice.[81] They also found smaller baseline collateral diameters in the hindlimb, similar to our finding among pial collaterals in 2KO mice.

Findings in our 2KO genetic model of metabolic syndrome might be viewed at variance with two previous studies. Pial collateral number was increased in 10 week-old GK rats that develop a type of lean Type 2 diabetes,[10] while in the present study pial collateral number (and diameter and collateral-dependent flow immediately after ligation in the hindlimb) were decreased in the 2KO mice. This contrast might arise from the different pathophysiology of the two disease models plus unknown genetic variants present in the GK rat. Likewise, in 4–5 month-old $Lepr^{db/db}$ mice (leptin receptor deficient mice on the same background as the $Leptin^{ob/ob}$ leptin-deficient mice studied herein) that were studied as a model of Type 2 diabetes, no differences were found in pial collateral number.[11] However, these data are similar to our findings in 8 months-old $Leptin^{ob/ob}$ obese mice that, however, have almost twice the body weight and half the blood glucose as in the former study. It is likely that differences in age and phenotype/pathophysiology contribute to these discrepancies.

The above previous and current studies show that different CVRFs have variable effects on inhibiting recovery of perfusion, collateral remodeling, and collateral rarefaction. Those risk factors associated with the most severe rarefaction also have the most severe perfusion deficit and tissue injury following acute arterial obstruction. This is in agreement with our previous studies in mice, as well has studies by others in humans, showing the importance of variation in abundance of the native pial collateral circulation in determining outcome after acute ischemic stroke.

Why are collaterals so susceptible to rarefaction? A consideration of the unique hemodynamic environment in which collaterals reside offers a potential clue. In the absence

of obstruction, flow and thus shear stress in the central-most segment of collaterals that cross-connect the crowns of adjacent arterial trees is very low and disturbed, ie, it slowly oscillates in direction, resulting in little or no net flow—at least in the brain and skeletal muscle of the anesthetized mouse.[32] Since the kinetic energies of the colliding flows are converted to potential energy, circumferential wall stress is increased. In addition, these low-flow conditions favor oxygen content in the lumen of collaterals to be low and toward equilibration with tissue PO_2 due to diffusion to surrounding tissue. It is well known that chronic low and disturbed shear stress, high wall stress, and low oxygen favor endothelial dysfunction, oxidative stress, inflammation, cell proliferation and thus athero- and arteriosclerosis in larger arteries (ie, an accelerated aging-like phenotype),[82] whereas in the microcirculation, collateral-like intra-tree arteriole anastomoses that can appear transiently during development are predominantly pruned away in most but not all tissues, eg, intestinal mesentery.[32,83] By contrast, in healthy adult tissue, collaterals persist even though they reside in this "at-risk environment." However, we postulate that this makes them particularly vulnerable, much like "canaries in a mine shaft", to further stresses to the vascular wall caused by chronic hypertension, hyperglycemia, dyslipidemia, and natural aging. Our finding of markers of endothelial dysfunction (decreased eNOS and phospho-VASP) in collaterals but not in the femoral artery or aorta of mildly hypertensive mice is consistent with this hypothesis. Moreover, under normal conditions endothelial cells are one of the least proliferative cell types, with turnover estimated at   43 days.[84] As such, endothelial cells undergo replicative senescence under conditions of sustained proliferation, which then leads to premature apoptosis.[85,86] As collateral endothelial cells reach there replicative maximum, preferential loss, or rarefaction, of collateral wall cells would be expected. In line with this prediction, pial collateral tortuosity increased significantly between 4 and 8 months-age in the hypertensive mice, followed by progressive collateral rarefaction and gradual decrease in tortuosity thereafter. This recapitulates the sequence observed in our previous study on aged C57Bl/6 mice, in which tortuosity reached a maximum at 16 months-age, followed by incremental collateral rarefaction and decreased tortuosity in the collaterals that remained present.[8]

In conclusion, the present study demonstrates that cardiovascular risk factors when present beginning at a young age cause—compared to aging alone[8]—"premature" rarefaction of the native collateral circulation, resulting in greater tissue injury in ischemia. Hypertension and metabolic syndrome caused the most severe rarefaction. If risk factor-induced rarefaction also occurs in humans as indirect findings suggest,[42, 43, 66, 74–76] loss of these natural bypass vessels, combined with the elevated risk of atherosclerosis and increased propensity for thrombosis and peripheral vasoconstriction, create a "perfect storm" for poor overall prognosis. These considerations thus add additional weight to the already prodigious rationale for adopting lifestyles and treatments to mitigate modifiable CVRFs. Further elucidation of the mechanisms underlying collateral rarefaction, beyond endothelial dysfunction and proliferative changes as identified herein, will require additional investigation. However, identifying the mechanisms that endow collaterals with their hallmark increased proliferation and tortuosity may lead to insights into how collateral rarefaction might be lessened or prevented.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Non-standard abbreviations and acronyms

Akita	*Ins2*^{Akita} mouse strain with spontaneous mutation of insulin receptor-2 causing destruction of beta cells of pancreas thus diabetes mellitus
B6	C57BL/6J inbred mouse strain
CVRFs	cardiovascular risk factors
EdU	5-ethinyl-2′-deoxyuridine
FAL	femoral artery ligation
L-NAME	L-N^{G}-nitro-arginine methylester
MCA	middle cerebral artery
NO	nitric oxide
PCA	posterior cerebral artery
RTG	renin over-expressing transgenic mouse strain with hypertension
WT	wildtype
2KO	*Lep*$^{ob/ob}$;*Ldlr*$^{-/-}$ "double knockout" (2KO) model of metabolic syndrome

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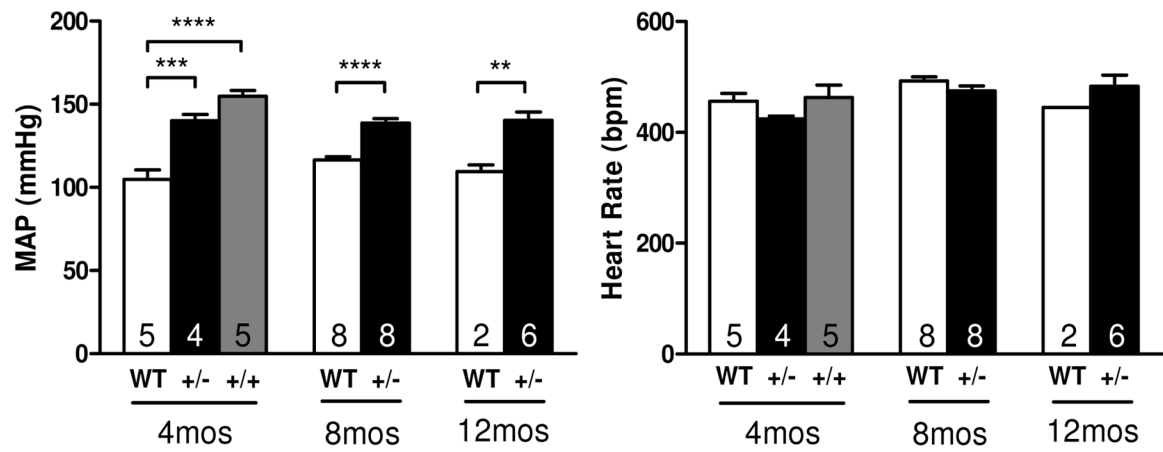
Figure 1. Mean arterial pressure (MAP, via carotid artery, isoflurane anesthesia) and heart rate in wildtype littermates (WT) and renin transgenic (RTG) heterozygous (+/−) and homozygous (+/+) mice at the indicated ages (mos, months)
Values are mean ± SEM, number of animals (n) indicated in columns, *,**,*** p<0.05, 0.01, 0.001 levels of significance in this and subsequent figures.
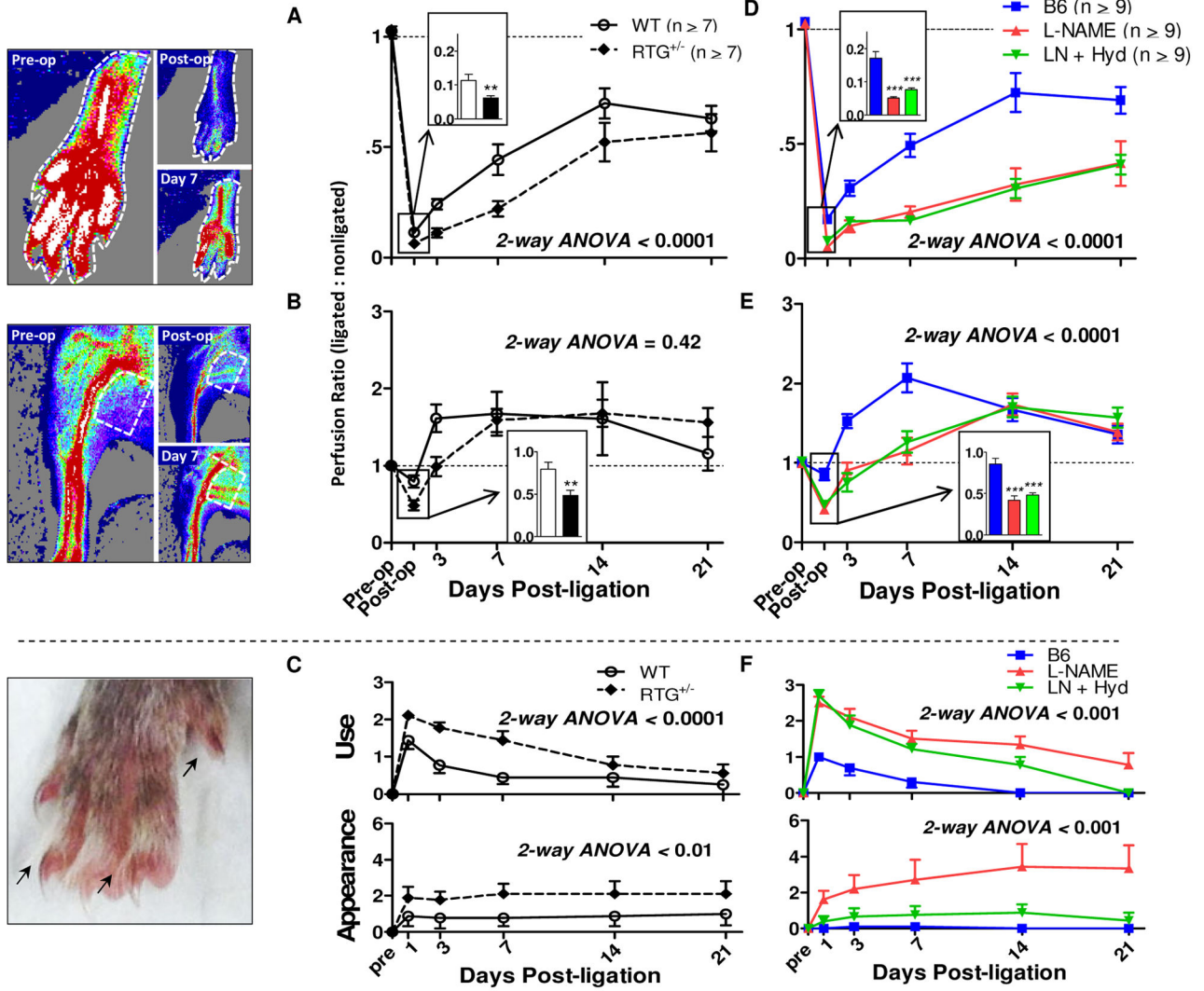
Figure 2. Hypertension causes greater ischemia and impaired recovery of perfusion after femoral artery ligation (FAL)

A, Laser Doppler imaging (LDI) of plantar perfusion (dotted lines identify region of interest in representative figure), which correlates with whole limb perfusion, is reduced in 8 months-old RTG^{+/−} mice immediately after FAL (post-op; inset bar graph), indicating reduced native collateral conductance. Lower recovery of perfusion on subsequent days is consistent with reduced collateral remodeling (confirmed in Figure 5). **B,** Perfusion in RTG^{+/−} adductor region, which contains the major collateral network recruited by FAL (note flow induced after FAL in the two parallel-running collateral pathways in the gracilis muscles), is lower immediately after FAL (inset) and trends higher after day-7, consistent with the conclusions above for panel A. **C,** Ischemic nail-beds in RTG^{+/−} mouse one day after FAL (arrows in representative figure). Hindlimb use-impairment and ischemic appearance scores are greater in RTG^{+/−} mice. **D–F,** Similar results were obtained in 8 months-old C57BL/6 mice treated with L-NAME (LN) for 6.5 months and having similar hypertension as in RTG^{+/−} mice (20 mmHg increase, Online Table 1). Concomitant
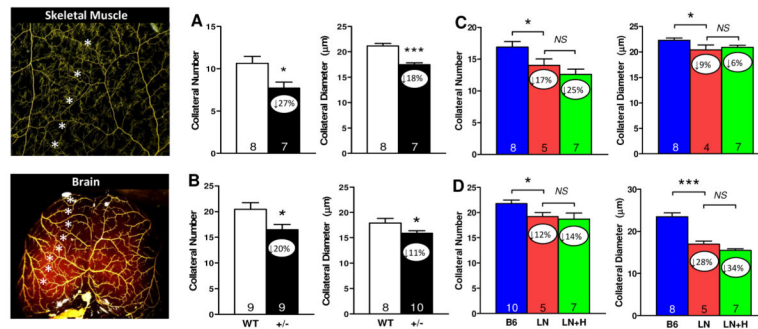
administration of hydralazine (Hyd) normalizes the hypertension (Online Table 1), yet the perfusion deficit persists, suggesting that nitric oxide synthase inhibition rather than hypertension is the primary cause of deficient collateral conductance in L-NAME treated mice.

Figure 3. Chronic hypertension causes collateral rarefaction in skeletal muscle and brain
A, B, mouse groups shown in Figure 2. Eight months-old RTG$^{+/-}$ mice have fewer native collaterals (asterisks) of smaller diameter in abdominal wall skeletal muscle and brain (decreases shown at top of bars are relative to WT, here and in subsequent figures). **C, D,** Similar effects in L-NAME treated mice (LN) are not prevented by concomitant treatment with hydralazine (H). *NS*, non-significant.
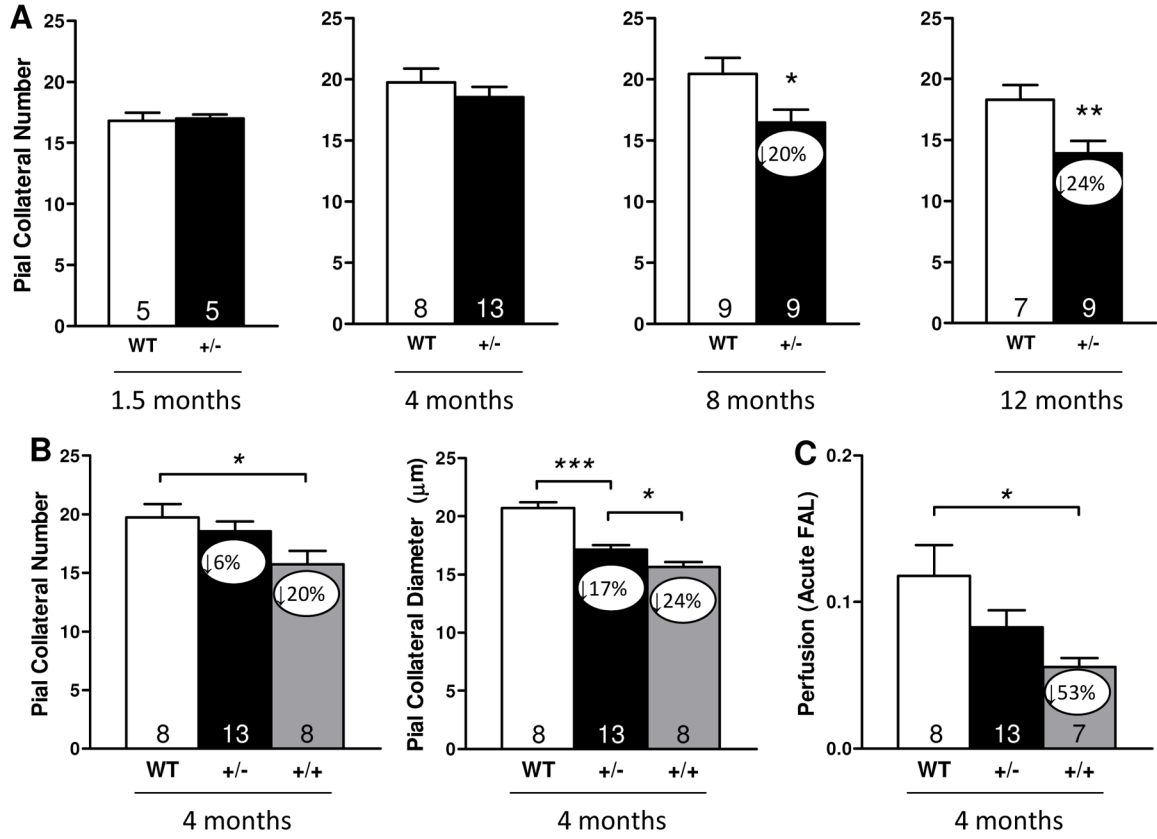
Figure 4. Collateral rarefaction increases with duration and severity of hypertension
A, B, RTG^{+/−} mice are born with the same number of native collaterals as WT (data not shown). By 4 months-age, collateral diameter is reduced; reduction of both number and diameter is evident with longer duration of hypertension. RTG^{+/+} mice with more severe hypertension evidence earlier collateral rarefaction. **C,** Consistent with collateral rarefaction shown in Figure 3 in skeletal muscle, plantar perfusion (by LDI) immediately after FAL in 4 months-old mice is inversely related to the severity of hypertension.
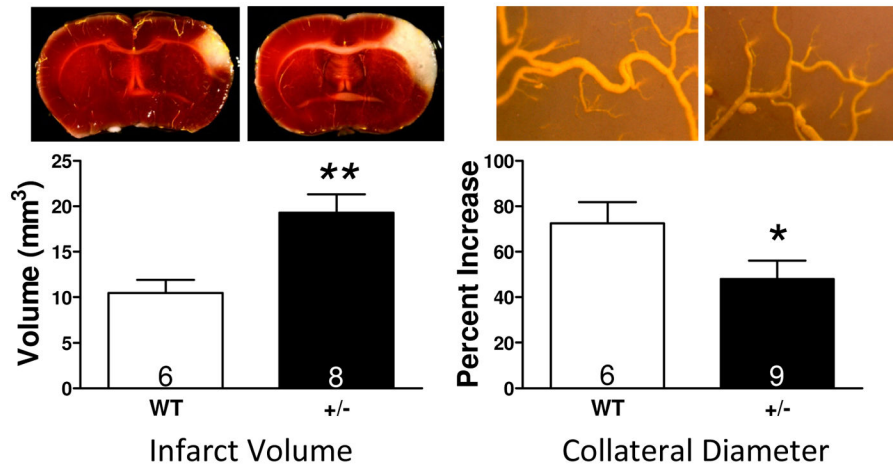
Figure 5. Larger infarct volume and less outward remodeling of collaterals evident 3 days after permanent middle cerebral artery occlusion in 8 months-old RTG^{+/−} hypertensive mice

Remodeling is given as percent increase from baseline for collaterals between MCA and ACA trees (shown in representative figure).
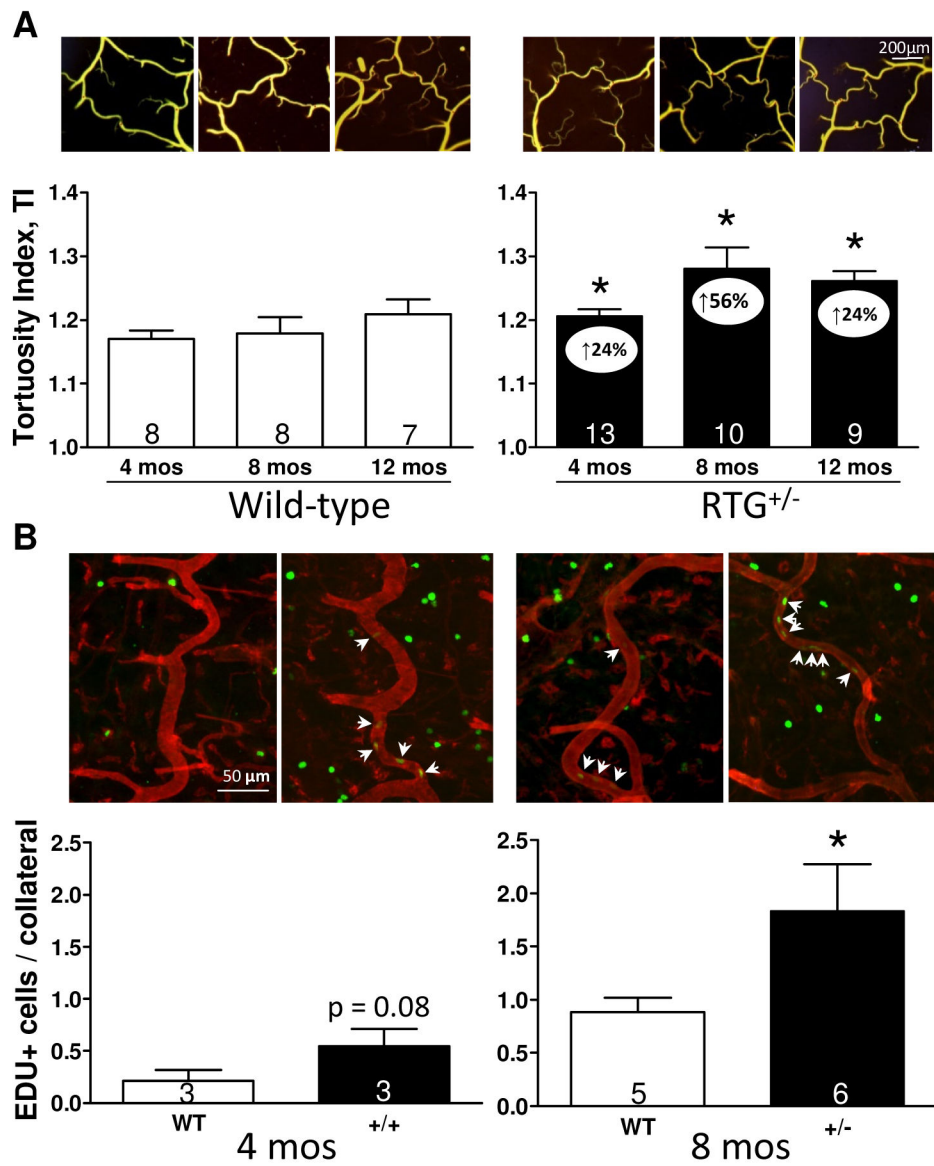
Figure 6. Tortuosity and proliferation in vascular wall cells are greater in native collaterals of hypertensive mice

Tortuosity (**A**) and proliferation of endothelial cells (**B**, arrows, EdU^{+} cells) in collaterals between MCA and ACA trees are increased during times shown in Figure 4 when rarefaction is occurring in RTG hypertensive mice.
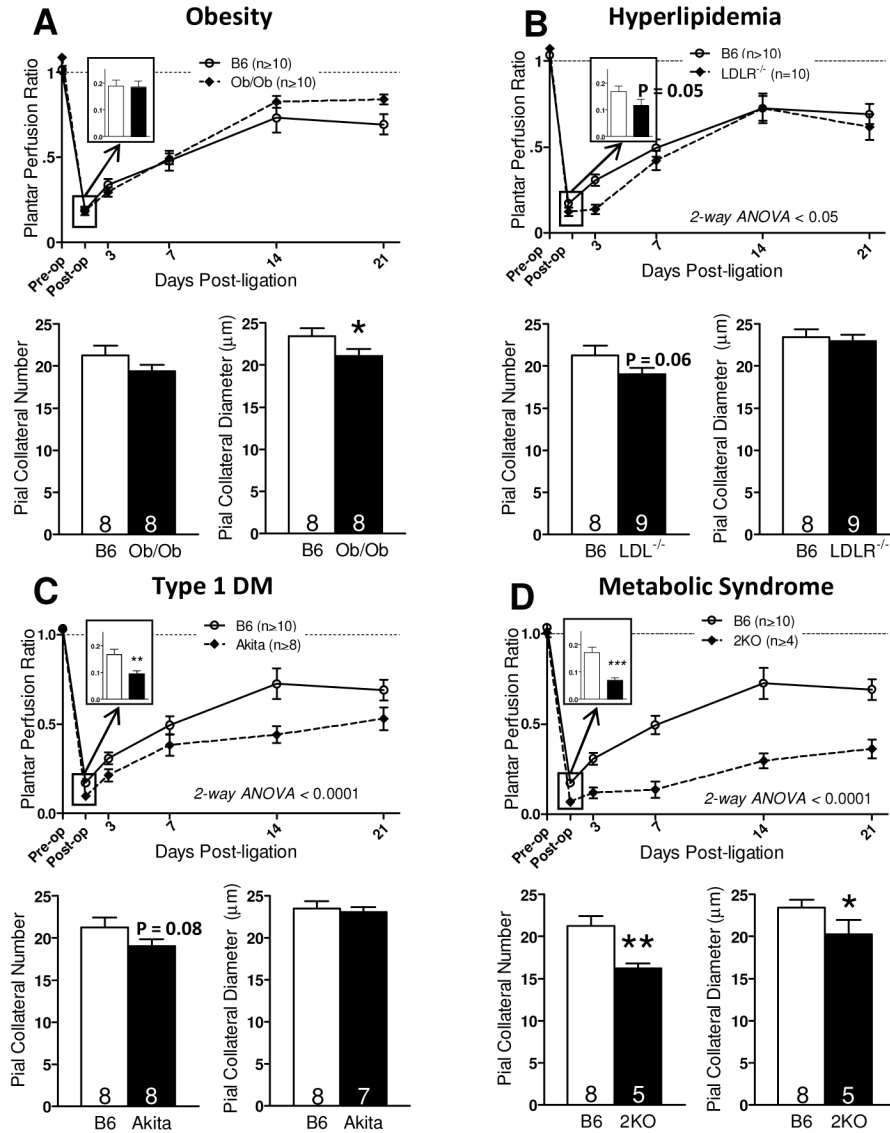
Figure 7. Cardiovascular risk factors in addition to hypertension cause rarefaction of the native collateral circulation
A, Eight months-old obese mice (Ob/Ob) have no deficit in hindlimb perfusion immediately after FAL (post-op, inset) or recovery of perfusion over subsequent 21 days, but have decreased collateral diameter. **B,** Eight months-old hyperlipidemic mice (LDLR$^{-/-}$) have lower perfusion immediately after FAL and reduced recovery, and trend toward fewer collaterals. **C,** Eight months-old mice with Type 1 diabetes mellitus (DM, Akita) have lower perfusion immediately after FAL, impaired recovery, and trend towards fewer collaterals (see Online figure 4 and its legend showing significant rarefaction in a different model of Type 1 diabetes). **D,** Eight months-old mice with metabolic syndrome (2KO) have lower perfusion immediately after FAL, severe impairment in recovery of perfusion, and greater hindlimb use-impairment and ischemic appearance (Online Figure 5), and reduced collateral number and diameter.