Impact of Mouse Strain Differences in Innate Hindlimb Collateral Vasculature

Armin Helisch, Shawn Wagner, Nadeem Khan, Mary Drinane, Swen Wolfram, Matthias Heil, Tibor Ziegelhoeffer, Ulrike Brandt, Justin D. Pearlman, Harold M. Swartz, Wolfgang Schaper

Objective—To assess the importance of genetic background for collateral artery development.

Methods and Results—C57BL/6, BALB/c and 129S2/Sv mice were studied after femoral artery ligation by laser Doppler imaging, visible light oximetry, time-of-flight–magnetic resonance imaging, and treadmill testing; C57BL/6 and BALB/c also underwent electron paramagnetic resonance (EPR) oximetry, x-ray angiography, and histology. C57BL/6 had the least initial distal ischemia and most complete recovery. BALB/c had the most severe initial ischemia and poorest recovery. BALB/c had some vasodilatory reserve in their ligated limbs not seen in the other strains at 3 weeks. By in vivo TOF-magnetic resonance angiography, C57BL/6 had larger preexistent and developed collaterals. By x-ray angiography, C57BL/6 had a higher collateral-dependent filling score and number of visible collaterals immediately after femoral ligation and a higher number of visible collaterals at 1 week but not at 4 weeks. EPR oximetry and histology revealed hypoxia and tissue damage in regions of collateral growth of BALB/c but not C57BL/6 mice. In C57BL/6 BrdUrd uptake in the thigh was limited to larger vessels and isolated perivascular cells. Proliferative activity in collateral arterioles was similar in both strains.

Conclusions—Genetic differences in preexistent collateral vasculature can profoundly affect outcome and milieu for compensatory collateral artery growth after femoral artery occlusion. (Arterioscler Thromb Vasc Biol. 2006;26:520-526.)

Key Words: angiogenesis ■ collateral circulation ■ hypoxia ■ mouse strains ■ vascular biology

Pre-existent interarterial anastomoses have been identified in the coronary, cerebral, and peripheral circulation of various species.1 The number and size of these anastomoses varies between species and tissues, resulting in different degrees of protection after arterial occlusion.1 Pre-existing collateral arterial connections have been found in human hearts without evidence for coronary artery disease.1-3 Re-modeling of preexistent arterioles into mature collateral arteries has been observed in dog hearts,4 hindlimbs of rabbits,5-7 rats,8 and mice,9 and this process has been suggested to be the dominant mechanism responsible for the restoration of blood flow after arterial occlusion.10-12

Previously, we identified differences in perfusion recovery by laser Doppler imaging (LDI) after femoral artery occlusion in inbred strains of mice and attributed these to marginal differences in growth rates of collateral arterioles.9 However, this study was limited by the fact that LDI and visible light oxygen spectrometry were the only comparative methods of in vivo assessment and histology was confined to isolated gracilis muscle collaterals with minimal surrounding tissue. Diameters of preexistent collaterals on the gracilis muscle were not significantly different between strains.

In this study we examined collateral artery development and related parameters after femoral artery ligation in C57BL/6, 129S2 and BALB/c mice using the most comprehensive approach ever attempted in this model, including magnetic resonance blood flow quantification and angiography, electron paramagnetic resonance (EPR) oximetry, functional testing, and histology on complete cross-sections of medial and caudal thigh muscles. Application of these techniques resulted in new insights about the importance of strain-dependent variations in the innate collateral circulation in this model.

Methods

Animals were handled in accordance with the American Physiological Society guidelines for animal welfare and the Bioethics Committee of the State of Hessen, Germany. We studied 11- to 13-week-old male BALB/c, 129S2/Sv, and C57BL/6 mice from Harlan (Borchen, Germany and Indianapolis, Ind) and Charles River Laboratories (Sulzfeld, Germany). Operations were performed under general anesthesia with intraperitoneal xylazine (25 mg/kg) and ketamine (150 mg/kg). Right (for stress testing both) femoral arteries were ligated (60 silk, Ethicon) just distal to the a. femoris profunda and proximal to the a. genus descendens. Before anesthesia for serial
measurements, spontaneous movement of the right hind limbs was scored as either “0” (dragging of foot), “1” (no dragging but no active plantar flexion), “2” (moderately to severely reduced plantar flexion), or “3” (normal or only mildly abnormal use).

In Vivo Studies

Most serial measurements were performed under general anesthesia with intraperitoneal xylazine (15 mg/kg) and ketamine (85 mg/kg) at 37°C in a temperature controlled chamber or magnetic resonance spectrometer after 5 minutes equilibration time. EPR measurements and magnetic resonance angiograms were performed under isoflurane narcosis.

LDI and Reactive Hyperemia

Mouse feet were scanned with a laser Doppler imager (MLDI 5063; Moor Instruments Ltd, Devon, UK). Transient occlusion of blood flow to the limbs by inflatable cuffs was performed once on all animals to determine mean background flux values for each strain. Background values were subtracted from measured flux values before calculating right-to-left ratios. Three weeks after surgery, reactive hyperemia was assessed after 5 minutes of simultaneous bilateral cuff inflation to 300 mm Hg followed by sudden deflation.

Visible Light Oxygen Tissue Spectrometry

Hemoglobin oxygen saturation in hind feet was measured with a visible light spectrometer (AbTisSpec spectrometer; LEA Medizintechnik GmbH, Germany) using a specially designed circular probe.

Magnetic Resonance Flow Imaging

Blood flow was measured in 3 planes at calf-level in both hindlimbs using a spin labeling inflow sequence with a 7.05 Tesla Bruker PharmaScan horizontal bore magnet equipped with a 300 mT/m gradient system as described.14

Magnetic Resonance Angiography

In the fourth week after ligation, mice magnetic resonance angiography with a field of view of 2.54×2.54×2.54 cm were obtained as described.15 For general anesthesia, mice inhaled 1.6% isoflurane in 20% oxygen and air at 1.2 L/min via nasal cone. Three-dimensional maximum intensity projections viewable from multiple angles were analyzed and scored for collateral arterial vessel size and number by 2 independent blinded observers. The score differentiated “small,” “medium,” and “large” collaterals on a relative basis after the observers had overviewed the range of collateral sizes that occurred. Veins were differentiated from arteries by anatomic location and course.

Exercise Capacity

Exercise treadmill testing was performed on a rodent treadmill (University of Melbourne, Australia) after bilateral arterial ligation to ensure proper running without the possibility of compensation by the contralateral limb. Exercise treadmill testing began 7 days after surgery. Each test started with a 6° slope at a speed of 20 m/min for 15 minutes. Speed was increased by 5 m/min every 15 minutes until the mice were unable to keep pace. The same protocol was used for nonligated control animals.

EPR Oximetry

Tissue \( pO_2 \) was measured using 100 \( \mu \)g of oxygen-sensitive sterile charcoal powder, whose EPR spectra (line width) reflects the \( pO_2 \).16 Injected bilaterally into medial thigh or gastrocnemius muscles of BALB/c and C57BL/6 mice 2 weeks before surgery. Once implanted, the paramagnetic material remains at the site and is quite inert, permitting repeated tissue \( pO_2 \) measurements without invasion. Measurements were performed on a 1.2-GHz (L-band) EPR spectrometer while under inhalation narcosis with 1.25 to 1.5% of isoflurane with 26% oxygen. Right femoral artery ligations and left sided sham ligations were performed on day 0. Tissue \( pO_2 \) was measured serially for 14 to 21 days after surgery.

X-Ray Angiography

Seven or 28 days after right femoral artery occlusion the left femoral artery was ligated and angiograms were obtained (please see http://atvb.ahajournals.org).

Histology

BrdUrd uptake was evaluated on complete cross sections of medial and caudal thigh muscles at the level of the ligation site 7 days after surgery and continuous administration of BrdUrd.

Statistical Analysis

Please see http://atvb.ahajournals.org.

Results

Serial Measurements of Perfusion and Hemoglobin Oxygen Saturation in Hind Feet and Blood Flow at Calf Level

We used several complementary methods to assess collateral-dependent blood flow and related parameters to reduce the chance of experimental artifacts affecting the interpretation of our results and to gain insight into how these parameters relate to each other in this model.

One BALB/c mouse developed severe hindlimb necrosis on day 3 and had to be euthanized and a second one was found dead at day 14, reducing the numbers of animals in that strain available for measurements from 15 to 13 over time. BALB/c mice had the lowest relative LDI perfusion values immediately after femoral artery ligation and their recovery was the least complete (Figure 1A; Figures I and II, available online at http://atvb.ahajournals.org). C57BL/6 and 129S2 mice had higher relative perfusion immediately after surgery. C57BL/6 mice achieved almost full relative perfusion recovery at 14 days after surgery; 129S2 mice had a slower and less complete recovery.

Large vessel blood flow at calf level as measured by MRI immediately after femoral artery ligation was higher in the C57BL/6J and 129S2 groups (Figure 1b; Figure III, available online at http://atvb.ahajournals.org). Thereafter, it completely and rapidly recovered in C57BL/6 mice, whereas 129S2 and BALB/c mice behaved virtually identical reaching a plateau at ~60% of the nonligated side at 7 days.

Oxygen saturation obtained by visible light spectrometry mirrored the LDI findings, however, with faster initial recovery in all strains (Figure 1C).

For these studies animals of the same strain from 2 vendors were used. While there were no significant differences, there was a trend for faster recovery by LDI in C57BL/6J mice from Harlan compared with C57BL/6H mice from Charles River (data not shown). For the remaining studies only animals from Harlan were used.

Reactive Hyperemia

To assess, whether some of the observed differences in collateral-dependent perfusion are caused by differences in vasomotor tone the degree of reactive hyperemia was assessed 22 days after surgery. On the nonligated side, there was a uniform response in all strains with a 20% increase of LDI flux over baseline in the first minute (Figure IV, available online at http://atvb.ahajournals.org). On the ligated side, no hyperemic response occurred in C57BL/6 and 129S2
mice (Figure IVB). However, BALB/c mice, with more severe ischemia by LDI at baseline, had a hyperemic response on the ligated side. Overall, the right-to-left ratios of the maximal flux values on each side mildly decreased in C57BL/6J and 129S2, whereas there was no significant increase in BALB/c mice.

Serial Assessment of Functional Capacity
All mice regained full functional recovery as assessed by active hindlimb movement score. C57BL/6J recovered most rapidly, followed by 129S2 and then BALB/c mice (Figure 1D).

On exercise treadmill testing, nonligated C57BL/6J mice were able to perform a workload of 70.9 ± 3.5 J, 129S2 of 72.9 ± 6.5 J, and BALB/c of 102.1 ± 6.6 J (P < 0.05 versus 129S2 and C57BL/6J). After bilateral femoral artery ligation, C57BL/6J and 129S2 mice recovered their exercise tolerance better than BALB/c mice (Figure 1E).

Serial Cross-Sectional Areas and Final Weights of Distal Hindlimb Musculature
Swelling, as assessed by MRI, only occurred in the ischemic hindlimbs of BALB/c mice 3 days after surgery, followed by a gradual decrease of average cross-sectional areas to lower levels than on the nonligated side consistent with tissue atrophy (Figure 1F; Figure III). Right-to-left ratios of calf muscle weights on day 28 confirmed the MRI findings at this stage: C57BL/6J 0.96 ± 0.05, 129S2 0.89 ± 0.01, BALB/c 0.63 ± 0.03 (P < 0.001 for BALB/c versus both other groups, not significant for C57BL/6J versus 129S2). On the nonligated side, the corresponding muscle weights in C57BL/6J (239 ± 13 mg) and BALB/c (224 ± 5 mg) mice were higher than in 129S2 mice (170 ± 4 mg, P < 0.001).

Magnetic Resonance Angiography Assessment of Collaterals 3 Weeks After Surgery
Collateral vessels bridging the ligation site were identified subcutaneously, as well as in the quadriceps and occasionally more faintly in the medial or caudal muscle compartments of the thigh (Figure 2). Pre-existing collateral vessels were visible on the nonligated side. Two blinded observers identified 1 to 2 “large” quadriceps collateral vessels on the ligated side in 4 or 5 of 5 C57BL/6J mice (mean for observer A 1.0, observer B 1.4), but in none of the other strains (P < 0.05). On the nonligated side 2/5 C57BL/6J mice but none of the other groups had “large” quadriceps collateral.
vessels (by both observers). “Medium” and “large” collateral vessels combined in the quadriceps on the nonligated side were identified by observer A in all (mean 1.0) C57BL/6J mice, 2/5 (0.4) BALB/c and 3/5 (0.6) 129S2 mice (P=not significant). Observer B identified 1 to 2 large collaterals in 4/5 (mean 1.0) C57BL/6J mice, but none of the BALB/c (P<0.05 versus C57BL/6J, Mann-Whitney Rank Sum test), and 3 of 5 129S2 mice (mean 0.8).

Serial EPR Oximetry in Thigh (Region of Growing Collaterals) and Calf

Although there was a severe transient decrease of tissue oxygen tension (pO2) on the ligated side in the medial thigh of BALB/c, there was no evidence for hypoxia in the medial thigh of most C57BL/6 mice (Figure 3; Figure V, available online at http://atvb.ahajournals.org). In the calf, both groups had transient hypoxia; however, it was more severe and more prolonged in BALB/c mice. Immediately after femoral artery ligation, there was no overlap of oxygen saturation values in the thighs of BALB/c and C57BL/6 mice (Figure V), whereas there was some overlap between these strains by all other in vivo methods at that time point. (Figure 1; Figure II; data not shown).

X-Ray Angiography

Overall location and course of collateral vessels were preserved among strains (Figure 4; Figure VI, available online at http://atvb.ahajournals.org).

Results are summarized in the Table. Number of grown collaterals and calculated conductance scores were higher in C57BL/6 than in BALB/c at 7 but not 28 days after surgery. At both times, C57BL/6 had higher collateral-dependent filling scores and number of visible preexistent collaterals on the acutely ligated (left) side (Figure 4). Decreased collateral-dependent filling scores on day 28 are probably caused by a mildly higher viscosity of the gelatin-containing contrast agent.

Histology

Cross-sections of thigh muscles of C57BL/6 1 week after surgery and continuous BrdUrd administration revealed no uptake of BrdUrd by capillaries. However, some arteriolar/arterial vessels (of at least 27 μm diameter) had a large number of BrdUrd-positive nuclei in all of their wall layers (Figure 5A and 5B). Isolated BrdUrd-positive cells were found around growing vessels and rarely in the walls of large veins.

In BALB/c mice the same histological picture emerged in regions of normal appearing thigh muscle. In addition, multiple necrotic and regenerating areas with destroyed myofibers, myofibers with central nuclei, and large numbers of infiltrating BrdUrd-positive cells were seen (Figure 5C and 5D).

Interestingly, no significant difference in relative numbers of BrdUrd-positive cells in arterioles of C57BL/6 (0.56±0.07) and BALB/c mice (0.60±0.08) after 1 week of continuous administration of BrdUrd was found.
Discussion

Major findings of this study are that: (1) genetic mouse strain differences in preexistent interarterial connections exist; (2) these are of major importance for final recovery after femoral artery occlusion; (3) depending on collateral flow immediately after arterial occlusion, severe local hypoxia and necrosis can occur even in proximal hindlimbs where collaterals grow; (4) however, local resting hypoxia/ischemia is not essential for collateral growth as shown by C57BL/6 mice; (5) BrdUrd uptake in the thighs of C57BL/6 mice, and the preserved pattern of collateral growth in BALB/c and C57BL/6 support the concept that collateral arteries develop by remodeling of preexisting arterioles unrelated to de novo vessel growth processes. Furthermore, we demonstrate the feasibility and potential value of some newer techniques in this model, ie, assessment of collateral-dependent flow and direct in vivo visualization of collateral arteries by high-field MRI, evaluation of local tissue pO2 by EPR oximetry, and assessment of reactive hyperemia by LDI.

Comparison of mice with the poorest (BALB/c) and the best (C57BL/6) recovery of collateral-dependent flow and perfusion immediately after arterial occlusion and by in vivo imaging of collaterals by magnetic resonance angiography in the nonligated hindlimbs. However, as all of these in vivo methods can be affected by vasomotor tone, the final proof depended on angiographic assessment of collateral-dependent filling immediately after femoral artery ligation and perfusion-fixation. The observed differences in filling of collaterals and of the distal vasculature could be caused by differences in number or smallest diameters of innate collaterals. The very similar end results in terms of number and course of collaterals at later time points suggests that the differences immediately after ligation are most likely because of smaller minimal diameters of preexisting collateral connections in BALB/c mice. Because the contrast agent is too viscous to pass through capillary vessels, potential differences in capillary networks cannot explain the findings.

The importance of preexisting collaterals has been previously suggested by studies in pigs and dogs, in which the initial collateral dependent perfusion after coronary artery occlusion correlated with final recovery of perfusion.

Differences in preexistent collateral vasculature can strongly impact the milieu for growing collaterals as demonstrated by the dramatic differences between BALB/c and C57BL/6 mice with regard to tissue oxygenation in regions of collateral growth: severe transient hypoxia and evidence for tissue destruction in regions of collateral growth was only consistently found in the thighs of BALB/c mice. The absence of any pO2 decrease in thighs of most C57BL/6 mice with excellent collateral growth suggests that local hypoxia is not essential for collateral growth and proves that the lack of histological evidence of tissue damage in C57BL/6 is not primarily related to differences in tissue resistance to ischemia, as reported for the myocardium of inbred strains of rats but to differences in local tissue perfusion. Some previous studies did not find evidence for ischemia in the proximal hindlimbs of rabbits and rats after proximal femoral artery occlusion and a more aggressive arterial ligation approach; however, in these studies indirect markers for hypoxia were used, ie, upregulation of hypoxia inducible genes and ATP levels. By EPR oximetry and microspheres, evidence for hypoxia and ischemia in adductor and quadriceps muscles after femoral artery ligation in rabbits was found. The presence of local tissue hypoxia and necrosis in areas of collateral growth in BALB/c mice suggests that the arterial vessels which become functional collaterals play an important role also for local muscle perfusion. This is further supported by evidence for multiple

### Quantification of X-ray Angiograms

<table>
<thead>
<tr>
<th></th>
<th>C57BL/6</th>
<th>BALB/c</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Right (chronically occluded) side</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No. of collaterals</td>
<td></td>
<td></td>
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<tr>
<td>7 days</td>
<td>12.8±0.6</td>
<td>9.9±0.5</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>28 days</td>
<td>9.6±0.7</td>
<td>9.8±0.4</td>
<td>0.81</td>
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<tr>
<td>Conductance score</td>
<td></td>
<td></td>
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<tr>
<td>7 days</td>
<td>0.54 (0.40–0.74)</td>
<td>0.27 (0.26–0.36)</td>
<td>0.002</td>
</tr>
<tr>
<td>28 days</td>
<td>0.38 (0.24–0.57)</td>
<td>0.52 (0.32–0.60)</td>
<td>0.80</td>
</tr>
<tr>
<td><strong>Left (acutely occluded) side</strong></td>
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<tr>
<td>No. of pre-existent collaterals</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>7 days</td>
<td>5.9±0.4</td>
<td>3.6±0.4</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>28 days</td>
<td>3.5±0.2</td>
<td>1.4±0.4</td>
<td>0.001</td>
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<tr>
<td>Collateral-dependent filling score</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7 days</td>
<td>3.0 (3.0–3.0)</td>
<td>2.3 (2.0–2.5)</td>
<td>0.001</td>
</tr>
<tr>
<td>28 days</td>
<td>2.5 (2.5–2.5)</td>
<td>1.0 (1.0–1.4)</td>
<td>&lt;0.001</td>
</tr>
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Data are given as mean±SEM, or as median (interquartile range). N=10 for BALB/c at both time points, n=7 to 9 for C57BL/6.
creating total ischemia by external cuff inflation around the control. Furthermore, we performed LDI measurements after calculated as relative values using the nonligated leg as reference. The results of most of the in vivo measurements were performed at 37°C, which promotes peripheral vasodilation. Perfusion measurements to reflect collateral conductance, compared with measurements of flow, which may explain the lack of significant differences between BALB/c and C57BL/6 at 28 days.

The absence of evidence for hypoxia and angiogenesis in areas of collateral growth in C57BL/6 mice, combined with the presence of proliferative activity in small arteriolar vessels (<25 µm in diameter) in BALB/c and C57BL/6 on complete cross-sections of thigh muscles after cumulative BrdUrd administration, and the preserved pattern of collaterals among different strains of mice, support the concept that collateral growth occurs by remodeling of preexistent arteriolar vessels to collateral arteries independent of de novo vascular growth processes. Entirely ruling out any de novo formation of collateral vessels is difficult because of the incomplete visualization of small preexistent collateral arterioles. Interestingly, assessment of local tissue oxygenation in the areas of collateral growth by EPR oximetry appeared to be the best single in vivo method to discriminate between mice with good innate collateral circulation from mice with poor collateral circulation (Figure 1; Figures II and V).

Because the histological analysis was based on complete cross-sections of medial and caudal thigh muscles, it provided a more reliable and global assessment of collateral growth than when the analysis is limited to small specimens of tissue around isolated collateral vessels as we have performed previously.9 Perivasculare BrdUrd-positive cells were noted, and in BALB/c mice areas of necrosis had many more BrdUrd-positive cells. Most likely these are leukocytes that proliferated in the bone marrow before homing to regions of collateral growth. This is supported by previous data in the same model in which we found many CD45+ cells accumulating around growing collateral vessels after bone marrow transplantation from mice expressing green fluorescent protein.23 However, it cannot be entirely ruled out from our BrdUrd data that some perivascular cells proliferated in situ as recently proposed for tissue resident macrophages.25

Previously, the significance of genetic strain-related variations of arterial networks was suggested by cerebral ischemia studies. The posterior communicating arteries in the circle of Willis were found to be less often patent in BALB/c than in BDF and CFW mice, and this was associated with an increased risk of infarction after creating focal or multifocal cerebral ischemia.26 The higher susceptibility of C57BL/6

Figure 5. Histology of thigh muscles of C57BL/6 (A,B,E) and BALB/c mice (C,D,F). BrdUrd uptake (B,D) in C57BL/6 mice limited to wall cells of arterioles, and isolated perivascular cells (A,B). BALB/c mice in addition had large numbers of BrdUrd-positive cells in areas of tissue necrosis (C,D). A and C, red = BS-I lectin, blue = nuclei (DAPI). E and F, Hematoxylin & eosin-stained sections reveal evidence for myofiber destruction and regeneration (central nuclei) only in BALB/c (F), but not in C57BL/6 mice (E). muscular side branches from many of these vessels, eg, on the surface of the gracilis muscle.19,20 If these pre-existent interarterial connections are not large or not numerous enough, the intravascular pressure loss transmitted from their distal anastomoses after arterial occlusion apparently can result in an insufficient perfusion pressure for the surrounding muscle tissue.

One limitation of our study is that EPR oximetry can only be performed at rest. Thus, some intermittent hypoxia in the thighs of C57BL/6 mice with activity cannot be ruled out by our data. Furthermore, whereas the absence of measurable hypoxia in the thighs of most C57BL/6 mice after arterial ligation suggests that at least local hypoxia is not essential for collateral growth, it certainly does not rule out a modulating effect of hypoxia/ischemia on collateral growth.

Different from larger animal models,5,12,21,22 in mice collateral conductance cannot be directly assessed as this would require not just measurements of collateral flow but also of proximal and distal blood pressure. For distal flow and perfusion measurements to reflect collateral conductance, peripheral vascular resistance should be minimized, which is difficult to achieve in serial studies of living mice. Also, the hemodynamic response to anesthesia could differ between strains of mice.23 To address these concerns, measurements were performed at 37°C, which promotes peripheral vasodilation and results of most of the in vivo measurements were calculated as relative values using the nonligated leg as control. Furthermore, we performed LDI measurements after creating total ischemia by external cuff inflation around the hindlimbs. Surprisingly, on the ligated side the most ischemic stain, BALB/c, still had some capacity for perfusion increase over baseline, whereas the other 2 strains did not have any reactive hyperemia, suggesting that they were maximally vasodilated. On the nonligated side there was a uniform mild hyperemic response suggesting no differences in vasomotor tone. Thus, some of the differences in LDI perfusion, at least at later time points, may be caused by vasomotor tone differences on the ligated side between BALB/c and the other 2 strains of mice under the measurement conditions. A limitation of our reactive hyperemia study by LDI is the time resolution. We performed 1 minute scans and, therefore, may have underestimated the degree of early reactive hyperemia. However, spontaneous hindlimb function and exercise treadmill testing overall supported the validity of the in vivo resting measurements. X-ray angiography is not very sensitive for estimating collateral conductance, compared with measurements of flow, which may explain the lack of significant differences between BALB/c and C57BL/6 at 28 days.

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compared with 129/Sv mice to ischemia after transient bilateral common carotid artery occlusion was shown to be associated with poorly developed vascular connections in the circle of Willis of C57BL/6.37

Even though in this study we could not confirm any significant differences in proliferative activity of thigh arterioles by BrdUrd uptake, it may not imply that vascular growth differences between inbred mouse strains do not exist, as our method may not have been sensitive enough. Collateral artery growth appears to be a strongly flow-dependent process.28 Strain-related differences in flow-induced carotid artery remodeling have been described.29,30 However, in a model of mycoplasma pulmonis-induced growth of tracheal microvessels, C57BL/6 mice responded with an increased number of capillaries and venules, whereas in C3H mice the diameters of arterioles, capillaries, and venules increased.31 An additional problem in ischemia models, whereas in C3H mice the diameters of arterioles, capillaries, and venules increased.31 An additional problem in ischemia models, whereas in C3H mice the diameters of arterioles, capillaries, and venules increased.31

An additional problem in ischemia models, like the mouse femoral artery ligation model, is that little is known about the secondary effects of distal tissue necrosis on collateral growth. Conceivably, a major loss of collateral-dependent tissue caused by ischemia after arterial occlusion affects the peripheral vascular resistance which would impact blood flow through proximal collateral vessels and thus the driving forces for their growth. The results of this study highlight the need to consider the genetic background and the innate collateral circulation when designing and interpreting hindlimb ischemia studies. Differences in innate collateral circulation may make it impossible to interpret differences in final recovery of collateral-dependent flow and related parameters as caused by differences in collateral growth rates. However, genetic analysis of mouse strain-dependent variations in pre-existent collateral vessels, for example by strain intercross studies, might offer the opportunity for a better understanding of the mechanisms involved in collateral artery development.

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Methods:

**X-Ray Angiography**

Seven or 28 days after right femoral artery occlusion the left femoral artery was ligated. Via a cannula (0.8 mm outer diameter) in the descending thoracic aorta, at a pressure of 100 mmHg, distal vessels were rinsed for 2 minutes with phosphate buffered saline (PBS) containing 1 g/l adenosine (Fluka, Steinheim, Germany) and 4 mg/l papaverine (Paveron, Linden Arzneimittel, Germany) followed by 2% formaldehyde in PBS for 3 min, buffer for 2 minutes, and finally a two minute infusion of a contrast agent containing 5% gelatin and about 40% bismuth oxychloride17 at a temperature of 30 °C and a pressure of 200 mmHg. Mice were chilled in ice water. X-rays were taken with a Machlett-Balteau x-ray source (20 kV, 8 mA 2.5 minutes). Films were scanned at a resolution of 3600 × 1800 DPI, interpolated to 3600 DPI, (Microtek ArtixScan 1800f) and analyzed independently by two blinded observers. Numbers of collateral arterial connections bridging the ligation site were counted, their diameters measured at the level of the ligation site with a customized version of ImageJ permitting automatic border detection, and relative conductance scores were calculated using the formula:

\[
\text{Relative Collateral Conductance score} = \frac{\sum R_{CA}^4}{R_{FA}^4}
\]

where \( n \) is the number of collaterals, \( R_{CA} \) is the radius of a collateral arterial vessel, \( R_{FA} \) the radius of the ipsilateral femoral artery proximal to the ligation site.

To assess preexistent collaterals, the degree of distal hindlimb filling on the acutely ligated left side was scored independently by two blinded observers. A modification of the Rentrop Score18 was used, which classified collateral-dependent filling as 0 = no filling of collaterals and hindlimb vessels distal to the level of the femoral artery occlusion site, 1 = some filling of proximal thigh collaterals distal to level of arterial occlusion but no or only very faint filling of distal hindlimb vessels, 2 = markedly reduced filling of distal vessels, 3 = complete filling of distal hindlimb vessels.

**Histology**

5-bromo-2’-desoxyuridine (BrdU) (Sigma, Taufkirchen, Germany) was added to drinking water (80 mg/100ml) of BALB/c and C57 BL/6 mice (n=5 per group) one day before surgery and changed every three days. Seven days after surgery, mice underwent perfusion fixation and angiography. Complete cross-sections of medial and caudal thigh muscles at the level of arterial occlusion were frozen in liquid nitrogen after cryoprotection in 20% sucrose/PBS. Five µm sections were stained with a fluorescein-conjugated anti-BrdU monoclonal antibody (In Situ Cell Proliferation Kit, FLUOS, Roche Diagnostics, Mannheim, Germany), after pretreatment with ethanol, trypsin and hydrochloric acid according to instructions. Subsequently, staining with DAPI (Sigma) and TRITC-labeled BS-I lectin (0.01 mg/ml, Sigma) or a Cy3 conjugated monoclonal anti α smooth muscle actin antibody (Sigma) was performed. Uptake of BrdU by arterioles/arteries was quantified on 2 complete cross-sections of the medial and caudal thigh muscles at the level of the ligation.
Statistical Analysis
Data were analyzed with one way ANOVA or, if not normally distributed or variances unequal with Kruskall-Wallis one way ANOVA on ranks with the appropriate confirmation tests for the comparison of three strains, and student T-test or Mann-Whitney U rank-sum test for comparison of two groups (SigmaStat for Windows, version 3.0.1). Statistical significance was defined as p<0.05.

Results:

Figure 1. Representative laser Doppler images of distal hindlimbs at different time points.
**Figure II.** Individual laser Doppler values. For means and SEM, please see Fig 1a.

**Figure III.** MR-flow image at calf level of a BALB/c mouse, three days after surgery. Vessels with flow (high-signal intensity) are visible in the tail (T) and the left non-ligated side (L). Only minimal flow on the ligated side (R). The right calf is swollen. (See figure 1B for quantification of flow and figure 1F for quantification of cross-sectional area ratios).
Figure IV. Reactive hyperemia in feet quantified by LDI three weeks after surgery. After transient cuff inflation, six successive LDI scans were performed, each lasting one minute.
A, Relative flux values (compared to baseline before cuff inflation) non-ligated side.
B, Relative flux values in ligated side.
C, Right-to-left ratio of flux values before cuff inflation (R pre/ L pre), and ratio of maximal flux during hyperemia phase in ligated and non-ligated side (R max / L max). Values are mean±SEM, n=5 per strain.
Figure V. Individual EPR oximetry data on right medial thigh muscles. For means and SEM of right and left hindlimbs, please see Fig 3, upper panel.
Figure VI. X-ray angiogram of a BALB/c mouse 28 days after ligation of the right femoral artery (*). The left side is not ligated which allows for better filling of preexistent collateral vessels by the contrast agent (however collateral-dependent filling cannot be graded). The stem (origin) and target vessels of collateral arteries were identical for BALB/c, C57BL/6, and 129S2 mice. Subcutaneous collateral vessels are not visible on this image of a BALB/c mouse, as the skin was removed prior to immersion of these mice in Bone’s solution for fixation and bone demineralization. Collateral arterial connections in the cranial thigh muscles are fed by two arteries supplying the quadriceps muscle: a proximal one (arrowhead with open circle, ○) originating from the very proximal internal iliac artery, and the *a. circumflexa femoris lateralis* (arrowhead with triangle, ▲), a branch of the external iliac artery. Collaterals from these arteries anastomose distally with vessels related to the *a. genus descendens* (empty white arrowhead) and a more lateral artery (arrowhead with asterisk, *) that connects to the popliteal artery (arrowhead with pound sign, #). Collateral arteries in the medial and caudal thigh muscles originate from the internal iliac artery (arrowhead with closed circle, ●) and the deep (caudal) femoral artery (arrowhead with square, ■). These collateral vessels anastomose distally with the saphenous artery (arrowhead with empty square, □) and frequently are somewhat tortuous. On the non-ligated side preexistent collateral arteries can be identified in the same pattern as on the ligated side, even though not as well filled with contrast agent; they are smaller than on the ligated side and not tortuous.