Role of PECAM-1 in Arteriogenesis and Specification of Preexisting Collaterals

Zhongming Chen, Janet Rubin, Ellie Tzima

Rationale: Hemodynamic forces caused by the altered blood flow in response to an occlusion lead to the induction of collateral remodeling and arteriogenesis. Previous work showed that platelet endothelial cell adhesion molecule (PECAM)-1 is a component of a mechanosensory complex that mediates endothelial cell responses to shear stress.

Objective: We hypothesized that PECAM-1 plays an important role in arteriogenesis and collateral remodeling.

Methods and Results: PECAM-1 knockout (KO) and wild-type littermates underwent femoral artery ligation. Surprisingly, tissue perfusion and collateral-dependent blood flow were significantly increased in the KO mice immediately after surgery. Histology confirmed larger caliber of preexisting collaterals in the KO mice. Additionally, KO mice showed blunted recovery of perfusion from hindlimb ischemia and reduced collateral remodeling, because of deficits in shear stress–induced signaling, including activation of the nuclear factor κB pathway and inflammatory cell accumulation. Partial recovery was associated with normal responses to circumferential wall tension in the absence of PECAM-1, as evidenced by the upregulation of ephrin B2 and monocyte chemoattractant protein-1, which are 2 stretch-induced regulators of arteriogenesis, both in vitro and in vivo.

Conclusions: Our findings suggest a novel role for PECAM-1 in arteriogenesis and collateral remodeling. Furthermore, we identify PECAM-1 as the first molecule that determines preexisting collateral diameter. (Circ Res. 2010;107:1355-1363.)

Key Words: PECAM-1 ■ arteriogenesis ■ collateral vessels ■ shear stress ■ cyclic stretch

Arteriogenesis, the outward remodeling of arteriole–arteriole anastomoses, is necessary to restore blood flow to tissue distal to an occlusion. Although angiogenesis, capillary growth from preexisting capillaries, is important for distributing existing flow, it is the caliber, number, and growth of conduit arteries (collaterals) by arteriogenesis that determines tissue viability. A number of studies have shown that hindlimb flow subsequent to femoral artery occlusion is primarily determined by these collaterals.1 Notably, the number and size of these anastomoses varies greatly between species and tissues, resulting in different degrees of protection after arterial occlusion.2 Additionally, genetic mouse strain differences in preexisting collaterals exist, which are of major importance for final recovery after femoral artery occlusion.3 Despite a growing number of putative arteriogenic factors, little is known about the exact mechanisms that regulate collateral remodeling, and virtually nothing is known about the genetic program and signaling mechanisms that regulate preexisting collateral networks.4

Mechanistically, arteriogenesis is a complex process that requires proliferation of endothelial cells (ECs) and mural cells; upregulation of adhesion molecules, like intercellular adhesion molecule (ICAM)-1 and vascular cell adhesion molecule (VCAM)-1; chemokines, like monocyte chemoattractant protein (MCP)-1; and membrane-associated ligands like ephrin B2 in collateral arterioles.5,6 Mechanical hemodynamic forces are pivotal triggers for arteriogenesis. As a consequence of a pressure drop distal to the site of occlusion, the pressure difference between the ends of the collaterals is enhanced, resulting in increased blood flow, and, consequently, a rise in both shear stress and circumferential wall tension.7 Although shear stress is thought to be responsible for collateral remodeling during arteriogenesis, the role of circumferential wall tension in arteriogenesis is less well defined.8,9 In this context, the expression of several regulators of arteriogenesis, such as the transcription factor activator protein (AP)-1, MCP-1, and ephrin B2, is regulated by circumferential stretch.5,10 The upregulation of these genes promotes smooth muscle cell (SMC) migration and monocyte recruitment and activation, which are critical regulators of arteriogenesis and collateral vessel growth.11
Vascular ECs are ideally positioned to serve as transducers, to relay hemodynamic and biochemical changes into molecular events in the other layers of the vascular wall. EC surfaces are equipped with numerous mechanoreceptors capable of detecting and responding to shear stress, including caveolae, ion channels, integrins, receptor Tyr kinases, the apical glycocalyx, primary cilia, heterotrimetric G proteins, and intercellular junctions. In this context, we previously identified a mechanosensory complex comprising platelet endothelial cell adhesion molecule (PECAM)-1, vascular endothelial (VE)-cadherin, and vascular EC growth factor receptor (VEGFR)2 that mediates EC responses to shear stress. Based on the significant role of PECAM-1 in transducing stress in ECs in vitro and flow-mediated vascular remodeling in vivo, we hypothesized that PECAM-1 plays an important role in arteriogenesis and collateral remodeling in ischemia.

Methods
PECAM-1 knockout (PECAM-1−/−) mice were kindly provided by Dr P. Newman (Blood Research Institute, Blood Center of Wisconsin, Milwaukee). PECAM-1−/− C57BL/6 mice and PECAM-1−/− mice had been backcrossed for >12 generations onto a C57/BL6 background. Cell culture, shear stress, 18 and mechanical stretch (15% strain, 1 Hz for 24 hours)19 assays were performed as described.

Unilateral hindlimb ischemic surgery procedure was as described previously.20 Laser Doppler Imaging, postmortem angiography, morphometric analysis, blood vessel silver staining, measurement of leukocyte density and capillary density, quantitative real-time PCR, immunofluorescence, Western Blot assays, and statistical analyses are described in detail in the expanded Methods section, available in the Online Data Supplement at http://circres.ahajournals.org.

Results
PECAM-1 Regulates Acute Plantar Perfusion and Recovery After Hindlimb Ischemia
To determine the role of PECAM-1 in arteriogenesis, we subjected PECAM-1−/− (KO) and wild-type (WT) littermates to hindlimb ischemia by ligation of the femoral artery, which triggers growth of preexisting collaterals from the deep femoral artery. Blood perfusion of hind paws (plantar) was monitored with a laser Doppler imaging system before surgery (pre), immediately after surgery (acute), and 7 days (7d), and 3 weeks after surgery (Figure 1A). Plantar perfusion was quantified from the Doppler images and normalized to sham control side of the same animal for the comparisons of different time points. Surprisingly, we observed much higher acute plantar perfusion in PECAM-1−/− mice than in WT mice, although plantar perfusion was higher in both genotypes after 3 weeks (Figure 1B). The appearance and use scores of both genotypes were similar during the 3-week recovery stages (data not shown). Quantitation of recovery revealed a 65.5% increase in plantar perfusion in PECAM-1−/− mice from acute to 3 weeks after surgery versus a 189.8% increase in WT mice (Figure 1B and Online Figure I), suggesting impaired recovery in the absence of PECAM-1.

In addition to the less severe femoral artery ligation model, we also performed a more severe ischemia model, proximal lateral caudal femoral artery ligation, in a separate group of mice (Online Figure II). PECAM-1−/− mice showed blunted perfusion recovery compared to WT mice, similar to what we observed with the less severe model.

PECAM-1 Regulates the Lumen Diameter of Preexisting Collateral Vessels
Because conductance of the native collateral circulation regulates plantar perfusion immediately after surgery, we hypothesized that there are anatomic differences in preexisting collaterals. We performed microangiography and counted the number of arterial vessels crossing a line drawn through the center of the thigh collateral zone to determine whether PECAM-1−/− mice have more preexisting collaterals and arterioles. Quantification revealed no differences between the 2 genotypes (not shown). Next, we measured the diameter of collaterals in the anterior and posterior gracilis muscles by histomorphometry (Figure 2A). Baseline diameter of PECAM-1−/− collaterals was significantly bigger compared to WT animals. This suggests that the higher perfusion in PECAM-1−/− mice immediately after surgery is not attributable to more collaterals, but reflects wider collaterals (Figure 1B).
Interestingly, quantitation revealed wider collaterals in the intestine of PECAM-1⁻/⁻ mice (Online Figure III). Consistent with the recovery of plantar perfusion in the WT, collateral lumen diameter significantly increased 3 weeks after surgery. In contrast, the increase in collateral diameter was attenuated in PECAM-1⁻/⁻ compared to WT (14.8% versus 58.1%; Online Figure I).

The larger diameter of collateral vessels in the PECAM-1⁻/⁻ mice may reflect differences in the number, size, or orientation of ECs. We counted the number of ECs in collateral cross sections and found that the numbers were similar in both genotypes (data not shown). Next, we considered differences in size and orientation of ECs. For these studies, we isolated collateral vessels from gracilis muscles and EC borders were visualized by silver staining. In WT mice, collateral ECs displayed an elongated phenotype and were uniform in their orientation (Figure 2B). In contrast, PECAM-1⁻/⁻ collateral ECs were shorter, wider, and less elongated. We measured the length and width of ECs in collateral intima and confirmed that the EC width was significantly larger in PECAM-1⁻/⁻ mice (16.6 μm) than in WT mice (13.6 μm), and the length was significantly shorter in PECAM-1⁻/⁻ (69 μm) than in WT mice (89 μm; Figure 2C). Interestingly, the ratio of collateral EC width (16.6/13.6 = 1.22, KO versus WT) is equal to the ratio of diameter of preexisting collaterals (42.8/35.0 = 1.22, KO versus WT). These observations suggest that the differences in diameter of preexisting collaterals reflect differences in width and orientation of collateral ECs.

Angiogenesis in PECAM-1⁻/⁻ Mice

Although baseline collateral conductance and their subsequent enlargement are the primary determinants of plantar perfusion after surgery, baseline capillary density and ischemic angiogenesis could also contribute to plantar perfusion. Interestingly, recent reports have shown a role for PECAM-1 in angiogenesis, because Matrigel implants, tumor angiogenesis, and retinal angiogenesis were inhibited in PECAM-1⁻/⁻ mice. To address this possibility, we measured capillary density in the gastrocnemius muscle of sham or ligated animals (Online Figure IV, A and B). The capillary density before ligation is similar in WT and PECAM-1⁻/⁻ mice. This strengthens the suggestion from the preceding analyses that larger preexisting collateral diameter in PECAM-1⁻/⁻ mice, rather than baseline capillary density, accounts for the smaller drop in perfusion immediately after surgery. After 3 weeks of ischemia, WT mice showed a significant increase in capillary density, whereas PECAM-1⁻/⁻ mice exhibited a trend (P=0.085) to increase their capillary density relative to sham controls.

Collateral Remodeling in PECAM-1⁻/⁻ Mice

The attenuated increase in perfusion recovery (Figure 1B) and collateral diameter (Figure 2A) in PECAM-1⁻/⁻ mice...
suggested a role for PECAM-1 in arteriogenesis. This was particularly interesting in light of the requirement for PECAM-1 in shear stress signaling.14,23–27 To gain further insight into the mechanisms that regulate arteriogenesis, we evaluated proliferation and activation of the inflammatory response, because both are known to play an important role in collateral remodeling. Figure 3A illustrates the degree of EC proliferation as determined by PCNA staining of collaterals. Quantification of PCNA-positive ECs showed that there are significantly more proliferating ECs in WT mice than in PECAM-1−/− mice 3 days after hindlimb ischemia (Figure 3B).

Because monocytes/macrophages are known to play an important role in hindlimb ischemia and PECAM-1 is known to facilitate leukocyte transendothelial migration,28 we investigated the effects of PECAM-1 genetic deletion on leukocyte infiltration during hindlimb ischemia. Collaterals were immunostained for CD45, SMC α-actin and DAPI (Figure 4A). On day 3 after hindlimb ischemia, PECAM-1 deletion significantly decreased the number of CD45-positive cells as compared to wild-type littermates, suggesting that PECAM-1 deletion reduces inflammatory cell infiltration (Figure 4B).

To identify a mechanism for the decrease in infiltration of CD45+ cells in the ischemic tissue of PECAM-1−/− mice, we examined the nuclear factor (NF)-κB pathway. Importantly, the NF-κB pathway is activated by shear stress and PECAM-1 is important for flow-induced NF-κB activation.14,15,29 In particular, we examined activation of NF-κB by assaying nuclear translocation of the p65 subunit (Figure 5A) and downstream intercellular adhesion molecule (ICAM)-1 and vascular cell adhesion molecule (VCAM)-1 upregulation. As shown in Figure 5B and 5C, ECs from WT collaterals showed nuclear accumulation for the p65 subunit of NF-κB, whereas ECs from PECAM-1−/− mice showed cytoplasmic localization for NF-κB. Additionally, although we did not observe any differences in VCAM-1 or ICAM-1 staining in sham collaterals, expression of both cell adhesion molecules in ligated collaterals was much lower in PECAM-1−/− compared with WT animals (Figure 5B and 5C). Taken together, these results demonstrate that lack of PECAM-1 signaling leads to a disruption of the NF-κB pathway and a reduction in inflammatory cell accumulation during collateral remodeling.

Responses to Cyclic Stretch

PECAM-1−/− mice have impaired collateral remodeling, yet show some perfusion recovery, albeit blunted compared to
WT mice. Although we cannot exclude a role for other mechanosensors in the attenuated perfusion recovery in the PECAM-1/−/− mice, we considered the complex hemodynamic environment in collaterals after ligation. Collective evidence suggests that shear stress and circumferential wall strain act together to alter the hemodynamics which govern arteriogenic remodelling.30 We therefore examined the role of PECAM-1 in mediating EC responses to circumferential stretch.

First, we used an in vitro system to apply cyclic stretch to ECs that express (PE-RC) or lack (PE-KO) PECAM-1. PE-RC and PE-KO ECs were exposed to cyclic stretch (1Hz, 15% strain for 24 hours) to allow alignment of actin stress fibers, which was assessed by staining with TRITC-phalloidin (Figure 6). These assays showed that ECs expressing PECAM-1 oriented their cytoskeletons perpendicular to the stretching direction, consistent with previous reports.31–33 Similarly, PECAM-1 null ECs were also oriented perpendicular to the stretch direction, suggesting that PECAM-1 null ECs are able to respond to stretch.

We further tested this hypothesis by examining expression of ephrin B2 and MCP-1, which are 2 proteins previously shown to be upregulated during arteriogenesis because of circumferential wall strain.6–10 MCP-1 and ephrin B2 abundance in sham collaterals was rather low; however, we noticed a marked increase in MCP-1 and ephrin B2 expression in response to ligation in collaterals from both genotypes (Figure 7A and 7B). Although some groups reported upregulation of MCP-1 and ephrin B2 by shear stress,34 others reported only a transient upregulation35 or even downregulation of their expression by shear stress.36–38 We examined MCP-1 and ephrin B2 expression at the mRNA and protein levels in ECs subjected to cyclic stretch (15% strain, 1Hz) or laminar shear stress (12 dynes/cm²) for 24 hours. We observed a robust increase in MCP-1 and ephrin2 mRNA levels in both PE-RC and PE-KO ECs in response to cyclic stretch compared with static controls (Figure 7C). Prolonged cyclic stretch also resulted in an increase in ephrin B2 protein levels in both cell types (Figure 7D); however, we did not observe changes in MCP-1 expression in cell lysates (data not shown), possibly because of secretion of MCP-1 in the culture media.39 Importantly, we did not observe upregulation of MCP-1 or ephrin B2 by shear stress in either cell type. These results therefore demonstrate that PECAM-1 is not required for expression of ephrin B2 and MCP-1 during arteriogenesis, possibly because of a lack of requirement of PECAM-1 for EC responses to circumferential stretch.

**Discussion**

Physical forces are pivotal triggers for arteriogenesis,30 Mechanical forces caused by the altered blood flow in response to an occlusion lead to the induction of collateral remodelling. PECAM-1 is thought to be involved in flow mechanosensing or transduction, based on changes in its phosphorylation with altered flow and in vitro and ex vivo experiments showing PECAM-1–dependent activation of flow-mediated intracellular signaling pathways.14,23–27 More recently, our group demonstrated a role for PECAM-1 in flow-mediated vascular remodeling and intima-media thickening in vivo.15 In light of the view that arteriogenesis is a predominantly shear stress–mediated remodeling process and the importance of PECAM-1 in transduction of shear stress signaling, we hypothesized that PECAM-1/−/− animals would have impaired ischemia-induced arteriogenesis. Our data show that PECAM-1 contributes to multiple steps involved in collateral remodelling, in particular, activation of the NF-κB pathway and downstream inflammatory cell accumulation. Consistent with these findings, PECAM-1 mediates shear stress–induced NF-κB activation in vitro and in vivo.14,15,29 A possible explanation for the impaired recovery might be deficits in shear stress–induced signaling in the PECAM-1/−/− animals. Another equally plausible possibility is that the PECAM-1/−/− mice already have higher perfusion acutely, thus meeting tissue...
perfusion demands even in the face of reduced collateral remodeling. Likewise, it is possible that PECAM-1−/− collaterals experience lower shear stress because of their larger size, thus lacking a key stimulus for remodeling. To address this, we created a shunt between the distal stump of the occluded femoral artery and the accompanying vein to increase shear stress. Importantly, although the higher shear stress greatly stimulated arteriogenesis in the WT, PECAM-1−/− mice showed reduced perfusion recovery (data no shown), suggesting impaired remodeling even in the presence of increased shear stress.

It is thought that hemodynamic forces and, in particular, fluid shear stress is the primary morphogenic physical factor that induces collateral remodeling. ECs are equipped with numerous mechanoreceptors capable of detecting and responding to shear stress, and, thus, regulating perfusion recovery in response to ischemic insult, including caveolae, ion channels, integrins, receptor Tyr kinases, the apical glycocalyx, primary cilia, heterotrimeric G proteins, and intercellular junctions. It is therefore possible that in the absence of PECAM-1, other mechanosensors kick in and facilitate the blunted, albeit significant, perfusion recovery. Although we cannot rule out this possibility, we considered that the vessel wall is exposed to both shear stress and circumferential stretch during arteriogenesis. Based on Poiseuille’s equation, progressive stenosis of the main artery will lead to an increase in resistance hence a significant drop in pressure distal to the site of occlusion. As a consequence, the pressure difference between both ends of the collateral arterioles is enhanced, resulting in increased flow and, consequently, a rise in both shear stress and circumferential wall tension, which may act in concert on the collateral blood vessels. There is accumulating evidence that these altered hemodynamics to which the arteriolar vessel wall is exposed to initiate arteriogenesis. Whereas a requirement for PECAM-1 in shear stress responses is well established, its role in circumferential stretch is unknown. Our data show that PECAM-1 is not only dispensable for stretch-induced EC alignment, but also for stretch-induced expression of ephrin B2 and MCP-1, which are 2 proteins previously shown to be upregulated during arteriogenesis because of circumferential wall strain.

An unexpected finding of this study was that PECAM-1−/− animals show increased perfusion immediately after surgery. This increased perfusion is not attributable to more collaterals but, instead, reflects wider collaterals. Although virtually nothing is known about the genetic and environmental factors that specify collateral formation, recent reports have identified 2 genes that specify preexisting collateral numbers (VEGF and CLIC4). Here, we identify PECAM-1 as the first molecule that determines preexisting collateral diameter in skeletal muscle. We hypothesized that the larger diameter of collateral vessels

![Figure 6. Effects of cyclic stretch on EC alignment in vitro. A, PECAM-1 reconstituted (PE-RC) or knockout (PE-KO) ECs were exposed to cyclic stretch (15% strain, 1 Hz, 24 hours) and stained with TRITC-phalloidin, or β-catenin (red) and DAPI (blue) (inset images). Arrows show the direction of stretch. Bar, 50 μm. B, Quantification of orientation of F-actin stress fibers. Values are means ± SE.](http://circres.ahajournals.org/content/1360-1105/6/5/1360/F6.large.jpg)
in the PECAM-1−/− mice may reflect differences in the morphology or orientation of ECs. We therefore developed a method to isolate collaterals and visualized EC morphology. Surprisingly, WT collateral ECs were elongated and uniformly oriented, whereas KO ECs were less elongated and wider, thus contributing to the larger lumen diameter. Importantly, it has been proposed that flow-evoked remodeling processes determine the number, and possibly diameter, of preexisting collaterals during embryonic development.44 Our own work has pointed to PECAM-1 as an important regulator of shear stress–induced cell alignment.14 In contrast, ECs are able to align in response to cyclic stretch independently of PECAM-1. Importantly, PECAM-1 has also been shown to regulate the activity of Rho family GTPases,45 which are known to be master regulators of the cytoskeleton and, thus, regulate processes such as cell shape, adhesion, and migration.46 Consistent with this, our own preliminary data show decreased Rho activation in the absence of PECAM-1 (E.T., unpublished data, 2010). It is therefore possible that the wider collateral diameter in the PECAM-1−/− mice is attributable to differential activation of Rho GTPases, which, in turn, regulate cell shape via their effects on the cytoskeleton. Alternatively, the endothelial nitric oxide synthase (eNOS)/NO pathway might be worthy of consideration. An interesting recent study reported dilated vessels in the retinal vasculature of PECAM-1−/− mice, which mechanismistically might be associated with dysregulation of eNOS.22 Moreover, PECAM-1 has been shown to regulate eNOS activity, which in turn affects vessel structure.26,27,47 Interestingly, it has been reported that the activity of Rho GTPases can be controlled by the level of NO via changes in their phosphorylation.48,49 Further studies are required to define when and how PECAM-1 regulates collateral formation, as well as the downstream pathways that stabilize and maintain collaterals.

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**Disclosures**

None.

**References**


Collaterals are small arterioles that serve as alternate routes of blood supply in the face of an obstruction by connecting 2 larger arteries. The density and diameter of preexisting collateral networks and their ability to grow and remodel are critical factors that determine restoration of blood flow. Mechanical forces (shear stress and circumferential stretch) caused by the altered blood flow in response to an occlusion are critical regulators of collateral remodeling. Vascular endothelial cells are equipped with numerous mechanoreceptors capable of detecting and responding to changes in blood flow, including PECAM-1. In this study, we demonstrate impaired perfusion recovery from femoral artery ligation in PECAM-1–deficient mice. This was associated with deficits in shear stress–induced signaling, such as activation of the NF-κB pathway and inflammatory cell accumulation, but normal responses to circumferential stretch. Unexpectedly, PECAM-1–deficient animals showed increased perfusion immediately after ligation, because of the presence of larger diameter collateral vessels. Further studies are required to define when and how PECAM-1 regulates collateral formation, as well as the downstream pathways that stabilize and maintain collaterals.
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SUPPLEMENTAL MATERIAL.

Detailed Methods

Cell culture, shear stress and cyclic stretch assays  PECAM-1 knockout (PE-KO) cells and cells reconstituted (PE-RC) with full-length PECAM-1 were prepared as described.\textsuperscript{1, 2} Levels of PECAM-1 in reconstituted cells are similar to wild-type levels.\textsuperscript{2} Laminar shear stress assay was described previously.\textsuperscript{3} Briefly, confluent cells were incubated in starvation media (full medium with 0.5% fetal bovine serum) overnight before flow experiments. Cells on 10μg/ml fibronectin-coated slides were exposed to shear stress using the cone and plate flow chamber system for 24h at 12 dynes/cm\textsuperscript{2}. Mechanical stretch assay was described previously.\textsuperscript{4} Briefly, cells were plated on 6-well Bioflex 10μg/ml fibronectin-coated plates (Flexcell International, Hillsborough, NC). Uniform biaxial strain was applied (15% magnitude, 1Hz for 24 hours) using the Flexcell FX-4000 system. Static controls were cells not subjected to shear stress or cyclic stretch. To visualize F-actin filaments and cell boarders, cells were stained with TRITC-labeled phalloidin (1:100) and β-catenin (1:100, Sigma-Aldrich, St. Louis, MO). The angle of actin filaments relative to the direction of stretch was measured using NIH Image J package. The angle average and S.E were calculated to compare the F-actin alignment in both genotypes.

Western blotting  Cells were washed with PBS and lysed in 10x sample buffer (1ml/100mm dish, or 125ul/well). Immunoblotting was performed using anti-ephrin B2 (1:300, R & D systems, Minneapolis, MN) and mouse anti-α-tubulin (1:1000, Sigma-Aldrich) and Alexa 680-conjugated donkey anti–goat (Invitrogen, Carlsbad, CA) followed by IRDye800-conjugated goat anti–mouse antibodies (Rockland, Gilbertsville, PA). Membranes were scanned with an Odyssey laser scanner and quantified with Image J package.

Animals  Wild-type C57/BL6 (PECAM-1\textsuperscript{+/+}) mice were purchased from Charles River Laboratories International, Inc (Wilmington, MA). PECAM-1\textsuperscript{−/−} C57BL/6 mice were kindly provided by Dr. P. Newman (Blood Research Institute, Blood Center of Wisconsin, Milwaukee, WI). PECAM-1\textsuperscript{+/−} C57BL6 mice and PECAM-1\textsuperscript{−/−} mice had been backcrossed for >12 generations onto a C57BL6 background.\textsuperscript{5, 6} The animals were bred in house and used in accordance with the guideline of the National Institute of Health and for the care and use of laboratory animals (approved by the Institutional Animal Care and Use Committees of the University of North Carolina at Chapel Hill). Male PECAM-1\textsuperscript{−/−} and age-matched littermates (PECAM-1\textsuperscript{+/+}, 10-14 weeks) were used for all experiments. To genotype animals, DNA was isolated from ears at weaning and PCR performed. All analyses were conducted by observers blinded to animal phenotype.

Unilateral hindlimb ischemia  The surgery procedure was performed on the right side as described previously.\textsuperscript{7} Briefly, animals were anesthetized with 1.125% isoflurane supplemented with oxygen, and body temperature was maintained at 37°C. Hair was removed from the hindquarters with a depilating cream. The femoral artery was exposed aseptically through a 2mm incision and isolated from vein and nerve. The femoral artery was ligated with 7-0 ligatures proximal to the bifurcation of the popliteal artery and distal to the lateral caudal femoral artery (LCFA) for the less severe ischemia mode. The incision was closed after the wound was irrigated with sterile saline.

Laser-Doppler Imaging  The animals were placed for 5min at a 37°C chamber before the measurements to avoid vasoconstriction by anesthetic heat loss. A Laser Doppler imager was used to estimate relative blood flow. Ratios of occluded over non-occluded values were compared. Noninvasive measurements of superficial hindlimb perfusion were obtained before ligation, immediately after ligation (acute), 7 and 21 days after ligation.\textsuperscript{8} Plantar perfusion was quantified within anatomically defined regions of interest (ROIs). All ROIs were drawn by an
investigator blind to animal genotype. Data are reported as perfusion ratios of ligated vs. sham control side.

**Postmortem angiography** Arterioles and collaterals were counted as described previously. Briefly, mice were perfusion-fixed with 4% paraformaldehyde (PFA) at 100 mmHg. The vasculature was injected with barium sulfate to provide X-ray angiograms. Films were digitized, and index of pre-existing collateral number in the acutely ligated limb was measured by counting arteries crossing a Rentrop-like line beginning at the midpoint between the proximal and distal ligations of the femoral artery and extending to the posterior edge of the thigh.

**Morphometry** Collateral arteries were harvested from mice as described previously. Briefly, animals were transcardially perfused at 100 mmHg with PBS containing 10 nmol/l sodium nitroprusside and 10U/ml heparin 3 weeks after hindlimb ischemia. PBS was followed by 2% PFA for 20 min. We harvested the anterior and posterior gracilis muscles which contain two preexisting collaterals. The midzone of the muscles (i.e., the 5-mm-wide centermost section) was trimmed. A section of the calf (gastrocnemius/soleus) muscle was also harvested for the examination of capillary density (described below). Samples were embedded in paraffin and 5μm thick cross sections were H.E. stained. Lumen diameter of collateral arteries was measured as previously described. Briefly, HE stained cross-sections within 0.5mm from the midzone of the collateral arteries in anterior and posterior gracilis muscles were digitized at 60x magnification. Lumen circumference (C) was measured interactively using NIH Image J package. Lumen diameter (D) was calculated from the circumference as D=c/π. For each mouse, four arteries were studied, two from the surgery-operated side and two from the sham control side. At least 4 cross-sections from each mouse were measured and the average lumen diameter of collateral vessel was used. Samples were selected by an observer blinded to wild-type vs. PECAM-1<sup>−/−</sup> genotype.

**Silver staining** The staining procedure was previously described and slightly modified. Mice were euthanized and perfused sequentially with 5ml PBS, 5ml 0.2% AgNO3 and 5ml PBS through abdominal aorta using 5ml syringes, followed by 60min fixation with 4% paraformaldehyde at 100mm Hg pressure. Femoral arteries and gracilis muscles were isolated from fixed tissue and cleared sequentially with 70%, 95%, 100% ethanol, and methyl salicylate (Sigma-Aldrich) for 60min each. Cleared arteries and muscles were mounted with methyl salicylate on glass slides and examined under microscope.

**Immunohistochemistry** We used antibodies to NFκB (1:200, BD Pharmingen, San Diego, CA), ICAM-1 (1:200, Santa Cruz Biotechnology Inc, Santa Cruz, CA), VCAM-1 (1:200, Santa Cruz), CD45 (1:100, BD Pharmingen), PCNA (1:1000, Abcam, Cambridge, MA), ephrin B2 (1:200, R & D systems, Minneapolis, MN), MCP-1 (1:200, Abcam), smooth muscle cell α-actin (SMC α-actin, 1: 1000, Sigma-Aldrich). Antigen retrieval was performed for cross sections with Retrogen (BD Pharmingen), except for NFκB, PCNA and ICAM-1 antibodies. Thyramide signal amplification (TSA, Perkins Elmar Inc, Waltham, MA) was performed for NFκB, ICAM-1, VCAM-1 and ICAM-1, CD45, ephrin B2 and MCP-1, per manufacturer’s instruction. Briefly, a primary antibody was incubated at 4°C overnight, followed by 60 minutes for biotinylated secondary antibody (1:500), and 30 minutes for ABC complex (Elite ABC kit, Vector Laboratories, Burlingame, CA). Cy3-thymamide was used to visualize the peroxidase-binding sites. To visualize collateral media, the slides were further incubated with mouse anti-SMC α-actin for 2 hours, followed by the incubation of Cy5-goat anti-mouse Ig G (1:100) for one hour. The slides were counter stained with DAPI to visualize cellular nuclei.

**Leukocyte density** Leukocytes in the adventitia and periadventitia of preexisting collaterals were detected with CD45 antibody as described above. CD45-positive cells having a blue nucleus surrounded by Cy3 fluorescence (from Cy3-thymamide) on their surface were counted by an observer blinded to the identity of the randomly arranged slides. Average leukocyte density was determined from 4 sections from each animal.
Capillary density  Capillary density was counted as described previously. Briefly, muscle fibers were harvested from the right and left side, from the m. adductor and m. gastrocnemius. The plasma membrane of capillary ECs in tissue sections was labeled with Alexa 633-conjugated wheat germ albumin (WGA, Invitrogen, CA). Micrographs were obtained with the Nikon fluorescence microscope using a 20x objective lens. Digitized images were analyzed with an image analysis package, Image J. The total number of capillaries was counted on 5 random optical fields for each mouse. Results were expressed in capillaries per square millimeters.

Real-time reverse transcription PCR  Total RNA was extracted from static or stretched ECs with Trizol reagents (Invitrogen, Inc, Carlsbad, CA). Total RNA (5μg) was reverse-transcribed into cDNA with (0.5μg) dT17 and SuperScript II (Invitrogen) per manufacturer’s instruction. The primer pairs were as following (“-F”: forward, “-R”: reverse): α-tubulin-F, TGC AAC CAT CAA GAC AAA GC; α-tubulin-R, CAC AGT GGG AGG CTG GTA GT; ephrin B2-F, CAG CTT GTT TAA CGG CAG TGT; ephrin B2-R, CAG CAA TTT GGC AAC CTT TT; MCP-1-F, TGC ATC CAC TAC CTT TTC CA; MCP-1-R, AAG GCA TCA CAG TCC GAG TC. SYBR Green I based real-time PCR (Absolute SYBR Green ROX Mix, Thermo Fisher Scientific, Surrey, UK) was performed in a Rotor Gene thermal cycler (Qiagen, Foster City, CA) with the following thermal parameters: 95°C 15 min, followed by 40 cycles of 95°C for 30sec, 60°C for 30sec, 72°C for 30sec. Data were analyzed using relative real-time PCR quantification based on the ΔΔCt method. α-tubulin was the endogenous reference gene for ephrin B2 and MCP-1, and the control was static ECs.

Statistical analysis  Values are presented as means ± SE. Differences was determined by Student t-test (between two groups) and one-way ANOVA (among multiple groups). A value of $P<0.05$ was considered to indicate statistical significance.
Supplemental Figures and Figure legends

Online Figure I. Perfusion recovery and collateral diameter changes. (A) Ratio of plantar perfusion (ligated vs. sham control side) acutely and 3 weeks after ligation. (B) Collateral lumen diameter of sham controls and 3 weeks after ligation. Values are means ± SE. * p<0.05.
Online Figure II. Plantar perfusion after severe hindlimb ischemia. Proximal lateral caudal femoral artery ligation was performed. Ratio of plantar perfusion (ligated vs. sham control side) quantified from the Doppler images. Values are means ± SE. * p<0.05, compared with the respective time point of PECAM-1+/+; # p<0.05, compared with the respective acute time point.
Online Figure III. Intestinal collateral diameter and EC size. Intestinal collateral caliber (A) and EC size (B) are quantified in both genotypes. Values are means ± SE.
Online Figure IV. Capillary density in *gastrocnemius* muscle during arteriogenesis. (A) Capillary density in *gastrocnemius* muscle 3 weeks after hindlimb ischemic surgery. Sham control is the non-surgery side. Cross-sections were stained with Alexa 633-labeled WGA (red) and DAPI (blue). Images were taken with a 20x objective. Scale bar: 100μm. (B) Capillary density was counted from the images and values are means ± SE; n, number of animals.
Supplemental References

Reference List


