Endothelial Nitric Oxide Synthase Activity Is Essential for Vasodilation During Blood Flow Recovery but not for Arteriogenesis

Barend Mees, Shawn Wagner, Elena Ninci, Silvia Tribulova, Sandra Martin, Rien van Haperen, Sawa Kostin, Matthias Heil, Rini de Crom, Wolfgang Schaper

Objective—Arteriogenesis is the major mechanism of vascular growth, which is able to compensate for blood flow deficiency after arterial occlusion. Endothelial nitric oxide synthase (eNOS) activity is essential for neovascularization, however its specific role in arteriogenesis remains unclear. We studied the role of eNOS in arteriogenesis using 3 mouse strains with different eNOS expression.

Methods and Results—Distal femoral artery ligation was performed in eNOS-overexpressing mice (eNOS(tg)), eNOS-deficient (eNOS(-/-)) mice, and wild type (WT) controls. Tissue perfusion and collateral-dependent blood flow were significantly increased in eNOS(tg) mice compared with WT only immediately after ligation. In eNOS(-/-) mice, although tissue perfusion remained significantly decreased, collateral-dependent blood flow was only decreased until day 7, suggesting normal, perhaps delayed collateral growth. Histology confirmed no differences in collateral arteries of eNOS(tg), eNOS(-/-), and WT mice at 1 and 3 weeks. Administration of an NO donor induced vasodilation in collateral arteries of eNOS(-/-) mice, but not in WT, identifying the inability to vasodilate collateral arteries as main cause of impaired blood flow recovery in eNOS(-/-) mice.

Conclusions—This study demonstrates that eNOS activity is crucial for NO-mediated vasodilation of peripheral collateral vessels after arterial occlusion but not for collateral artery growth. (Arterioscler Thromb Vasc Biol. 2007;27:1926-1933.)

Key Words: endothelial nitric oxide synthase ■ arteriogenesis ■ mouse ■ hind limb ■ vasodilation

The stimulation of vascular growth has become an important therapeutic goal for prevention and treatment of tissue ischemia in cardiovascular disease and is referred to as therapeutic neovascularization. Three distinct processes of vascular growth can contribute to the recovery of blood flow and preservation of tissue: arteriogenesis, ie, collateral artery growth, angiogenesis, ie, sprouting of capillaries from preexisting blood vessels, and vasculogenesis, ie, formation of blood vessels from endothelial progenitors. From a therapeutic point of view, it is essential to isolate the distinct mechanisms of vascular growth, because these occur in different types of tissue and vessels and are regulated by separate stimuli. For example, in the experimental ischemic hind limb model, arteriogenesis is initiated and stimulated in collateral vessels in the upper part of the hind limb by an increase in fluid shear stress. In contrast, in the lower part of the limb, both angiogenesis and vasculogenesis are mainly driven by tissue ischemia. Clinical trials based on stimulation of therapeutic VEGF- and FGF-mediated angiogenesis have not shown convincing results. Therefore, in this study we focus on arteriogenesis, as this is the most upstream mechanism and the most efficient one to provide bulk flow to the ischemic area after occlusion or stenosis of a major artery.

A potential mechanism for inducing therapeutic neovascularization is to increase the production of endothelial NO. eNOS activity has been shown essential for neovascularization. eNOS(-/-) mice display a decreased neovascularization in response to severe ischemia. Recent studies have attributed this to impaired arteriogenesis, angiogenesis, vasculogenesis, or a combination of these. However, in these studies the different competing or complementary mechanisms of vascular growth could not be isolated because of the use of a severe murine ischemia in vivo model, which causes substantial damage to the lower limb. Thus, the role of eNOS in each specific mechanism could not be elucidated. In addition, the role of eNOS in arteriogenesis has been paradoxical. Increased shear stress is known to upregulate the expression of...
eNOS augmenting endothelial NO production.\textsuperscript{11–13} In contrast, NO has also been shown to inhibit expression of adhesion molecules and smooth muscle cell proliferation, which are both indispensable for arteriogenesis.\textsuperscript{11,14,15} Besides, given the importance of eNOS in regulating vascular tone and blood flow, other mechanisms could be involved.

In this study, we investigated the role of eNOS in arteriogenesis in both eNOS\textsuperscript{−/−} and eNOStg mice using a hind limb model, which caused only minimal ischemia in the lower limb, to specifically analyze arteriogenesis. Using this murine arteriogenesis-specific model several research groups have recently described different molecular and cellular mechanisms of arteriogenesis.\textsuperscript{16–18} Two lines of eNOStg mice have been previously generated in our laboratory and overexpress the human eNOS gene.\textsuperscript{19,20} We have recently reported that in the eNOStg mice eNOS expression is functional and restricted to the endothelial lining in all blood vessels. In eNOStg mice eNOS protein and eNOS activity (20-fold greater) levels in the vasculature as well as NO-production (1.8-fold increased) are significantly enhanced, causing a lower blood pressure, lower plasma cholesterol levels, and less atherosclerosis.

In the present study, we first compared eNOStg and WT mice and only found beneficial effects of eNOS overexpression on blood flow recovery immediately after ligation and no further favorable effects on collateral artery growth. Subsequently, we analyzed arteriogenesis in eNOS\textsuperscript{−/−} and WT control mice and confirmed previously published data showing impaired blood flow recovery and increased ischemic tissue damage in eNOS\textsuperscript{−/−} mice. However, we found no differences in collateral growth between eNOS\textsuperscript{−/−} and WT mice. Interestingly, we discovered that in eNOS\textsuperscript{−/−} mice blood flow recovery and clinical outcome after distal femoral ligation were impaired by the inability to sufficiently vasodilate collateral peripheral vessels, and not because of impaired arteriogenesis.

Materials and Methods

Animal Experiments

Transgenic mice overexpressing eNOS-GFP (eNOStg) were generated as previously described.\textsuperscript{22} Briefly, an eNOS-GFP fusion gene was made by inserting a DNA fragment encoding the enhanced green fluorescent protein (GFP) in frame at the stop codon of the complete human eNOS gene and used to perform microinjections of fertilized mouse oocytes. Hemizygous eNOStg mice were used that expressed the human eNOS gene fused to GFP under the regulation of the mouse oocytes. Hemizygous eNOStg mice were used that expressed human eNOS gene and used to perform microinjections of fertilized fluorescent protein (GFP) in frame at the stop codon of the complete human eNOS gene. The eNOStg mice expressed the human eNOS gene and used to perform microinjections of fertilized fluorescent protein (GFP) in frame at the stop codon of the complete human eNOS gene.

Mouse Model of Femoral Ligation

The surgical procedure was performed as previously described.\textsuperscript{7} Briefly, mice were anesthetized with a mixture of ketamin (20 mg/kg) and xylazin (110 mg/kg). After minimal incision in the right mid-thigh, the right superficial femoral artery was dissected and ligated just distally to the origin of the deep femoral artery.

Limb Function and Muscle Atrophy

For a clinical evaluation of the function of the ischemic hind limb the active movement of the right foot was scored (1= no use; 2= standing; 3= normal use without spreading toes; 4= normal use), as previously described.\textsuperscript{21} For evaluating the extent of atrophy the weights of the excised left and right m. gastrocnemius were determined.

Tissue Perfusion

Relative hemoglobin oxygen saturation measurements were performed using an AbTisSpec spectrometer (LEA, Medizintechnik) placing the probe alternately on the left and right foot. Laser Doppler perfusion imaging (LDI; Moor Instruments Ltd) was used for recording serial relative blood flow measurements. The whole region of the foot was analyzed on either side. All measurements were performed in a preheated chamber (37°C) after 5 minutes of warming and under the influence of a mixture of ketamin (15 mg/kg) and xylazin (82 mg/kg). Measurements are expressed as right-to-left ratios.

Collateral-Dependent Blood Flow

Blood flow in the three main arteries of both m. gastrocnemius was analyzed by Magnetic Resonance Imaging (MRI), as previously described.\textsuperscript{22} For further details, see supplemental methods. A subset of mice from WT and eNOS\textsuperscript{−/−} groups was studied after intraperitoneal injection of 5 mg/kg of SNAP (S-nitroso-N-acetylpenicillamine; Sigma).

Histology

For evaluating capillary density, cryosections (7 μm) were cut from the m. gastrocnemius and stained with an antibody against lectin (BS-1; Sigma). Capillaries and muscle fibers were counted and data expressed as capillary-to-muscle fiber ratio. We used 2 different techniques for collateral artery morphometry. In 1 set of mice, for ultrastructure, collateral vessels in the m. adductor were counted and dissected, and embedded as previously described.\textsuperscript{7} Ultra-thin sections (1 μm) were cut and stained with toluidine blue. Collateral arteries (ligated side) and preexisting arterioles (non-ligated side) were then measured with NIH software, and subsequently diameters, wall areas, and wall thicknesses were calculated. In another set of mice, the complete m. adductor was dissected and embedded. Cryosections (7 μm) were cut and immunostained with an antibody against α-SM-actin (Sigma). In both ligated and non-ligated muscles arterioles were identified and measured as above. For further details, see online data supplement.

Statistics

Statistical analysis of all data were performed using 1-way ANOVA followed by a multiple comparison test. Data are reported as means±SEM. Statistical significance was accepted when \( P<0.05 \) (2-tailed).

Results

eNOS-Overexpressing Mice

Tissue Perfusion

Hemoglobin oxygen saturation measurements revealed that oxygen saturation, a marker for tissue perfusion, was only significantly increased in eNOStg mice immediately after ligation, compared with WT (R/L post-ligation: 0.56±0.06 versus 0.24±0.05 in eNOStg and WT, respectively, \( P<0.001; \) Figure 1A). Equally, LDI measurements of the feet only showed a significant increase in relative blood flow in eNOStg mice immediately after ligation, compared with WT
maximal vasodilation in eNOS tg mice but no beneficial effects of eNOS overexpression on arteriogenesis.

**eNOS-Deficient Mice**

**Limb Function and Muscle Atrophy**
Limb function, assessed by a foot movement score, recovered completely to normal within 2 weeks in WT mice, whereas limb function of eNOS−/− mice did not even reach normal levels during complete follow-up (Figure 2A). Similarly, toe necrosis and autoamputation were commonly seen in the eNOS−/− group, in contrary to the mice in the other groups (data not shown). Also, significant more m. gastrocnemius atrophy was seen in the eNOS−/− mice compared with WT mice (R/L muscle weight: 0.94±0.02 versus 0.78±0.04 in WT and eNOS−/−, respectively; P<0.01; Figure 2B).

**Tissue Perfusion**
Oxygen saturation remained significantly impaired in eNOS−/− mice during complete follow-up (Figure 2C). As expected, relative blood flow in the feet, assessed by LDI, remained equally impaired until 3 weeks after ligation (Figure 2D).

**Collateral-Dependent Blood Flow**
In contrast with tissue perfusion findings, a decrease in collateral-dependent blood flow was found only up to 7 days after surgery in eNOS−/− mice, when compared with WT, suggesting normal or possibly somewhat delayed collateral artery growth in eNOS−/− mice (R/L blood flow at day 7: 1.02±0.07 versus 0.69±0.08 in WT and eNOS−/−, respectively; P<0.01; at day 14: 0.81±0.04 versus 0.83±0.08 in WT and eNOS−/−; Figure 3A). In eNOS−/− mice the blood flow even continued to increase and was significantly higher than in WT mice at 3 weeks. To circumvent the effects of vasoconstriction in eNOS−/− mice, we systemically administered at day 7 the NO-donor SNAP to both WT and eNOS−/− mice and measured collateral-dependent blood flow. In WT mice, SNAP induced vasodilation and thus an increase of blood flow in the unligated leg, whereas in the ligated leg no increase in blood flow could be induced, as vasodilation was already at its maximal (or submaximal) level (Figure 3B). In eNOS−/− mice, however, SNAP induced vasodilation and increase of blood flow to the same extent in both legs. This suggested that normal functional collateral arteries had grown in the eNOS−/− mice, but that these were unable to vasodilate sufficiently because of impaired NO production.

**Collateral Morphometry**
Finally, to obtain anatomic data of collateral arteries we studied collateral artery growth in the adductor muscle of the 3 groups of mice using 2 different histological analyses.

**Cryosections**
In complete cross-sections of the adductor muscle conducting arterioles were identified and analyzed (Figure 4A). Diameters of preexisting arterioles in the nonligated adductor muscle did not differ between eNOS tg, WT, and eNOS−/− mice (in μm: 37±2 versus 33±3 versus 34±2 in eNOS tg, WT, and eNOS−/−, respectively; Figure 4B). Collateral diameters significantly and continuously increased after ligation,
as compared with preexisting arterioles, and no differences between collateral diameters from eNOS−/− mice were found at 1 and 3 weeks (in μm: 62±11 versus 76±11 versus 67±15 in eNOS−/−, WT, and eNOS−/−, respectively). Wall areas of preexisting arterioles of eNOS−/− mice were significantly smaller than in WT mice (in μm²: 557±51 versus 958±107 in eNOS−/− and WT, respectively; P<0.01; Figure 4C). In all 3 groups wall areas significantly and continuously enlarged after ligation. At 1 and 3 weeks after ligation no differences were found between the wall areas of collateral arteries from eNOS−/−, WT, and eNOS−/− mice (in μm²: 1098±195 versus 1610±240 versus 1598±321 in eNOS−/−, WT, and eNOS−/−, respectively, at 3 weeks after ligation). Analysis of the wall thickness of collateral arteries revealed the same significant and continuous enlarging after ligation in all 3 groups of mice (Figure 4D). The wall thickness of collateral arteries in eNOS−/− mice was smaller than in WT mice, both before and after ligation. However, no differences in wall thickness were observed between eNOS−/− and WT mice, neither before nor after ligation (in μm: 8.4±0.5 versus 7.8±0.5 in WT and eNOS−/−, respectively, before ligation and 11.8±1.3 versus 10.7±0.8, in WT and eNOS−/−, at 3 weeks after ligation).

Ultra-Thin Sections
In ultra-thin sections cut from the predilection area for collateral artery growth in the adductor muscle, preexisting arterioles or collateral arteries were identified and analyzed (Figure 5A). We did not find any significant differences in diameters and wall area between preexisting arterioles in eNOS−/−, WT, and eNOS−/− mice (Figure 5B and 5C). Three weeks after ligation diameters and wall areas from collateral arteries were significantly enlarged in all 3 groups of mice, as compared with preexisting arterioles. However, as in cryo-sections, between groups no differences were found in collateral artery size. These findings confirmed the above blood flow data and indicate that collateral artery growth was normal in conditions of either deficient or increased eNOS activity.

Capillary Density
Capillary density measurements in the calf muscle confirmed the arteriogenesis-specificity of our hind limb model. In unligated calf muscles of eNOS−/− and eNOS−/− mice capillary density was decreased compared with WT mice (in capillary/fiber ratio: 1.01±0.06 versus 1.23±0.05 versus 1.00±0.04, for eNOS−/−, WT, and eNOS−/−, respectively; P<0.01; Figure 5D). After ligation the capillary/fiber ratio in calf muscles in both eNOS−/− and WT mice did not change, suggesting the absence of an ischemic stimulus for angiogenesis or vasculogenesis in these mice. In eNOS−/− mice, however, a significant 1.8-fold increase in capillaries was seen in the ligated leg, implying the presence of angiogenesis and tissue ischemia.
increase in capillary density. However, in both studies a limb of rats resulted in improved blood flow recovery and study adenoviral eNOS-gene transfer in the ischemic hind and inducing both arteriogenesis in the upper limb and causing large ischemia and tissue damage in the lower limb much more severe hind limb ischemia model was used, overexpression on blood flow recovery after arterial occlusion were found during complete follow-up. In another previously published study performed with a different transgenic artery ligation (n=13). Measurements are expressed as arbitrary intensity units, vs before SNAP, vs WT.

**Discussion**

In the present study we evaluated the role of eNOS in a hind limb model, specific for arteriogenesis. Firstly, to evaluate the possible benefits of elevated eNOS activity on collateral growth we compared transgenic mice overexpressing human eNOS with WT control mice. We only found a beneficial effect of overexpression of eNOS in the acute phase of blood flow recovery after femoral occlusion. At all later time points during follow-up blood flow recovery was equal in eNOStg and WT mice. Histological analysis revealed no differences in collateral artery growth between the 2 groups of mice. In a previously published study performed with a different transgenic mouse overexpressing (bovine) eNOS beneficial effects of eNOS overexpression on blood flow recovery after arterial occlusion were found during complete follow-up. In another study adenoviral eNOS-gene transfer in the ischemic hind limb of rats resulted in improved blood flow recovery and increase in capillary density. However, in both studies a much more severe hind limb ischemia model was used, causing large ischemia and tissue damage in the lower limb and inducing both arteriogenesis in the upper limb and angiogenesis and vasculogenesis in the lower limb. Therefore, beneficial effects of eNOS overexpression could not be extrapolated to 1 single type of vascular growth. Our findings suggest that the beneficial effects of eNOS overexpression on blood flow recovery in a severe hind limb ischemia model are not the result of increased arteriogenesis, but rather increased angiogenesis or vasculogenesis. Besides, the beneficial effect of eNOS overexpression in our study immediately after femoral occlusion suggests an important role for NO-mediated vasodilation in the initial phase of blood flow recovery, when vascular growth cannot have reached a substantial level yet.

Previous studies suggesting a positive role for eNOS in arteriogenesis have used different models, either the severe murine hind limb ischemia model or an exercise based-model in rats. However, in these studies arteriogenesis and angiogenesis were both present and not analyzed separately. The distal femoral artery ligation model used in our study is an in vivo model specific for collateral artery growth. In earlier studies of our group minimal hypoxia and ischemia where found in the lower leg. Capillary density data from our study also suggest the absence of an ischemic stimulus in the lower leg, because no increase of capillary growth was seen after femoral occlusion in control animals. Therefore, our present study is the only study on eNOS and collateral artery growth using an in vivo model specific for this type of vascular growth based on acute increase of shear stress after arterial occlusion.

As we did not find beneficial effects of eNOS overexpression on arteriogenesis, we were interested to evaluate the effect of absence of eNOS activity on arteriogenesis and thus compared eNOS<sup>−/−</sup> and WT mice using the same hind limb model and measurements. In earlier studies using eNOS<sup>−/−</sup> mice and the severe hind limb ischemia model it was already demonstrated that eNOS<sup>−/−</sup> mice had a very poor blood flow recovery, suggested to be caused by impaired angiogenesis or vasculogenesis. Equally, in our study distal femoral artery ligation caused a significantly reduced blood flow recovery as well as tissue damage in eNOS<sup>−/−</sup> mice, despite our observations of an increase in angiogenesis and normal arteriogenesis in eNOS<sup>−/−</sup> mice compared with WT. We demonstrated that the impaired blood flow recovery was not caused by impaired arteriogenesis, but by insufficient vasodilation in the early recovery phase, before collateral arteries were completely formed.

Using MRI, a relatively new technique which measures absolute (and largely deep muscular) blood flow and is less influenced by changes in skin blood flow as LDI, we found in eNOS<sup>−/−</sup> mice a decreased collateral-dependent blood flow only in the first week after ligation. In eNOS<sup>−/−</sup> mice, collateral-dependent blood flow continued to increase during 3 weeks of follow up, whereas in WT mice blood flow remained the same or was slightly decreased after 2 weeks. We suspect that after 2 weeks vasodilation is reduced in WT mice as growing collaterals adequately provide bulk flow to the distal leg, resulting in equal (or slightly decreased) net collateral blood flow after 3 weeks. In the eNOS<sup>−/−</sup> mice, however, in the absence of vasodilation collateral blood flow continues to increase as collaterals continue to enlarge.
Consequently, administration of the NO-donor SNAP confirmed the insufficient vasodilation of collateral arteries in eNOS\(^{-/-}\) mice caused by the absence of eNOS activity.

To obtain conclusive anatomic data of collateral arteries we extensively studied collateral artery growth in the adductor muscle after maximal vasodilation using 2 different histological analyses. Cryosections were immunohistochemically stained and used to study collateral artery growth in the general overview of the whole adductor muscle. For ultra-thin sections only the predilection area for collateral artery growth was isolated. This isolation procedure and the thinness of these sections permitted us to analyze a smaller number of vessels in more detail. Both histological analyses revealed normal collateral artery growth in eNOS\(^{-/-}\) mice after 1 and 3 weeks, suggesting a crucial role for vascular tonus in the early phase after femoral ligation. Finally, the tissue damage

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**Figure 4.** A, Representative photographs of cryosections with a collateral artery (A) in the adductor muscle of eNOS\(^{tg}\), wild-type, and eNOS\(^{-/-}\) mice at different time points. Note the accompanying vein (V). Red=artery wall stained with anti--SMC actin, blue=nuclei stained with Dapi (magnification 400×). B, C, and D, Morphometric measurements of diameter, wall area, and wall thickness of collateral arteries and preexisting arterioles (n=5). *\(P<0.05\), **\(P<0.01\), and ***\(P<0.001\) vs preexistent, *\(P<0.05\), **\(P<0.01\), and ***\(P<0.001\) vs WT.
in the lower limbs of eNOS−/− mice appeared irreversible, because at 3 weeks of follow up muscle weights were decreased, tissue perfusion of the feet remained impaired, and collateral-dependent blood flow was still increasing. This irreversibility stresses the essential role of NO-mediated vasodilation.

Our findings confirm that eNOS activity is essential for an effective restoration of blood flow after femoral artery occlusion, however not by stimulation of arteriogenesis but by inducing NO-dependent vasodilation of the collateral-dependent peripheral vessels. Because arteriogenesis itself is not an immediate process and thus cannot protect tissue from ischemic injury in the acute phase, the combination of both adequate vasodilation (of peripheral collateral vessels) in the acute phase and vascular (ie, collateral artery) growth for continuation is necessary for successful blood flow recovery and tissue salvage.

Clinical trials aiming for therapeutic neovascularization by stimulating vascular growth using different growth factors have shown disappointing results.5,6 These disappointing results might be attributable to the preexistent endothelial dysfunction in patients with atherosclerosis or diabetes. Endothelial dysfunction causes decreased vasoreactivity as a result of a reduced bioavailability of NO. Therefore, there might be (partial) analogies between these patients and eNOS−/− mice, in which arteriogenesis is not impaired but the inability to sufficiently vasodilate resulted in severe ischemic damage. However, similar to the approach to stimulate vascular growth only, stimulation of vasodilation only might not be sufficient to adequately restore blood flow distally.

Figure 5. A, Representative photographs of ultra-thin sections with a collateral artery (A) in the adductor muscle of eNOSGt, wild-type, and eNOS−/− mice, 21 days after femoral artery ligation (magnification 630×). Note the accompanying vein (V) or nerve (N). In all 3 collaterals at least 1 smooth-muscle cell nucleus (*) can be identified. B and C, Morphometric measurements of diameter and wall area of collateral arteries and preexisting arterioles (n=5). #P<0.05, ##P<0.01, and ###P<0.001 vs preexistent. D, Capillary density measurements in the calf muscle (n=5). Measurements are expressed as capillary/fiber ratio, *P<0.05 and ###P<0.001 vs preexistent, *P<0.05 and **P<0.01 vs WT.
from the occlusion in a clinical situation of chronic ischemia in the lower limb. A combination of stimulation of vascular growth and vasodilation could be essential for successful therapeutic neovascularization, specifically in conditions where endothelial dysfunction is present (ie, hypercholesterolemia and diabetes). We are currently investigating this hypothesis using both diabetic and hypercholesterolemic mice.

In summary, eNOS overexpression did not have beneficial effects for collateral artery growth. eNOS deficiency caused severe impaired blood flow recovery after arterial occlusion caused by insufficient vasodilation of collateral-dependent vessels, whereas collateral artery growth was intact. Therefore, eNOS activity is essential for an effective restoration of blood flow after femoral artery occlusion, however not by stimulation of arteriogenesis but by inducing NO-dependent vasodilation of the collateral-dependent peripheral vessels.

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Disclosures
None.

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Correction

In the article by Mees et al, which appeared in the September 2007 issue of the journal (Arterioscler Thromb Vasc Biol. 2007;27:1926–1933. DOI: 10.1161/ATVBAHA.107.145375), there was an error in Figure 2. The correct version of the figure appears below. The online version of the article has been corrected.
Transgenic mice overexpressing eNOS-GFP\(^1\)

A genomic DNA fragment including 6 kb of 5’ sequence, the complete eNOS gene including its native promotor, and 3 kb of 3’ sequence was isolated from a human genomic cosmid library. At the STOP codon of the eNOS gene, a linker was introduced that allowed the in-frame insertion of a BamHI-NotI DNA fragment encoding eGFP which was derived from the pEGFP-N1 plasmid (BD Biosciences Clontech). A solution of 1–2 µg/ml DNA was used for microinjection of fertilized oocytes from FVB donor mice and transplanted into the oviducts of pseudopregnant B10 x CBA mice. Founder mice and offspring were genotyped by PCR. Mice were backcrossed to C57Bl6 for at least ten generations (>99% C57Bl/6). Transgenic mice used in the present study were hemizygous.

Collateral-dependent blood flow

A 7.05T Bruker PharmaScan MR small animal scanner was used for imaging blood flow in the m. gastrocnemius, as previously described\(^2-5\). A fast gradient echo sequence with a slice thickness of 0.62 mm and a repetition time of 20 ms was used to obtain six axial slices along the length of the muscle with an in-plane resolution of 100 x 100 µm for a 2.56 x 2.56 cm imaging area with 16 averages resulting in a total imaging time of 5 minutes. A 90-degree flip angle was used to obtain signal from primarily just the blood flow in the larger vessels. For the
parameters used, the signal intensity obtained is linearly proportional to the blood flow.

A specially designed mouse imaging quadrature probe was used to achieve an adequate signal-to-noise ratio for all experiments. The probe contained a water jacket to allow for mouse heating while in the animal scanner. The ambient temperature was set to 37°C. All mice were preheated for at least five minutes at 37°C before any imaging was done. Right-to-left blood flow ratios were calculated from the intensities obtained from the corresponding right and left m. gastrocnemius. The six slices were averaged to obtain an average blood flow ratio for each mouse at each time point.

**Perfusion Fixation and Tissue Sampling**

At day 7 and 21 after surgery, mice were euthanized. After administration of ketamin/xylazin and heparin (625 IU), we cannulated the left ventricle and perfused the mouse under constant pressure of 100 mm Hg during ten minutes with PBS buffer, containing 0.5% albumin and 0.1% adenosin to achieve maximal vasodilation. Subsequently, we perfused with the fixative, 2% paraformaldehyde, for another ten minutes. For cryo-sections, the complete m. adductor and m. gastrocnemius of both legs were excised. The tissue was post-fixed in 2% paraformaldehyde and cryo-protected overnight in a 10% sucrose solution at 4°C. After 24 hours the tissue was embedded in Tissue-Tek (Sakura Finetek) and snap-frozen in methylbutane chilled with liquid nitrogen. For ultra-thin sections, the predilection place for the main collateral arteries in the adductor
muscle was identified and a superficial rectangle containing these collaterals (right leg) or the pre-existing arterioles (left leg) was excised, as previously described\textsuperscript{6}. The tissue was post-fixed in 3% glutaraldehyde, then in 1% OsO\textsubscript{4}, dehydrated in alcohols and embedded in epon using the Lynx Microscope Tissue Processor (Reichert).

**Capillary Density**
Using the Leica CM 3000 Cryo-microtome cryo-sections (7 µm) were cut from the upper-, middle- and lower part of the m. gastrocnemius. For identifying capillaries and not interfering with the GFP signal in the eNOS-GFP mice we stained sections with a monoclonal TRITC-conjugated antibody against lectin (BS-1, Sigma). The immunoreactions were visualized with a Leica DMLD fluorescence microscope and photographed with the accompanying digital camera. In three fields per section and three sections per part of the muscle the amount of capillaries and muscle fibers, identified by auto-fluorescence, was counted using the NIH software. The mean capillary-to-muscle fiber ratio was calculated per muscle.

**Collateral Artery Morphometry**
*Ultra-thin sections*
Ultra-thin sections were cut from all four parts of the excised rectangle of adductor muscle with the Ultracut Microtome (Reichert) to a thickness of 1 µm. Sections were stained with toluidine blue and analyzed with the Leica DMLD microscope. Criteria for identifying a collateral artery were characteristic location,
proliferating endothelial and smooth-muscle cells, surrounding vein and/or nerve, continuous internal lamina elastica and size. Using the NIH image software we measured the internal and external perimeter of the intima/media, which enabled us to calculate diameter, wall area and wall thickness.

Cryo-sections

Serial cryo-sections (7 µm) were cut and immediately stained with toluidine blue for identifying the first level of insertion of collateral arteries into the superficial femoral artery. From this level on consecutive sections were cut, mounted and immunostained with a monoclonal CY3-conjugated antibody against α-SM-actin (Sigma). Collateral arteries (ligated side) and pre-existing arteries (non-ligated side) were identified by the presence of a pronounced α-SM-actin positive media. Internal and external perimeters of intima/media were measured as in semi-thin sections and subsequently diameter and wall area were calculated.
References


