Published in final edited form as:

Microcirculation. 2011 April ; 18(3): 163–171. doi:10.1111/j.1549-8719.2011.00085.x.

A new method for *in vivo* visualization of vessel remodeling using a near-infrared dye

Marie Billaud¹, Jeremy A Ross¹, Mark A Greyson¹, Anthony C Bruce², Scott A Seaman², Katherine R Heberlein^{1,3}, Jenny Han¹, Angela K Best¹, Shayn M Peirce², and Brant E Isakson^{1,3,*}

¹ Robert M. Berne Cardiovascular Research Center, University of Virginia School of Medicine, PO Box 801394, Charlottesville, VA 22908

² Department of Biomedical Engineering, University of Virginia School of Engineering

³ Department of Molecular Physiology and Biological Physics, University of Virginia School of Medicine

Abstract

Intro—Vascular obstructive events can be partially compensated for by remodeling processes that increase vessel diameter and collateral tortuosity. However, methods for visualizing remodeling events *in vivo* and with temporal comparisons from the same animal remain elusive.

Methods—Using a novel infrared conjugated polyethylene glycol dye, we investigated the possibility of intravital vascular imaging of the mouse ear before and after ligation of the primary feeder artery. For comparison, we used two different mouse models known to have impaired vascular remodeling post ligation (i.e. aged and PAI-1^{-/-} mice). The results obtained with the infrared dye were confirmed using immunofluorescence labeling of the ear microvasculature with confocal microscopy.

Results—After ligation, increases in vessel diameter (between 10% and 60%) and tortuosity (approximately 15%) were observed in C57Bl/6 mice using both the infrared dye and the immunofluorescence technique. However, aged C57Bl/6 and PAI-1^{-/-} mice did not show vascular remodeling following ligation.

Conclusion—Vascular remodeling can be visualized and accurately quantified using a new infrared dye *in vivo*. This analysis technique could be generally employed for quantitative investigations of changes in vascular remodeling.

Keywords

vascular remodeling; *in vivo* imaging; plasminogen activator inhibitor-1; aging

Introduction

Microvascular remodeling is the result of complex processes that include changes in the structure and function of the vessel wall via modulation of cell adhesion, migration, proliferation, production and degradation of extracellular matrix (ECM) and cell death (for review, see ¹). These processes usually occur during pathological conditions as adaptive processes. For example, when an artery is stenosed or occluded, arteriogenesis and

* to whom correspondence should be addressed, bei6n@virginia.edu, T: 434-924-2093, F: 434-924-2828.

angiogenesis will occur as processes of microvascular remodeling in order to maintain blood supply in the region downstream². However, this effect is not observed in aged populations of humans³. Indeed, it has been reported that patients with peripheral arterial disease exhibit impaired vascularization and vascular remodeling leading to critical limb ischemia and amputation. According to a recent study in the United States, approximately one in five patients above the age of 65 hospitalized for peripheral arterial disease undergoes limb amputation⁴. Aged mice also exhibit impaired response to ischemia induced by vascular occlusion and have reduced capacity to remodel microvascular networks in order to restore perfusion⁵.

Vascular remodeling can also be reduced by dysregulation of the plasminogen activation system⁶. Plasminogen activator inhibitor-1 (PAI-1) was initially described as an enzyme capable of regulating the plasminogen activator system and was shown to play a role in vascular remodeling⁷. However, the effects of PAI-1 on vascular remodeling are currently undefined due to conflicting results that suggest PAI-1 can both promote and prevent tumor growth and tumor angiogenesis⁷, a phenomenon that is sometimes referred to as the '*PAI-1 paradox*'⁸.

Because vascular remodeling plays a critical role in several vascular diseases (e.g., occlusion, atherosclerosis, restenosis and pulmonary hypertension^{9–11}), there has been an emphasis on developing techniques to quantify vasculature remodeling *in vivo*. Although current *in vivo* techniques utilizing microCT, ultrasound, and microPET produce good resolution, they require specialized equipment and a great expense¹². In the present study, we demonstrate a new technique to visualize changes in the mouse ear vasculature after ligation by injecting an infrared dye attached to a polyethylene glycol (PEG) molecule and imaging on a Li-cor scanner. To test the feasibility of the method, we compared ear vasculatures of 10–16 week old C57Bl/6 mice to both aged C57Bl/6 mice and PAI-1^{-/-} mice after arterial occlusion and found significant differences in both vessel diameter and tortuosity between study groups. This new method was validated using confocal microscopy to visualize and quantify changes in the microvasculature following immunolabeling of excised ears with antibody directed against smooth muscle α -actin¹³. We believe this analysis method will be helpful for obtaining rapid, high-resolution, quantifiable images of *in vivo* vessels undergoing remodeling.

Materials and Methods

Animals

All mice were treated in accordance with approved animal protocols by the University of Virginia Animal Care and Use Committee. Young male C57Bl/6 and PAI-1^{-/-} (Jackson Laboratory) were all aged 10–16 weeks, and aged male C57Bl/6 mice were 52 weeks old (National Institute of Aging).

Visualization of the ear vasculature using infrared dye 800 IR PEG Dye

Animals were anesthetized with an intraperitoneal injection of ketamine/xylazine/saline (15 ml/kg of body weight) prior to each intervention described below. The day before the first control image was taken, depilatory cream was applied topically to the right ear and then circumferentially around the ear using a cotton-tipped applicator in order to remove the surrounding hair. Caution was taken to avoid placing excessive pressure on the ear during the hair removal as we noted this resulted in damage to the vasculature and necrosis of the ear. The depilatory cream was exposed to the ear for approximately 8–10 seconds and then gently washed off using dampened sterile gauze pads. On day 0, the mice were injected intravenously with an infrared dye attached to a polyethylene glycol (PEG) molecule (800

IR PEG; Li-Cor) at 5 pmol/g (unless otherwise stated) via the lateral tail vein. The mice were immediately placed supinely on the odyssey imaging system purchased from Li-cor and their right ears were simultaneously excited with 2 diode lasers at 785 nm and 685 nm excitation to measure the 800 IR PEG and the background noise, respectively. Fluorescence emitted either by the 800 IR PEG or the background were split into two fluorescent signals after passing through a dichroic mirror. The fluorescent signals travelled through two independent optical paths and were focused on separate silicon avalanche photodiodes and detected. The two colors were imaged simultaneously in a single scan with a resolution of 42 μm .

Visualization of immunostained ear vasculature using confocal microscopy

Mice were anesthetized and hair was removed from the right ear as described above. The right ear was harvested at either one or seven days after ligation or sham surgery was performed and placed in PBS and the external layer of the epithelium was removed under a stereomicroscope. Ears were permeabilized in PBS containing 0.1% saponin for 72 hours at 4°C. The 0.1% saponin/PBS solution was changed three times during the incubation. Immunolabeling was performed as previously described¹³ using monoclonal anti-smooth muscle α -actin antibody pre-conjugated to CY3 to label smooth muscle cells. Ears were imaged using a Nikon TE 2000-E2 confocal microscope equipped with a 10x objective.

Ligation of the primary feeder artery of mouse ear

A mouse ear is usually supplied by three neurovascular bundles containing an artery, vein, and nerve¹⁴. The central artery has the highest caliber and characteristically branches into two primary arterioles within 10 mm of exiting the neck. On the right ear, the site 5 mm proximal to this branch point was chosen for ligation (Fig. 3). Following sterile preparation and using aseptic technique, an incision of 5 mm proximal to major branch point of primary artery was performed. The artery was carefully dissected away from the vein and a 9-0 nylon suture was passed between the artery and the vein. The suture was tied around the artery and the process was repeated with another suture distally. The artery was then cut between the sutures. Caution was taken not to damage surrounding tissue or other contents of the neurovascular bundle. The incision site was then closed with a 9-0 nylon suture and the mouse was allowed to recover by injecting pre-warmed 0.9% saline solution subcutaneously. For sham surgery, the procedure was carried out exactly as mentioned above, except that no ligation of the artery was performed after sutures were gently looped around the vessel. Ligation was performed on two groups of mice: one group of mice underwent ligation one day before processing the mice either for scanning or for harvesting and immunolabeling, and the other group of mice underwent surgery 7 days before processing the mice so that all post-ligation experiments were performed on the same day (Fig. 2A).

Data collection

Using Metamorph, the primary branch point distal to the ligation was marked and used as a reference point for measurements. Control and post-ligation vessel diameters were measured in both left and right primary bifurcation at 10 pixels distal to the bifurcation site for the images obtained with the Li-Cor (Fig. 3A), and at 200 μm distal to the bifurcation site for the images obtained with the confocal microscope (Fig. 3B left panel). Control and post-ligation images were overlaid in Adobe Photoshop using the main feeder artery as the reference point and vascular changes in vessel diameter between post-operation and control were calculated as percentage difference. Tortuosity was measured one or seven days after ligation using Metamorph software. The actual length of the collateral vessel between the two arteries and the straight line distance between the two arteries were measured and the resulting ratio of these two numbers was our Collateral Ratio. Statistical significance was

calculated using one-way ANOVA test with a p-value of 0.05 considered significantly different.

Results

Pharmacokinetics of 800 IR PEG Dye

Total fluorescence of the ear using the 800 nm laser (800 IR PEG dye) and the 700 nm laser (background) were quantified for each dose (0.1 pmol/g, 1 pmol/g, and 10 pmol/g) as a function of injection at day 0 (Fig. 1A–B). Results show that the $t_{1/2}$ of the 800 IR PEG dye is approximately one week; after this amount of time, approximately half of the original fluorescence had either decayed or the dye was excreted by the animal (Fig. 1A). There was no change in the background fluorescence in the 700 channel, indicating minimal bleed-through of the 800 IR PEG dye into that channel (Fig. 1B). These results suggested that the optimal balance between rapid washout and clear imaging occurred when using approximately 5 pmol/g of the 800 PEG dye. Using this concentration, we obtained the representative images in Fig. 1C–E. Using 5 pmol/g of 800 IR PEG dye is therefore sufficient to visualize the mouse ear vasculature immediately after injection, while allowing near complete dye clearance within two weeks.

Visualization of vascular remodeling using 800 IR PEG Dye after ligation

We hypothesized that we could use the dye to visualize vessel remodeling in the mouse ear. To prove this, we used the experimental timeline defined in Fig. 2A. Control scans of the right ear were taken at day 0. As the dye clearance requires two weeks, post ligation scans had to be performed two weeks after controls scans (day 14). Therefore, the ear right main feeder artery was ligated either seven days or one day, prior to post-ligation scans (Fig. 2A and 2B). Representative images of the mouse ear vasculature before (control), one day or seven days after the ear surgery are shown in Fig. 2C and 2D, respectively. Control (green) and post-ligation (red) images were merged to assess the vascular changes post-ligation. As illustrated on Fig. 2C and D, 90 to 95% of the dye was retained in the vasculature immediately after injection of the dye.

Vessel remodeling after ligation

In order to quantify vascular remodeling in the mouse ear, the diameters of both the left and right bifurcation of the main ear arteries immediately after bifurcation of the ligated feed artery were measured one or seven days after ligation. One day after ligation, the young C57Bl/6 mice exhibited an approximate 65% increase in vessel diameter as compared to sham operated mice, which was significantly greater than changes in the sham, aged, or PAI-1^{-/-} mice (Fig. 4A). This trend was still apparent seven days after ligation (Fig. 4B), although there was a larger degree of variability observed in the left bifurcated vessel and the increase in vessel diameter was not as great seven days post-ligation as that seen one day after ligation. The overall trend of decreased vessel diameter changes in response to ligation in the aged and PAI-1^{-/-} mice is in agreement with previously published results^{5, 15}.

In addition to changes in vessel diameter, modification in tortuosity of collateral vessels is another common vascular remodeling event observed after ischemic injury¹⁶. We compared collateral length ratios in mouse ears either one (Fig. 5A) or seven (Fig. 5B) days after ligation. We found increased collaterals in the C57Bl/6 mice as compared to the aged C57Bl/6 and PAI-1^{-/-} mice regardless of the number of days after ligation (Fig. 5A and 5B). This data is consistent with previously reported data on a lack of vascular remodeling in aged and PAI-1^{-/-} mice^{6, 17}. In summary, these data suggest the use of the 800 IR PEG dye may be a novel tool to quantify vessel remodeling and also confirm previously reported

observations of microvascular remodeling in different mouse populations in this specific tissue bed.

Confocal microscopy of immunostained ear vasculature

In order to validate the model system described above, the same ligation experiments were reproduced on C57/Bl6 and PAI1^{-/-} mice and immunostained ear vasculature was visualized by confocal microscopy. One day after ligation, the arterial diameter of C57/Bl6 mice was increased by 25%, which was greater than increased in diameter observed in the sham and the PAI1^{-/-} groups (Fig. 6A left panel). The diameters of both left and right bifurcation of the main ear feeder artery of C57/Bl6 (left bifurcation: $51.42 \pm 3.3 \mu\text{m}$; right bifurcation: $51.87 \pm 2.9 \mu\text{m}$) were significantly increased compared to sham (left bifurcation: $37.71 \pm 6.6 \mu\text{m}$; right bifurcation: $39.71 \pm 4.3 \mu\text{m}$, Fig. 6B left panel). As previously observed with the 800 IR PEG dye, only the right bifurcation in the C57/Bl6 group exhibited a significant increase in diameter compared to sham and PAI1^{-/-} groups at day 7 (Fig. 6A and B right panels). Interestingly, the arterial diameters of the left and right bifurcation of the main ear feeder artery of the PAI1^{-/-} mice were significantly higher compared to the C57/Bl6 mice (left bifurcation: $49.09 \pm 3.5 \mu\text{m}$ versus $40.37 \pm 2.11 \mu\text{m}$; right bifurcation: $46.9 \pm 3.48 \mu\text{m}$ versus $40.99 \pm 2.8 \mu\text{m}$). This observation reinforces the absence of increase of the right bifurcation diameter observed in PAI1^{-/-} mice seven days after ligation (Fig. 6B right panel). Furthermore, this observation can also explain the absence of significant differences between arterial diameter from C57/Bl6 and PAI1^{-/-} groups one day after surgery (Fig. 6B left panel).

Tortuosity was also measured on the immunolabeled ears one or seven days after ligation of the main ear feeder artery in C57/Bl6 mice and PAI1^{-/-} mice. Using this technique, the collateral ratio between each group was unchanged one day after ligation (Fig. 6C left panel). Although there was a larger degree of variability observed seven days after ligation, the overall trend shows that the collateral ratio in C57/Bl6 mice was higher than the collateral ratio in sham and PAI1^{-/-} groups (Fig. 6C right panel). In summary, these results confirm the validity of our new *in vivo* method using the 800 IR PEG dye.

Discussion

Thrombotic occlusion of arteries often causes severe ischemia in the affected tissues, which can lead to numerous vascular changes. Therefore, significant efforts have been made to visualize microvascular remodeling processes induced by thrombotic inclusion. In this study, we developed a new method for the visualization of *in vivo* vascular remodeling. Using a model of arterial ligation in mouse ear, we optimized the use of an infrared dye in an attempt to measure vessel diameter and tortuosity of the ear microvasculature. Both parameters were noticeably increased after arterial ligation in young C57Bl/6 mice. Because vascular adaptation to arterial obstruction is known to be dysfunctional in aging population and in mice lacking PAI-1 gene expression, we used these two different mouse models to verify our visualization method. Our results show that vascular remodeling is highly impaired both in aged (52 weeks) C57Bl/6 and PAI-1^{-/-} mice. Collectively, these data demonstrate the feasibility of using this particular imaging technique as a mean of monitoring and quantifying vessel remodeling *in vivo*.

Although there are several well-established models for evaluating arteriogenesis by inducing ischemia and assessing the resulting vascular changes, there are distinct drawbacks to these methodologies. Immunohistochemical studies, for example, require that the tissue of interest is harvested for visualization and therefore no initial (pre-intervention) image can be generated. In addition, these values are obtained from the outside of the vessel whereas our values are obtained from inside the vessel. Besides this method, the vasculature can be

visualized *in vivo*, but this requires specialized equipment (e.g., microCT or microPET). Furthermore, these techniques have resolution limits that are above what we were able to measure using our approach. Using an 800 IR PEG Dye, we investigated the possibility of intravital vascular imaging of the mouse ear before and after ligation. We initially investigated the basic pharmacokinetic properties of this dye. Our results show that the dye is retained in the vasculature for one day before it leaks into the extracellular space (Fig. 1). Furthermore, the $t_{1/2}$ of the PEG dye is approximately one week. This has important consequences for the development of future experimental protocols in that serial injections of the dye must be separated by at least several weeks to ensure adequate clearance; therefore, a clear second (post-intervention) image can be captured without background generated by dye leakage into extracellular space. These optimization experiments allowed us to conclude that the optimum concentration of dye is 5 pmol/g and a series of injections must be separated by two weeks to obtain satisfactory images of the ear vasculature.

The data presented in Fig. 4 illustrate that the method developed in this study can be used to serially and noninvasively track vascular changes over a relatively large vascular bed in a single mouse following a localized ligation. In young C57Bl/6 mice on post-ligation day 1, the significant data points illustrate that the vascular changes occurred are due to ligation of the main feeder artery, not simply to surgical manipulation of the vessels and tissue beds (C57Bl/6 vs Sham). This is key to interpreting our data as it is well known that surgical procedures, as well as ligation, can cause acute hypoxia and increased blood flow downstream of the ligation site, resulting in vasodilation^{18, 19}. Thus, we can infer that vasodilation is an early response to ligation in the mouse ear that may help to maintain adequate blood flow. Additionally, the maintenance of vasodilation and the development of vessel tortuosity at post-operative day 7 further illustrate prolonged structural changes following ligation and thus strengthen our assertion that the observed changes are not due to surgical manipulation.

Compared with 10–16 week old C57Bl/6 mice, 52 week old C57Bl/6 mice did not show any significant vascular changes in response to ligation. It was hypothesized that 52 week old mice would have decreased vascular response to ligation⁵. Increased arterial stiffness and impaired vascular endothelial function are the two most clinically important events that occur with vascular aging in humans. Together they contribute to age-associated complication of atherosclerosis and peripheral vascular diseases²⁰. In addition, endothelial nitric oxide synthase (eNOS) and NO production have been shown to be reduced with aging, which, in combination with increased arterial stiffness, can impair dilation of the senescent vasculature, thereby limiting vascular remodeling after ligation³. Hypoxia-inducible factor (HIF)-1 is another key molecule in vascular adaptation to ligation as it induces the expression of several growth factors which consequently trigger vascular remodeling²¹. Several studies show that HIF-1 expression decreases with aging, this phenomenon is, in part, responsible for the impaired vascular remodeling in response to ischemia in old population²¹.

Plasminogen activator inhibitor-1 appears to be a key factor at the intersection between thrombosis, fibrin deposition, extracellular matrix degradation, as well as the balance between cell death and proliferation and has thus been suggested to be involved in the vascular remodeling processes⁶. However, the role of PAI-1 in promoting or preventing changes in vessel structure is defined as paradoxical, where PAI-1 is proangiogenic at physiological concentrations and antiangiogenic at lower and higher levels¹⁵. This is consistent with our results showing that PAI-1^{-/-} did not undergo any vascular changes in response to ligation. Interestingly, several studies demonstrated that PAI-1 level is increased in aging population, which is coherent with our results demonstrating that vascular remodeling was reduced in aged mice^{22, 23}.

As with the development of any novel method, the advantages and drawbacks of the procedure must be defined. We believe that our current method is useful in that it provides for noninvasive monitoring of vascular changes in a single mouse over an extended period of time with interventions. Traditionally, the comparison of vascular responses requires animal sacrifice and does not allow for an internal control as our method does. In addition, our procedures are relatively easy to learn and use instruments that are easily accessible to many laboratories. However, we did note that the images we obtained with the 800 IR PEG dye did not allow for distinction between arterial and venous structures, thus we can not easily localize vascular changes to either vascular component. Additionally, the pharmacokinetics of the 800 PEG IR dye requires two weeks between scans and the procedures are therefore time intensive. This new method using the 800 IR PEG dye might only be applicable to superficial vasculatures (such as ear vasculature) due to the use of a flat bed scanner, while deeper vasculature such as hind limb vasculature, might be difficult to image using our method. Furthermore, this new method could be clinically relevant as it could be used as a model of local ischemia in the skin which can appear for example during cutaneous ulcer.

The data presented in Fig. 6 validates the results we obtained with the 800 IR PEG dye. The overall trend of increased vessel diameter in the C57/Bl6 mice and absence of increased vessel diameter in the PAI-1^{-/-} mice observed with the 800 IR PEG dye was confirmed using the traditional immunolabeling technique. Interestingly, the absence of increased vascular diameter of the left bifurcation observed in C57/Bl6 mice 7 days after ligation with the 800 IR PEG dye was confirmed using the immunolabeling technique. However the results obtained with the 800 IR PEG dye allowed us to detect an approximately 65% increase of vessel diameter in response to ligation in c57/Bl6 mice whereas the immunofluorescence technique allowed us to detect only 30 to 40% increase in vessel diameter. Although there is a large difference in these values, the trends were exactly the same between methods. Taken in this manner, it is possible that our method would allow for detection of very small changes in vascular diameter that would not be distinguishable using the common immunolabeling method.

In summary, we find the 800 IR PEG dye a useful tool for visualizing and quantifying vascular changes in the ear following arterial ligation. In addition, the results we obtained with PAI-1^{-/-} mice and aged mice are consistent with the literature, validating our model and method.

Acknowledgments

This work was supported by an AHA SDG (BEI) and NIH HL088554 (BEI).

References

1. Peirce SM, Skalak TC. Microvascular remodeling: a complex continuum spanning angiogenesis to arteriogenesis. *Microcirculation*. 2003; 10(1):99–111. [PubMed: 12610666]
2. Lehoux S, Levy BI. Collateral artery growth: making the most of what you have. *Circ Res*. 2006; 99(6):567–569. [PubMed: 16973912]
3. Isenberg JS, Hyodo F, Pappan LK, Abu-Asab M, Tsokos M, Krishna MC, Frazier WA, Roberts DD. Blocking thrombospondin-1/CD47 signaling alleviates deleterious effects of aging on tissue responses to ischemia. *Arterioscler Thromb Vasc Biol*. 2007; 27(12):2582–2588. [PubMed: 17916772]
4. Rowe VL, Lee W, Weaver FA, Etzioni D. Patterns of treatment for peripheral arterial disease in the United States: 1996–2005. *J Vasc Surg*. 2009; 49(4):910–917. [PubMed: 19341885]
5. Zhuo Y, Li SH, Chen MS, Wu J, Kinkaid HY, Fazel S, Weisel RD, Li RK. Aging impairs the angiogenic response to ischemic injury and the activity of implanted cells: combined consequences

- for cell therapy in older recipients. *J Thorac Cardiovasc Surg.* 139(5):1286–1294. e1281–1282. [PubMed: 19931095]
6. Diebold I, Kraicun D, Bonello S, Gorlach A. The 'PAI-1 paradox' in vascular remodeling. *Thromb Haemost.* 2008; 100(6):984–991. [PubMed: 19132221]
 7. Stefansson S, McMahon GA, Petitclerc E, Lawrence DA. Plasminogen activator inhibitor-1 in tumor growth, angiogenesis and vascular remodeling. *Curr Pharm Des.* 2003; 9(19):1545–1564. [PubMed: 12871067]
 8. Sobel BE. Increased plasminogen activator inhibitor-1 and vasculopathy. A reconcilable paradox. *Circulation.* 1999; 99(19):2496–2498. [PubMed: 10330378]
 9. Schoenhagen P, Ziada KM, Vince DG, Nissen SE, Tuzcu EM. Arterial remodeling and coronary artery disease: the concept of “dilated” versus “obstructive” coronary atherosclerosis. *J Am Coll Cardiol.* 2001; 38(2):297–306. [PubMed: 11499716]
 10. Gibbons GH, Dzau VJ. The emerging concept of vascular remodeling. *N Engl J Med.* 1994; 330(20):1431–1438. [PubMed: 8159199]
 11. Humbert M, Morrell NW, Archer SL, Stenmark KR, MacLean MR, Lang IM, Christman BW, Weir EK, Eickelberg O, Voelkel NF, Rabinovitch M. Cellular and molecular pathobiology of pulmonary arterial hypertension. *J Am Coll Cardiol.* 2004; 43(12 Suppl S):13S–24S. [PubMed: 15194174]
 12. Zagorchev L, Mulligan-Kehoe MJ. Molecular imaging of vessels in mouse models of disease. *Eur J Radiol.* 2009; 70(2):305–311. [PubMed: 19304428]
 13. Taylor AC, Seltz LM, Yates PA, Peirce SM. Chronic whole-body hypoxia induces intussusceptive angiogenesis and microvascular remodeling in the mouse retina. *Microvasc Res.* 79(2):93–101. [PubMed: 20080108]
 14. Demicheva E, Hecker M, Korff T. Stretch-induced activation of the transcription factor activator protein-1 controls monocyte chemoattractant protein-1 expression during arteriogenesis. *Circ Res.* 2008; 103(5):477–484. [PubMed: 18669921]
 15. Devy L, Blacher S, Grignet-Debrus C, Bajou K, Masson V, Gerard RD, Gils A, Carmeliet G, Carmeliet P, Declercq PJ, Noel A, Foidart JM. The pro- or antiangiogenic effect of plasminogen activator inhibitor 1 is dose dependent. *FASEB J.* 2002; 16(2):147–154. [PubMed: 11818362]
 16. Bailey AM, O'Neill TJ, Morris CE, Peirce SM. Arteriolar remodeling following ischemic injury extends from capillary to large arteriole in the microcirculation. *Microcirculation.* 2008; 15(5):389–404. [PubMed: 18574742]
 17. Rivard A, Fabre JE, Silver M, Chen D, Murohara T, Kearney M, Magner M, Asahara T, Isner JM. Age-dependent impairment of angiogenesis. *Circulation.* 1999; 99(1):111–120. [PubMed: 9884387]
 18. Lombard JH, Duling BR. Relative importance of tissue oxygenation and vascular smooth muscle hypoxia in determining arteriolar responses to occlusion in the hamster cheek pouch. *Circ Res.* 1977; 41(4):546–551. [PubMed: 902360]
 19. Unthank JL, Nixon JC, Burkhart HM, Fath SW, Dalsing MC. Early collateral and microvascular adaptations to intestinal artery occlusion in rat. *Am J Physiol.* 1996; 271(3 Pt 2):H914–923. [PubMed: 8853325]
 20. Seals DR, Moreau KL, Gates PE, Eskurza I. Modulatory influences on ageing of the vasculature in healthy humans. *Exp Gerontol.* 2006; 41(5):501–507. [PubMed: 16537099]
 21. Bosch-Marce M, Okuyama H, Wesley JB, Sarkar K, Kimura H, Liu YV, Zhang H, Strazza M, Rey S, Savino L, Zhou YF, McDonald KR, Na Y, Vandiver S, Rabi A, Shaked Y, Kerbel R, Lavalley T, Semenza GL. Effects of aging and hypoxia-inducible factor-1 activity on angiogenic cell mobilization and recovery of perfusion after limb ischemia. *Circ Res.* 2007; 101(12):1310–1318. [PubMed: 17932327]
 22. Yamamoto K, Takeshita K, Kojima T, Takamatsu J, Saito H. Aging and plasminogen activator inhibitor-1 (PAI-1) regulation: implication in the pathogenesis of thrombotic disorders in the elderly. *Cardiovasc Res.* 2005; 66(2):276–285. [PubMed: 15820196]
 23. Serrano R, Villar M, Gallardo N, Carrascosa JM, Martinez C, Andres A. The effect of aging on insulin signalling pathway is tissue dependent: central role of adipose tissue in the insulin resistance of aging. *Mech Ageing Dev.* 2009; 130(3):189–197. [PubMed: 19063913]

Figure 1. Dose-response of 800-PEG in mouse ears

The 800-PEG dye was tail-vein injected into mice and the integrated fluorescent intensity from the mouse ears was measured at time points listed in the 800 nm wavelength (A) and the 700 nm wavelength (B). Representative image of a merged 800 nm (green) and 700 nm (red) image from a C57Bl/6 is demonstrated in C–E. Note the clearance of the 800-PEG after 14 days. Scale bar in C is 2 mm.

Figure 2. Arterial ligation of mouse ear main feeder artery and *in vivo* visualization

In A, a schematic representation of the timeline of events is listed. Photographs of mouse ears are demonstrated in B, with enlargement demonstrated by blue box. The asterisk in each image is the site of ligation, the arrow is the artery, and the arrowhead is the vein. Note the double suture (pink) in far right image. Representative images from a C57Bl/6 are shown one day after ligation (C) or seven days after ligation (D). Note that although both the control image and the scan after ligation were imaged using 800-PEG, the scan after ligation (either Day 1 or Day 7) have been pseudocolored (control, green; post-ligation, red) to show potential differences in the merged images. Scale bar in C is 3 mm.

Figure 3. Sites of measurements performed on mouse ear

(A) Cartoon representing a mouse ear, the point of ligation is marked by an asterisk (*). Vessel diameter measurements on the Li-cor scans (Fig. 4) were performed 10 pixels downstream the first bifurcation of the main ear feeder artery, both on left and right bifurcations. The collateral ratios (Fig. 5) were obtained by measuring the tortuosity of bridging vessels between two main arteries of the mouse ear. (B) Representative images of ear vasculature labeled for α -actin. The diameter of left and right bifurcations of the main ear feeder artery were measured at 200 μm downstream the bifurcation (arrows on the left panel). The point of ligation is marked by an asterisk (*). Right panel: representative image of a collateral arteriole used for tortuosity measurements (arrow). Scale bar = 100 μm .

Figure 4. Vessel diameter measurements after ligation using the 800 PEG dye technique
The percent diameter as compared to control scan from the left and right main artery after bifurcation were measured either one day (A) or seven days (B) after ligation. $*=p<0.05$ when compared to C57.

Figure 5. Collateral ratio measurement of tortuosity

The tortuosity ratio of collateral vessels in each mouse paradigm was randomly measured either one day (A) or seven days (B) after ligation. *= $p < 0.05$ when compared to C57.

Figure 6. Validation of the 800 IR PEG dye technique using immunofluorescence coupled to confocal microscopy

(A) The percent diameter change as compared to control ears from the left and right bifurcation of the main artery were measured one day (left panel) and seven days (right panel) after ligation. (B) The diameters of the left and right bifurcation of the main artery were measured either one day (left panel) or seven days (right panel) after ligation of the main ear feeder artery. (C) The tortuosity ratio of the collateral vessels in C57 mice and PAI^{-/-} mice was randomly measured one day (left panel) and seven days (right panel) after ligation of the main ear artery. * = $p < 0.05$ when compared to C57.