Assessing Large-Vessel Endothelial Permeability Using Near-Infrared Fluorescence Imaging—Brief Report

Erik Lundeberg, Anne M. Van Der Does, Ellinor Kenne, Oliver Soehnlein, Lennart Lindbom

Objective—Loss of endothelial barrier function in arterial blood vessels is characteristic of vascular pathologies, including atherosclerosis. Here, we present a near-infrared fluorescence (NIRF) imaging methodology for quantifying endothelial permeability and macromolecular uptake in large arteries in the mouse and evaluate its applicability for studying mechanisms of vascular inflammation.

Approach and Results—To validate the NIRF methodology, macrovascular inflammation was induced in C57bl/6 mice by local tumor necrosis factor-α stimulation of the carotid artery or in apolipoprotein E–deficient mice by Western diet for 4 weeks. Evans blue dye, serving as plasma protein marker and fluorescent in the near-infrared spectrum, was given intravenously at different doses. Carotids and aorta were excised, and Evans blue dye fluorescence was assessed through whole vessel scan in an infrared imaging system. NIRF correlated to extraction–absorbance methodology for Evans blue dye quantification and was superior at discriminating plasma protein accumulation in tumor necrosis factor-α–stimulated carotids. NIRF allowed for focal quantification of increased arterial wall Evans blue dye uptake in apolipoprotein E–deficient mice. Importantly, NIRF left vessels intact for subsequent histological analysis or quantification of leukocyte subpopulations by flow cytometry.

Conclusions—The described NIRF methodology provides a sensitive and rapid tool to locate and quantify macromolecular uptake in the wall of arterial blood vessels in vascular pathologies in mice. (Arterioscler Thromb Vasc Biol. 2015;35:783-786. DOI: 10.1161/ATVBAHA.114.305131.)

Key Words: atherosclerosis ■ endothelium ■ imaging ■ inflammation ■ permeability

Atherosclerosis is a chronic inflammatory disease of large and medium-sized arteries and the major cause of cardiovascular disease. Increased arterial endothelial permeability and entry of macromolecules, such as lipoproteins, into the subendothelial space are considered critical events in atherosclerotic lesion formation. Interventions aimed at stabilizing the arterial endothelial barrier may, therefore, be beneficial in reducing atherosclerotic risk. For several decades, detection of the intravenously injected plasma albumin marker Evans blue (EB) in the arterial wall has been used to assess arterial endothelial permeability in various animal models. From these observations, it is concluded that focal patterns of EB uptake correlate to atheroprone regions in the arterial tree, coinciding with locally disturbed hemodynamics. EB also shares spatial similarities with aortic uptake of radiolabeled low-density lipoprotein, supporting its use as a marker of intramural distribution of atherogenic lipoproteins. Although regional variations in EB uptake may be assessed by color photography or fluorescence imaging, its quantification has required extraction of the dye from excised vessels and analysis by optical absorbance measurement, a time-consuming process rendering the tissue unsuitable for further analysis. Our objective was to overcome these disadvantages by introducing a rapid and sensitive method for quantification of EB accumulation in intact arteries in the mouse, using the fluorescent properties of EB as detected by near-infrared fluorescence (NIRF) imaging.

Materials and Methods
Materials and Methods are available in the online-only Data Supplement.

Results
To validate the NIRF methodology, a model of local tumor necrosis factor-α–induced acute inflammation of the mouse carotid artery was applied. Different doses of EB (0.01–1 mg/mouse) were injected intravenously to determine the optimal dose for detection by NIRF. At all EB doses NIRF

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distinguished fluorescence intensity above tissue autofluorescence in the aorta and carotids (Figure 1A). EB was subsequently extracted from the vessels and quantified by standard extraction–absorbance methodology. Acquired data correlated strongly to fluorescence intensity (Figure 1B), confirming the accuracy of NIRF to quantify EB in intact mouse arteries. Importantly, NIRF detected increased EB uptake in tumor necrosis factor-α–stimulated carotids (compared with the right untreated vessels) at all EB doses, whereas extraction–absorbance methodology detected differences at the highest EB dose only (Figure 1C), demonstrating the superior sensitivity of NIRF imaging. To assess the possibility of combined analysis of permeability changes and cell recruitment in the same vessel, scanned carotids were subjected to flow-cytometric analysis, revealing an increased number of vessel-associated neutrophils in tumor necrosis factor-α–stimulated carotids (Figure 1D). Color photograph densitometry (Figure 1A in the online-only Data Supplement) for EB quantification revealed weak correlation with extraction–absorbance data (Figure 1B in the online-only Data Supplement) and did not significantly distinguish treated from nontreated carotids (Figure 1C in the online-only Data Supplement). To demonstrate applicability in a relevant disease model, NIRF imaging was used to quantify diet-induced focal changes in arterial permeability in the apolipoprotein E–deficient (Apoe−/−) mouse. Compared with wild-type mice, Apoe−/− mice exhibited increased EB fluorescence in atheroprone regions of the aorta, a response that was further aggravated by Western diet (Figure 2A and 2B). To verify that NIRF reflected increased luminal EB uptake, longitudinal cryosections of the aortic arch were acquired. Confocal imaging revealed luminal EB fluorescence in the inner curvature of the aortic arch and also in association with lesions in Western diet–fed Apoe−/− mice (Figure 2C and 2D), whereas no luminal staining was seen in wild-type mice. Background adventitial EB fluorescence was seen in all mice although of low intensity (Figure 2C and 2D).

Discussion

Here, we present and validate a NIRF imaging methodology for quantitative assessment of large artery endothelial permeability in mice. Analysis of intravenously injected EB accumulated in the vessel wall is an established and physiologically relevant approach for assessing arterial endothelial permeability. Compared with traditionally used extraction–absorbance methodology for EB quantification in mouse

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<td><strong>Apoe</strong></td>
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<td><strong>EB</strong></td>
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**Figure 1.** Near-infrared fluorescence (NIRF) for Evans blue (EB) quantification in mouse vessels correlates to and is more sensitive than extraction–absorbance methodology and enables single-vessel multivariate analysis. Left carotid artery (n=25) was subjected to topical stimulation with tumor necrosis factor (TNF)-α, 4 hours before euthanasia and was compared with the right, unstimulated carotid. EB (1, 0.1, 0.01, or 0 mg) in PBS was injected intravenously 30 minutes before euthanasia. The excised aorta and carotids were subjected to NIRF imaging (A) followed by extraction–absorbance methodology for EB quantification; the acquired data, presented as fluorescence intensity (FI) and optical density (OD), respectively, exhibited strong correlation (B). NIRF showed superior sensitivity to detect increased EB uptake in TNF-α-stimulated carotids at the lower EB doses (notice large spread in OD, indicating low sensitivity; C). NIRF left vessels intact for subsequent flow cytometry revealing an increase in neutrophils (Gr-1+ F4/80- cells) in stimulated carotids (D). Correlation was analyzed using Pearson correlation test. Stimulated and nonstimulated carotids from individual mice were compared using paired *t* test. *P*<0.05. Representative images are shown. Scale bar, 1 mm.
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Figure 2. Near-infrared fluorescence (NIRF) imaging detects focal luminal Evans blue (EB) uptake in a model of vascular inflammation. Apoe<sup>−/−</sup> (n=5) and wild-type (WT) (n=6) mice were fed Western diet (WD) or chow diet (CD) for 4 weeks. NIRF imaging revealed focal uptake of EB in Apoe<sup>−/−</sup> aortas (A) and EB fluorescence in the inner curvature of the aortic arch was quantified (B). Confocal images of longitudinal cryosections from aortic arch show EB distribution (C); Apoe<sup>−/−</sup> mice fed WD (left) and WT mice fed chow (right). D, A macroscopically visible lesion (left), associated EB uptake on NIRF scan (middle), and confocal image of cryosections of the same lesion (right). In confocal images, red=EB fluorescence; green=tissue autofluorescence. Macroscopic scale bar, 1 mm. Microscopic scale bar, 100 μm. Representative images are shown.

arteries, NIRF imaging is more sensitive, faster, and permits secondary multivariate analysis, in agreement with previous observations on EB leakage in ischemic rat brain. The high sensitivity of NIRF imaging allows for drastic lowering of the injected EB dose, which is advantageous as traditionally used EB doses of 50 to 100 mg/kg are known to affect blood pressure and pulse rate in small animals. It is not possible to fixate or conjugate EB to other macromolecules than albumin and, therefore, future research will evaluate other permeability tracers to allow a broader range of application and optimize sensitivity with minimal physiological interference. The NIRF methodology described here is limited by its inability to precisely discern the location of macromolecules in the vessel wall. Alternative histological methods for assessment of arterial permeability allow simultaneous analysis of intramural macromolecular distribution but require extensive manipulation of the tissue. In vivo imaging modalities, such as MRI, have been used for studying large arterial macromolecular uptake in mice and human. Although providing intriguing possibilities of repeated measurements and translational studies, these modalities are costly and have relatively low throughput. Most knowledge on the regulation of the endothelial barrier has been acquired using in vitro systems or microvascular models, and its relevance in the macrocirculation is obscure. Studies in small and large animals and in humans have shown association between increased endothelial permeability and sites of atherosclerosis development, suggesting common underlying mechanisms. Defining physiologically relevant molecular pathways in the macrocirculation is important because large-vessel endothelial permeability could provide a novel therapeutic target and also facilitate nanobased drug delivery to reduce atherothrombotic risk. Here, the current NIRF methodology provides a convenient high throughput tool for the study of arterial endothelial permeability and associated biological events. This method will be used to investigate the role of arterial endothelial permeability in the development and progression of vascular pathologies, such as atherosclerosis.

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Disclosures
None.

References
There are little in vivo data available on mechanisms regulating arterial endothelial permeability, and its impact on vascular pathologies such as atherosclerosis. We have established a near-infrared fluorescence imaging methodology for the assessment of arterial endothelial permeability in mice using the near-infrared fluorescent properties of the plasma albumin marker Evans blue. Compared with traditionally used methodology for Evans blue quantification, near-infrared fluorescence is sensitive, fast, and leaves the vessels intact for secondary multivariate analysis. Near-infrared fluorescence imaging provides a convenient and accessible tool for the investigation of arterial endothelial permeability that could ultimately aid in the development of novel treatment strategies in vascular disease.

**Significance**
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Materials and Methods

Animals
All animal protocols were carried out as approved by the regional ethical committee for animal experimentation. The study included female wild type (WT) C57bl/6 mice (Harlan, The Netherlands) and apolipoprotein E deficient (APOE^{-/-}) mice on a C57bl/6 background (B6.129P2-Apoel^{tm1Unc} N11, Taconic, Denmark) aged 6-9 weeks. Animals were kept in a temperature controlled room (21±2 °C), exposed to light-dark cycles of 12 h each, and were allowed ad libitum access to water and food.

Local TNF α stimulation
WT mice (n=25) were anaesthetized by an intraperitoneal injection of Ketamine (100 mg/kg) and Xylazine (5 mg/kg) mixture. A ventral midline incision (4–5 mm) was made in the neck. The left common carotid artery (LCA) was exposed by blunt dissection. TNF α (mTNFα, Cat No: 315-01A, 0.1 μg in 100 μl PBS, PreproTech, United Kingdom) was injected into the carotid sheath bellow the carotid bifurcation using a 23 gauge needle. The incision was then closed with a 6-0 suture and mice were monitored until recovery in a chamber on a heating pad. A single subcutaneous injection of buprenorphine (0.1 mg/kg, RB Pharmaceuticals Limited, Berkshire, GB) was given directly following surgery for pain relief. Animals were sacrificed four hours following inflammatory stimulation.

Vascular inflammation
APOE^{-/-} (n=5) and WT (n=6) mice were divided into four groups and fed either Western diet (WD, 21% fat, 0,2% cholesterol) (Cat No: E15721-34, ssniff Spezialdiäten GmbH, Soest, Germany) or chow diet (CD) for four weeks.

EB injections
Thirty minutes before sacrifice, injection of Evans blue dye (Cat No: E2129, Sigma, Schnelldorf, Germany) (100 μl, 1 mg, 0.1 mg, 0.01 mg or 0 mg in PBS) was performed intravenously by the lateral tail vein. The dose 0.1 mg was used when nothing else is stated.

Blood and vessel harvest
Mice were euthanized by isoflurane overdose and the abdominal cavity and chest were opened by blunt dissection. The vena cava was visualized and cut below the heart and blood was collected in eppendorf tubes for determination of serum EB. Animals were pressure perfused (100 mmHg) with 20 ml of PBS through the left ventricle. The aorta was carefully exposed and excised from the cusp to the diaphragm. The right and left common carotids were excised separately to equal lengths from the carotid bifurcation.

Detection of EB in serum
The collected blood was spun at 3000 rpm for 10 min. Twenty μl of serum was 10-fold diluted in PBS and subjected to absorbance spectrophotometry (Multiskan FC Microplate Photometer, Thermo Fisher Scientific, Waltham, MA, USA) at 620 nm. Values were expressed as optical density (OD).

EB detection in vessels by color photograph analysis
Freshly harvested specimens were mounted on objective glass with PBS as mounting medium. Low power microscopic images were obtained using the 10x objective on a stereomicroscope (M650; Wild Heerbrugg) equipped with a Nikon D5000 camera. Densitometry of EB stained vessels has been used to assess arterial endothelial permeability in large animals. Densitometry was performed on acquired photographs using ImageJ software (ImageJ 1.48a, National Institutes of Health, Bethesda, MD, USA). Images were converted to 8-bit grayscale, background was subtracted and pixel intensity over vessels was
recorded as integrated density as specified by the manufacturer. Data was presented as arbitrary units (AU). When stated AU was normalized to serum EB concentration.

**EB detection in vessels by NIRF methodology**

NIRF imaging was performed using the LiCor Odyssey infrared imager with Application software version 3.0.30 (LiCor, Lincoln, NE, USA). EB fluoresces with excitation peaks at 470 and 540 nm and an emission peak at 680 nm. Near infrared fluorescence analysis is ideal for biological tissue imaging because of minimal auto-fluorescence. To make use of EB’s fluorescent properties in the near-infrared spectrum we used the 700 channel (excitation by solid-state diode laser at 685 nm and emission 700–750) with the focal plane of the microscope head set at 1 mm. Freshly harvested specimens were mounted on objective glass with PBS as mounting medium. The specimens were scanned using laser intensity L0.5. Scans were analyzed by marking specimens using the automated drawing tool provided by the Odyssey Application software (Figure 1) and fluorescence intensity (FI) over the defined area was recorded as integrated intensity as specified by the manufacturer. In some samples, FI was normalized to serum EB concentration by dividing with OD from serum analysis, thereby correcting for minor variations in EB dose injected. When quantifying focal EB uptake in the aortic arch of APOE mice normalized FI was expressed per area with background fluorescence subtracted. Background fluorescence was defined as normalized FI per area over the right common carotid from the same animal. The right common carotid was chosen as it is a lesion-protected vessel segment where we did not detect any focal uptake of EB, (Figure 1).

**EB quantitation by extraction-absorbance methodology**

Following NIRF analysis specimens were dried overnight at 60°C and then incubated in 75 μl of formamid overnight. Vessel extracts were centrifuged at 3000 rpm for 10 min to remove particulate matter. Absorbance of the supernatants was assessed by spectrophotometry (Thermo Fisher Scientific) at 620 nm. Values were expressed as OD. When stated, OD was normalized to serum EB concentration.

**EB detection in cryosections by confocal microscopy**

Following NIRF analysis a subsample of aortic arches were transferred to optimal cutting temperature (O.C.T) medium and rapidly frozen in a dry ice – isopentane slush. Six μm cryostat sections were cut, allowed to dry at room temperature 30 min and subsequently fixed in 95% ethanol for 10 minutes. The O.C.T medium was quickly removed in distilled water, samples dehydrated in increasing concentrations of ethanol to xylene and mounted with Pertex (Histolab, Gothenburg, Sweden). Sections were imaged with 10× and 20× objectives on a Zeiss LSM 510 Meta confocal microscope at an excitation wavelength of 543 nm and a 650-nm long-pass filter for emission for EB fluorescence (Red) and 488-nm excitation wavelength and 505-550-nm long pass filter for emission for tissue autoflourescence (Green). Images were merged to discern where in the vessel EB was predominantly deposited.

**Leukocyte quantitation by flow cytometry**

Following NIRF analysis carotids were separately digested in 500 μl enzymatic cocktail; 125 U/ml collagenase type XI (Cat No:C7657), 60 U/ml hyaluronidase type I-s (Cat No: H3506), 60 U/ml DNase1 (Cat No: D4513), and 450 U/ml collagenase type I (Cat No: C0130) (all enzymes obtained from Sigma) in PBS containing 20 mM Hepes at 37°C for 1 h. A cell suspension was obtained by gently mashing the carotid lysate through a 35-μm strainer. The strainer was washed with 1 ml PBS and the samples were spun for 5 min at 1300×g, resuspended in PBS supplemented with 0.5% bovine serum albumin and subsequently labeled with PECy5-conjugated Ly6G- (Cat No: 108410, clone: RB6-8C, BioLegend, San Diego, CA, USA) and FITC-conjugated F4/80 (Cat No: MCA497FB, Clone: A3-1, AbD Serotec, Düsseldorf, Germany) antibodies. Flow cytometric studies were performed using a
FACSort (BD biosciences, San Jose, CA, USA) and data were analyzed using CellQuest software (BD biosciences). Leukocytes were sorted by forward-side scatter and further categorized as neutrophils (Gr-1^-F4/80^-), inflammatory monocytes (Gr-1^+F4/80^+) or resident monocytes and macrophages (Gr-1^-F4/80^+). Results are expressed as cells per carotid.

**Statistics**
Statistical analysis was performed using GraphPad Prism version 6.00 for Windows, GraphPad Software, La Jolla California USA. Paired t-test, Pearson correlation coefficient (r²), and linear regression analysis were used to assess statistical significance where appropriate. P-values of ≤0.05 were considered significant.

**References**

Figure 1. NIRF scans were analyzed using the automated drawing tool provided by the Odyssey Application software to select either whole vessels (left) or defined areas of interest (right) and the fluorescence intensity over the marked area was recorded. Scale bar, 1mm.
Supplemental Figure I. Left carotid artery (n=12) (filled circle) was subjected to topical stimulation with TNFα, 4 h before sacrifice and was compared to the right, unstimulated carotid (empty circle). Evan’s blue (EB) (1; 0.1; 0.01 or 0 mg) in PBS was injected i.v. 30 min before sacrifice. The excised aorta and carotids subjected to color photograph densitometry (A) followed by extraction-absorbance methodology for EB quantification, presented as arbitrary units (AU) and optical density (OD), respectively, exhibited weak correlation (B). Color photograph densitometry did not detect a difference in EB uptake in TNFα-stimulated carotids at any EB dose (C). Correlation was analyzed using Pearson correlation test. Stimulated and non-stimulated carotids from individual mice were compared using paired t-test. Representative images are shown. Scale bar, 1 mm.