Localization of the permeability barrier to solutes in isolated arteries by confocal microscopy

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Van Haaren, Paul M. A., Ed VanBavel, Hans Vink, and Jos A. E. Spaan. Localization of the permeability barrier to solutes in isolated arteries by confocal microscopy. Am J Physiol Heart Circ Physiol 285: H2848–H2856, 2003. First published August 7, 2003; 10.1152/ajpheart.00117.2003.—Endothelial cells are covered by a surface layer of membrane-associated proteoglycans, glycosaminoglycans, glycoproteins, glycolipids, and associated plasma proteins. This layer may limit transendothelial solute transport. We determined dimension and transport properties of this endothelial surface layer (ESL) in isolated arteries. Rat mesenteric small arteries (diameter ~150 μm) were isolated and cannulated with a double-barreled 0-pipette on the inlet side and a regular pipette on the outlet side. Dynamics and localization of intraarterial fluorescence by FITC-labeled dextrans (FITC-Δs) and the endothelial membrane dye Dil were determined with confocal microscopy. Large FITC-Δ (148 kDa) filled a core volume inside the arteries within 1 min but was excluded from a 2.6 ± 0.5-μm-wide region on the luminal side of the endothelium during 30 min of dye perfusion. Medium FITC-Δ (50.7 kDa) slowly penetrated this ESL within 30 min but did not permeate into the arterial wall. Small FITC-Δ (4.4 kDa) quickly passed the ESL and accumulated in the arterial wall. Prolonged luminal fluorochrome illumination with a bright mercury lamp destroyed the ~3-μm exclusion zone for FITC-Δ148 within a few minutes. This study demonstrates the presence of a thick ESL that contributes to the permeability barrier to solutes. The layer is sensitive to phototoxic stress, and its damage could form an early event in atherosclerosis.

dressed in experimental studies (1, 21, 38) on microvessels and in new theoretical transport models (18). Vascular permeability forms an important parameter in the regulation of water and solute exchange between the circulation and tissues (10, 26). It is important that the intrusion of certain macromolecules into the arterial wall is limited. Inclusion of albumin and low-density lipoproteins into the subendothelial space forms part of the process of atherogenesis. Thus ESL dysfunction may contribute to the microvascular disease phenotype of atherosclerosis (4, 23, 24). An altered vascular permeability is one of the earliest detectable symptoms of several pathophysiological states, including atherosclerosis, diabetes, shock, and tumors.

This study addresses two basic biophysical properties of the ESL in resistance arteries: its thickness and its limiting effect on macromolecular transport. In capillaries, the gap between ECs and the single line of passing red blood cells (RBCs) forms a relatively easy way of quantifying the in vivo thickness of the ESL, which was found to be ~0.5–0.7 μm (13, 37, 38). Most likely, all ECs are covered with an ESL, but many of its properties in blood vessels other than capillaries still need to be quantified. We hypothesized that the ESL in larger vessels is thicker than that found in capillaries because there is more space available for the constituting molecules to polymerize into the lumen and there is less distortion of the ESL by blood cells being forced through a small lumen.

Direct in vivo observations of ESL thickness or transport properties of solutes in conduit vessels are difficult because of the wall thickness. We therefore studied ESL properties in pressurized resistance arteries in vitro, which were mounted on 0-pipettes to allow for rapid perfusate changes (20, 21, 25). Localization of the wall permeability barrier to FITC-dextran (FITC-Δs) of different sizes was performed using confocal microscopy. In addition, the effect of destruction of the ESL by light-dye treatment (LDT) (37) on the dye distribution was studied.

MATERIALS AND METHODS

Artery preparation. All experiments were performed according to institutional guidelines. Male Wistar rats (n = 24,

ENDOTHELIAL CELLS (ECs) are covered by a surface layer of membrane-associated proteoglycans, glycosaminoglycans, glycoproteins, glycolipids, and associated plasma proteins, known as the endothelial surface layer (ESL) (30). The functional properties of the ESL have been extensively described only recently. Biochemical research elucidates receptor functions of glycosaminoglycans within the ESL and the binding patterns of proteins to heparan sulfates (8, 31, 34). Bio-degradation of sialic acid, an important constituent of the ESL, by neuraminidase inhibits shear-induced nitric oxide production (12, 29). The role of the ESL in the control of vascular wall permeability has been ad-

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200–250 g) were decapitated, and the mesentery was excised and immediately put into cold (4°C) MOPS-buffered physiological saline solution (MOPS-PSS; see Solutions). A small artery was dissected from the mesentery and transported to the pressure myograph. Average internal diameter at 60 mmHg and full dilation was 148 ± 5 μm; no significant differences in diameter existed between the various groups of arteries in this study. Each rat provided one artery. Other vessels or organs from the same rat were used in other experiments.

**Myograph.** The isolated arteries were cannulated at one end with a double-barreled cannula, a 0.9-pipette (World Precision Instruments), and at the other end with a regular glass cannula. Arteries were pressurized and perfused with MOPS-BSA at input and output pressures of 65 and 55 mmHg, respectively. This pressure difference resulted in a flow through the arteries of 2.3 ± 0.4 μl/min, as measured by a μFlow liquid mass flowmeter (Bronkhorst Hi-Tec Holland). The arteries could be perfused with a solution containing fluorescent tracers via the second barrel of the pipette. Fluorescent perfusate was also pressurized to 65 mmHg. The superfuse was MOPS-PSS, of which PO2 was maintained at 10 mg/ml BSA. All chemicals were purchased from Sigma. All solutions were adjusted to pH 7.35.

**Solutions.** MOPS-PSS contained (in mM) 145 NaCl, 4.7 KCl, 1.17 MgSO4, 1.2 NaH2PO4, 2 CaCl2, 5 MOPS, 5 glucose, and 2 pyruvate. As perfuse MOPS-PSS was supplemented with 10 mg/ml BSA. All chemicals were purchased from Sigma. All solutions were adjusted to pH 7.35.

**Fluorescent probes.** FITC-Ds of different sizes (molecular mass 4.4 kDa (FITC-Δ4), 50.7 kDa (FITC-Δ50), and 148 kDa (FITC-Δ148)) were purchased from Sigma; the lipophilic membrane tracer 1,1'-dioctadecyl-3,3',3'-tetramethylindocarbocyanine perchlorate (DiI) was purchased from Molecular Probes. FITC-Ds were applied in concentrations of 36.0 mg FITC-Δ4/l solution, 45.0 mg FITC-Δ50/l solution, or 13.85 mg FITC-Δ148/l solution, resulting in a concentration of 1.0 × 10^-6 M FITC in all three cases. Although the dextran concentrations were not equal (in contrast to FITC concentrations), these concentrations were very low (10^-9–10^-8 M) compared with the concentration of another perfuse constituent present, namely, albumin (10^-4 M). Thus, we feel that the differences in dextran concentrations did not significantly influence their transport characteristics or other possible osmotic effects. DiI was dissolved as a 2.0 × 10^-2 M stock solution in DMSO before dilution by a factor of 2,000 in the fluorescent perfusate solution to a final concentration of 1.0 × 10^-5 M. This diluted solution is considered not to influence endothelial integrity or vascular permeability (3). Once incorporated in the endothelial membrane, DiI stays there for the remainder of the experiment. DiI is able to spread along the membrane of an EC but cannot migrate from one cell to another (15–17).

**Confocal microscopy.** Images of 512 × 512 pixels were recorded with a Leica Fluovert Confocal Laser Scanning Microscope (CLSM). Arteries were visualized from below through a cover glass, which formed the bottom of the cannulation chamber. Excitation was obtained by an Ar-Kr laser using the 488-nm line. A ×16/0.5 objective resulted in a pixel size of 611 × 611 nm in the plane of focus. Green (FITC-Δs) and red fluorescence (DiI) was detected using a 525-nm band-pass filter and a 610-nm long-pass filter, respectively, and measured with photomultipliers (PMTs). Cross-talk between both fluorescence channels was negligible. The detection pinhole was 25 μm wide. Images were recorded at the midplane of the arteries every 1.85 s during the first 1.8 min of dye perfusion. During the remainder of the dye perfusion period (2–30 min), images were recorded every minute. Arteries were not illuminated between measurements to prevent phototoxic damage (37) except in the LDT experiments.

For the LDT experiments, a newly obtained Leica DM-IRBE microscope equipped with a Leica TCS-S2 confocal unit was used. Excitation was obtained by an argon ion laser using the 488-nm line. A ×20/0.70 objective with a zoom factor of 2 resulted in a pixel size of 366 × 366 nm. Green (500–530 nm) and red fluorescence (625–750 nm) were detected using a prism and adjustable slits in front of the PMTs. The detection pinhole here was 20 μm wide. Arteries were perfused with fluorescent tracers for 40 min, and images were recorded every 3 s during the first 1.5 min of dye perfusion. Subsequently, images were recorded every minute. After 30 min of dye perfusion, LDT was started for 10 min using a bright mercury lamp, the light of which was guided through the FITC-filter block. During confocal image acquisition, the arteries were not exposed to the light of the mercury lamp.

**Image analysis.** Profiles in the radial direction, averaged along the length of the arteries, were made of all fluorescence images, recorded at midplane, with the image analysis software ImageJ (NIH). Measurement of the arterial diameter was based on the position of the endothelium, which was determined from the peak in the DiI fluorescence profiles, after subtraction of the luminal DiI fluorescence (i.e., the profile after 1.5 min). FITC-Δ fluorescence profiles, normalized to average midluminal fluorescence intensity in a 5-μm-wide window in the same image, were quantified in a region spanning from 10 μm abluminally to 15 μm laminally of the endothelium.

**Quantification of optical properties.** Rayleigh’s criterion (22) predicts a resolution for the CLSM of ~0.3 μm, somewhat smaller than the pixel size in the images. This resolution was improved by 512 repeated measurements because fluorescence profiles were averaged along the length of the arteries. The point-spread function (PSF) of the CLSM system was determined by recording a stack of images in the z-direction, i.e., along the optical axis) of fluorescent microspheres of 0.18 μm in diameter (Molecular Probes). The PSF in each direction was approximately Gaussian shaped with a full width at half maximum (FWHM) in the image plane of ~3 μm, which increased at increasing distances from the plane of focus. FWHM in the z-direction, i.e., the optical section thickness, was ~13 μm. Because DiI specifically labels EC membranes and the endothelium is ~0.2 μm thick, the average DiI fluorescence profile can be used as a line-spread function (LSF), the one-dimensional equivalent of the PSF (33). This LSF (shown in Fig. 3D) was slightly asymmetric and had a FWHM of 4.8 μm (see RESULTS and DISCUSSION). Using the PSF, we predicted such a LSF by calculating the fluorescence response of a thin cylinder wall, representing the endothelium, with a diameter in the range studied and uniform fluorescence. The peak position of the predicted LSF was within 0.1 μm of the cylinder wall position in the midplane; the predicted LSF was slightly more asymmetric than the experimental one and had a FWHM of 5.1 μm. Convolution of the experimental LSF with square-shaped FITC-Δ concentration distributions in radial direction results in predicted fluorescence profiles that, after being fit to the measured fluorescence profiles, provide an estimate of the position of the FITC-Δ front near the arterial wall (see Fig. 4).
This method was validated for a glass tube with an inner diameter of \(167 \pm 0.13 \, \mu m\) (mean \(\pm \) SD, \(n = 8\) pieces of \(~1\) cm broken from the same supply tube) and a wall thickness of \(~65 \, \mu m\). Tube diameter was accurately determined from scanning electron microscopic photographs of the tube ends. The tube was submerged in silicon gel, with a refractive index close to that of glass, on top of a cover glass, through which the tube was visualized from below. One end of the tube was glued to a \(\theta\)-pipette using silicon glue. The inner tube surface was confirmed to be smooth from CLSM measurements of the width (FWHM) of the fluorescence column of the tube filled with FITC-\(\Delta\), which varied only with a SD of \(0.12 \, \mu m\) \((n = \text{512 profiles along the tube})\).

Electron and confocal microscopes are well maintained by the institutional Center for Microscopical Research, and calibrations were regularly checked using standard calibration replica, revealing deviations smaller than \(0.1\%\). To determine the influence of refractive index differences between oil and water as submerging fluids for the glass tube and vessels, respectively, on measured distances in the plane of focus, a calibration micrometer was studied in the two media at the objective working distance position. Estimated pixel sizes were within \(0.1\%\) of each other and of the standard system value. Furthermore, we compared fluorescence profiles of the glass tube filled with a fluorescent dye soluble in both water and oil, namely, \(3,3'\)-dioctadecyloxacarbocyanine.

**Fig. 1.** Behavior of FITC-148-kDa dextran (FITC-\(\Delta148\); green, \(A\)) and DiI (red, \(B\)) in a cannulated small artery (diameter 157 \(\mu m\)). Top: autofluorescence before switching to dye perfusion; middle: fluorescence after 2 and 30 min of dye perfusion; bottom: differences between 30 and 2 min, where blue pixels represent negative values. \(t\), Time.
perchlorate (DiO; Molecular Probes), which has approximately the same wavelength as FITC. Despite the mismatch in the refractive index between the aqueous solution and glass, the resulting fluorescence profile in the midplane of the tube did not differ from the profile with matched refractive indexes. The positions of the inner tube wall as determined from the positions where the fluorescence is about one-third of maximal (see Fig. 4B) differed only by 0.07 ± 0.05 μm [two profiles averaged over 512 scan lines along the length of the tube, *P* = not significant (NS) vs. 0 μm] between both profiles. Thus possible similar refractive index mismatches between solvent, tube/arteries, or surroundings do not significantly influence measurements of distances or profile widths in radial direction in the plane of focus.

**Statistics.** Data are presented as means ± SE. Parameters describing fluorescence profiles for the different FITC-Δs were compared using ANOVA and Bonferroni post hoc tests. A paired *t*-test was used to test the effect of LDT. A value of *P* < 0.05 was considered significant.

**RESULTS**

Typical images of combined infusion of FITC-Δ148 (green) and DiI (red) are depicted in Fig. 1. Figure 1 shows autofluorescence (*top*), fluorescence images after 2 and 30 min (*middle*), and the increase in fluorescence between 2 and 30 min of dye perfusion (*bottom*). DiI accumulated in the endothelium, as is clear both in the 30-min image and, more pronounced, in the subtraction image. In contrast, no increase in FITC-Δ148 fluorescence was observed between 2 and 30 min. From images such as these, fluorescence profiles in radial direction at midplane of the arteries were determined. Typical profiles are shown in Fig. 2. For both colors, there is autofluorescence of the arterial wall (Fig. 2, *top;* note the different vertical scales in the panels). Endothelial DiI accumulation is clear from the fluorescence profiles and further accentuated by the difference signals shown in Fig. 2, *bottom,* regardless of which FITC-Δ was used in combination with DiI. Peak DiI fluorescence increased steadily from 2 to 30 min with 265 ± 20% (*P* < 0.05, 30 vs. 2 min, *n* = 24 experiments; no differences between the different groups of arteries). The shape of the DiI peaks, as expressed by their width (FWHM), remained constant within 5% over time.

The fluorescence profiles of the glass tube are also provided in Fig. 2. Behavior of the different FITC-Δs in the glass tube was identical. Note the similar difference profile of the glass tube filled with FITC-Δ4 and

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**Fig. 2.** Fluorescence profiles of arteries perfused with FITC-Δ148 (A), FITC-50-kDa dextran (FITC-Δ50; B), or FITC-4-kDa dextran (FITC-Δ4; C) and of the dummy perfused with FITC-Δ4 (D). *Top*: autofluorescence; *middle*: profiles after 2 and 30 min; *bottom*: increase in fluorescence between 2 and 30 min of dye perfusion. Dotted lines indicate the DiI peaks (A–C) or the inner glass tube wall (D). Note the different scales at the vertical axes of all panels. a.u., Arbitrary units. Dark gray lines, green fluorescence; light gray lines, red fluorescence.
the artery with FITC-Δ148, notwithstanding the difference in molecular size.

Average data (7 experiments/FITC-Δ) on the development of the fluorescence distributions near the endothelium are provided in Fig. 3. All FITC-Δ curves are normalized to midluminal fluorescence, which was constant within 2% for all time points and all FITC-Δs. Also depicted in Fig. 3, A–C, are the average normalized fluorescence levels at the midluminal position. The position of the endothelium, as determined from peak DiI fluorescence, is taken as position 0 μm and is indicated by the vertical dotted lines. DiI peak position remained constant over time; average SD of variations in this position was 0.24 ± 0.01 μm, i.e., <1 pixel, allowing accurate localization of the endothelium. Figure 3, A–C, shows that the smaller the dextran the more the fluorescence distribution extends over the endothelial position. For the large FITC-Δ148, there was no difference between 2 and 30 min, indicating that an equilibrium state is reached after 2 min (Fig. 3A), and practically all fluorescence falls inside of the DiI peaks. For the smaller FITC-Δs, the fluorescence profiles are still developing after 2 min, but outward migration of FITC-Δ4 between 2 and 30 min (Fig. 3C) is more pronounced than that for FITC-Δ50 (Fig. 3B). To directly compare the location of the different tracers, the FITC-Δ fluorescence profiles of the arteries after 30 min are replotted in Fig. 3D. Also shown is the FITC-Δ fluorescence profile in the dummy after 30 min (dashed line), where the tube wall position, known from the diameter measurement by scanning electron microscopy, is superimposed on the endothelial position. The average fluorescence distribution of DiI bound to the endothelium, normalized to peak fluorescence, is shown as well.

The FITC-Δ profiles after 30 min had similar shapes but were horizontally shifted. The arterial FITC-Δ50 profile coincided with the fluorescence profile from the dummy. The arterial FITC-Δ148 profile was found to be shifted toward the luminal side over ~2–3 μm, whereas the arterial FITC-Δ4 profile was shifted toward the abluminal side over ~1 μm.

The relation between an assumed dye concentration front, its fluorescence distribution in the image, and the DiI-derived LSF is demonstrated in Fig. 4A. Convolution of the concentration distribution (dashed line) with the LSF results in a sigmoidal FITC-Δ fluorescence profile (solid line) with a well-defined position relative to the dye front. Plotting the measured FITC-Δ148 data in Fig. 4A (symbols), such that an optimal fit

Fig. 3. Average normalized fluorescence after 2 and 30 min of perfusion with FITC-Δ148 (A) and after 2, 10, and 30 min of perfusion with FITC-Δ50 (B) or FITC-Δ4 (C). Fluorescence was normalized to midluminal fluorescence. Dotted lines indicate the endothelial cell (EC) position, determined from peak DiI fluorescence, and therefore negative x values indicate positions abuminally of the endothelium.

Profiles are means ± SE; n = 7 experiments for each FITC-Δ. FITC-Δ fluorescence profiles after 30 min from A to C are replotted in D, which also shows the fluorescence profile in the dummy after 30 min (dashed line) as well as the average fluorescence distribution of DiI, normalized to peak DiI fluorescence.
between data and predicted fluorescence profile is obtained, reveals that the FITC-Δ148 concentration front is a distance $X_s$ “shifted” from the location of the Dil peak (EC). The same procedure applied to the measured glass tube profile (Fig. 4B) revealed that the dye front in the tube coincides exactly with the inner tube wall ($X_s = 0.001 \mu m$).

Averaged over all vessel experiments, $X_s$ was $2.6 \pm 0.5 \mu m$ for FITC-Δ148 ($P < 0.05$ vs. 0 $\mu m$, $n = 7$ experiments). The shapes of the fluorescence distributions after 30 min are quite similar for all dextrans (see Fig. 3D), and therefore the same procedure was repeated for FITC-Δ50 and FITC-Δ4. For FITC-Δ50, $X_s$ was $0.1 \pm 0.9 \mu m$ ($P = NS$ vs. 0 $\mu m$; $P < 0.05$ vs. FITC-Δ148, $n = 7$ experiments). For FITC-Δ4, $X_s$ was $-0.7 \pm 0.4 \mu m$ ($P < 0.05$ vs. 0 $\mu m$; $P < 0.05$ vs. FITC-Δ148, $n = 7$ experiments).

As shown in Fig. 5, LDT shifted the FITC-Δ148 fluorescence profile outward, which was quantified by a change in $X_s$ from $3.2 \pm 0.3 \mu m$ ($P = NS$ vs. the FITC-Δ148 group without LDT) to $0.1 \pm 0.1 \mu m$ after 10 min ($P < 0.05$, 40 vs. 30 min; $n = 3$ experiments). Electron microscopy of sections of the arteries made after the experiments revealed no damage to the endothelium by LDT (Fig. 6). Endothelial thickness was $0.19 \pm 0.01 \mu m$ ($n = 66$ images) in general (Fig. 6A) and $0.64 \pm 0.03 \mu m$ ($n = 14$ images) in regions of nuclei (Fig. 6B).

**DISCUSSION**

We demonstrated by direct observation that FITC-Δ148 is excluded from a 2- to 3-μm-thick region luminal of the endothelium in isolated small arteries. In contrast, FITC-Δ50 and FITC-Δ4 were able to penetrate this region, and the latter also penetrated the arterial wall. The luminal exclusion zone was found to be sensitive to LDT.

**Comparison to literature.** Insights are evolving that vascular endothelium possesses a glycocalyx (ESL) forming an exclusion zone in which axial convective plasma transport is greatly impaired and that forms a barrier for radial transport of especially larger molecules through the vessel wall. Presence of the ESL was originally postulated to explain low levels of capillary hematocrit (7). More direct evidence was obtained from intravital microscopy demonstrating a gap between RBCs and the endothelium in the order of 0.5–1 μm (5, 13, 14, 36–38). Moreover, it has been demonstrated that molecular penetration rates in the ESL depend on the size and charge of the molecules (38).

The ESL thickness measured in the present study is larger than that measured in capillaries and arterioles of 10–15 μm. In capillaries, RBCs are partly compressing the ESL, because of their size in relation to the capillary diameter. Compression may still occur in small arterioles. White blood cells were found to completely compress the capillary ESL (37). These findings suggest that the ESL is a highly flexible and dynamic layer. Compression obviously will occur to a lesser extend in larger vessels. It remains, however, to be

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**Fig. 4.** A: example of fitting an arterial FITC-Δ148 fluorescence profile after 30 min of dye perfusion (○) with a predicted fluorescence profile (solid line) calculated from the convolution of an assumed square-shaped concentration profile (dashed line) and the Dil-derived line-spread function. Fitting resulted in a predicted dye front position relative to Dil peak position (dotted line), in this case characterized by a shift distance ($X_s$) of 2.0 μm. B: verification that fitting the measured glass tube profile (●) with a predicted fluorescence profile (solid line) resulted in a dye front that coincided with the inner tube wall. EM, electron microscopy.

**Fig. 5.** Average normalized fluorescence near the endothelium for arteries during the light-dye treatment (LDT) protocol. Arteries were perfused with FITC-Δ148 for 30 min, after which LDT was started and dye perfusion and measurements were continued for another 10 min. Profiles are mean ± SE; $n = 3$ experiments.
established how hemodynamic conditions, notably shear stress, affect ESL properties.

The existence of an ESL in larger arterioles (13–101 μm) with barrier properties similar to those in capillaries was postulated earlier from a 2.3-fold increase in permeability after treatment of the arterioles with pronase and heparinase (21), enzymes that degrade the ESL (7). Such permeability studies are usually based on the measurement of total integrated fluorescence in a window over the vessel including an area of extravascular space, even in an earlier study (2) that used confocal microscopy. Model interpretation of these enzyme data resulted in an estimate of ESL thickness up to 7 μm (21), roughly a factor of 3 larger than found presently from more direct observations of dye distributions. However, permeability studies are usually limited to a time period of ~2 min (2, 19–21, 25), whereas fluorescence profiles are still significantly changing after this time, as is clear from our experiments.

Criticism of the method. Because of the size of the arteries used, a long working distance objective with a relatively low numerical aperture was needed. Even in confocal microscopy, this results in blurring of the images and complicates the localization of the tracers.

The relative shifts between fluorescence profiles of different FITC-Δs and the effect of LDT on the FITC-Δ148 fluorescence profiles are not affected by the correction of these curves for optical distortions, because these shifts follow directly from comparison of the measured profiles (Figs. 3 and 5). However, estimation of the shift in concentration distributions with respect to the endothelium required fluorescent labeling of the endothelium with DiI and an estimation of the dye front with respect to the fluorescence profile.

DiI is able to spread along the membrane of an EC but cannot migrate from one cell to another, leaving the smooth muscle cells unlabeled (15–17). In histological sections of arteries that had been perfused with DiI, this dye was only found luminally of the internal elastic lamina. Electron microscopy pictures of the arteries used in the experiments demonstrated clearly that the endothelium was relatively thin (~0.2 μm) compared with the ESL thickness found (~2–3 μm).

Only ~10% of the endothelium was ~0.6 μm thick due to the presence of nuclei. In the localization of the endothelium, the question of whether DiI concentrates more at the luminal side or at the abluminal side of the endothelium is therefore not relevant. Peak DiI fluorescence at the endothelium increased steadily over time, but the shape of these DiI peaks as well as their position remained constant. Therefore, peak location was already clearly detectable after 2 min. LDT did not change the position or shape of the DiI profiles, indicating that the generated free radicals are solely destroying the ESL and not the ECs (see also below) and do not affect the determination of the endothelial position from the DiI measurements.

Under the assumption that the optical properties would be specific for the experimental setup, we used the LSF that resulted from the averaged DiI fluorescence profiles of all experiments. This average LSF had a FWHM of 4.8 μm, which is smaller than the average FWHM of all individual DiI peaks, 6.4 ± 0.6 μm. The difference results from the “bell” shape of the peaks. The spread in FWHM of the individual DiI peaks is most likely due to heterogeneity in DiI labeling of the ECs. If ECs at midplane (i.e., the plane of focus) are strongly labeled with DiI, the LSF will be narrow; if ECs out of the midplane are more strongly labeled, this will result in widening of the LSF and may in extreme cases result in a shift of the DiI peak from the midplane endothelial position. Simulation of the LSF using the PSF resulted in a predicted FWHM of 5.1 μm and a peak position that was within 0.1 μm of the assumed EC position in the plane of focus (see Quantification of optical properties). It should be noted that within an optical section thickness of 13 μm, the maximal horizontal deviation along the vessel wall circumference is in the order of 0.28 μm. In any case, a possible deviation of the DiI peak from the midplane EC position will be toward the luminal direction and will therefore result in an underestimation of the ESL thickness [see also Streekstra et al. (35)].

Like a fluorescent line source is imaged as a “blurred” band of fluorescence, a steep front of dye results in a graded smooth fluorescence profile in the image. The relation between dye front and fluorescence
profile can be predicted using the LSF, as was demonstrated for the glass tube as well as for an arterial FITC-Δ148 profile. For both cases, predicted curves, using the same LSF, tallied well with measured fluorescence profiles, supporting the concept that a sharp dye front does exist. In case of a symmetrical LSF, fluorescence intensity at the dye front position would have been half maximal instead of about one-third of maximal, as we found. The latter results from the fact that the LSF takes the curvature of the endothelium along the arterial wall circumference into account, which also reflects the curvature of the FITC-Δ core inside the vessels. This one-third maximal fluorescence at the dye front position was confirmed by calculations of fluorescence profiles from concentration distributions using the PSF instead of the LSF and was almost independent of the vessel diameter in the range studied. It should be noted that the full FITC-Δ148 fluorescence profile was inside of the Dil peaks (Fig. 3A), which makes it highly unlikely that the dye front had touched the endothelium.

Because the dye front will have reached the wall in the case of the glass tube, the accuracy of the prediction of the front position follows from its agreement with the inner tube wall position. Tube diameter was known within 0.1 μm from electron microscopy measurements. For the CLSM measurements, the center line of the tube profiles can be accurately determined because of symmetry of the profiles. The FWHM of 512 profiles measured along the length of the tube varied with a SD of 0.1 μm. CLSM calibration with a stage micrometer indicated by calculations that the full FITC-Δ fluorescence profile was inside of the Dil peaks (Fig. 3A), which makes it highly unlikely that the dye front had touched the endothelium.

The mechanisms for ESL disruption are comparable, in the sense that in both cases the disruption can be prevented by the radical scavengers superoxide dismutase and catalase (5, 36, 37). Support for the absence of damaging effects of LDT on the endothelium is indicated by 1) the absence of FITC-Δ148 at the abluminal side of the endothelium after LDT; 2) the constant shape of the Dil fluorescence profiles after LDT (an altered shape would be expected after disruption or swelling of ECs); and 3) histology performed on the arteries after LDT, which showed normal coverage of the arterial wall with ECs.

Implications of the study. An altered vascular permeability is one of the earliest detectable symptoms of several pathophysiological states, including atherosclerosis, diabetes, shock, and tumors. Consequently, considerable research has been devoted to the regulation of vascular wall permeability and its dependence on protein composition (11, 19), agonists (20, 25), temperature (9, 27), and pH (9). However, the exact localization of the permeability barrier had not yet been determined satisfactorily.

Current models of vascular permeability state that the EC layer forms the main permeability barrier allowing the exchange of water and solutes through pores and vesicles that are covered with a fibrous matrix (10, 26). However, at least in capillaries, it has been suggested that the ESL itself could form an additional barrier (37, 38), and the present study confirms this suggestion for resistance arteries. It is obvious that an ESL of several micrometers in thickness also has an effect on the interaction of blood cells with the vascular wall. Hence, the ESL can be seen as a line of first defense of the vascular wall (5, 6, 36). This view is supported by the notion that an increased vascular permeability is an early hallmark in many vascular pathologies, including atherosclerosis [see reviews by Ross (32) and Nielsen (28)].

Possibly many concepts on endothelial function in vascular regulation may have to be reconsidered with the knowledge that the endothelium can be covered by a layer many times thicker than the cells themselves.

In conclusion, we demonstrated the presence of a permeability barrier to solutes on the luminal side of the endothelium in isolated small arteries. This ESL, with a thickness of 2–3 μm, is likely to form an essential domain for transvascular transport and blood-vesel wall interaction. Its damage during oxidative stress could form a key initial event in atherosclerosis.

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