Wall permeability of isolated small arteries. Role of the endothelial surface layer
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chapter 2:

LOCALIZATION OF THE PERMEABILITY BARRIER TO SOLUTES IN ISOLATED ARTERIES BY CONFOCAL MICROSCOPY
2.1 ABSTRACT

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 in isolated arteries. Rat mesenteric small arteries (d=150 μm) were isolated and cannulated with a double-barreled θ-pipet on the inlet side and a regular pipet on the outlet side. Dynamics and localization of intra-arterial fluorescence by fluorescein-isothiocyanate (FITC)-labeled dextrans (FITC-Δs) and the endothelial membrane dye Dil were determined with confocal microscopy. Large FITC-Δ (148 kD) filled a core volume inside the arteries within 1 minute but was excluded from a 2.6 ± 0.5 μm wide region on the luminal side of the endothelium during 30 minutes of dye perfusion. Medium-sized FITC-Δ (50.7 kD) slowly penetrated this endothelial surface layer (ESL) within 30 minutes but did not permeate into the arterial wall. Small FITC-Δ (4.4 kD) 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 endothelial surface layer 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.

2.2 INTRODUCTION

Endothelial cells are covered by a surface layer of membrane associated proteoglycans, glycosaminoglycans, glycoproteins, glycolipids, and associated plasma proteins, known as the endothelial surface layer (ESL) (28). Only recently its functional properties are becoming more extensively described. Biochemical research elucidates receptor functions of glycosaminoglycans within the ESL and binding patterns of proteins to heparan sulfates (7;29;32). Biodegradation of sialic acid, an important constituent of the ESL, by neuraminidase inhibits shear-induced NO production (11;27). The role of the ESL in the control of vascular wall permeability has been addressed in experimental studies on microvessels (1;20;36) and in new theoretical transport models (17). Vascular permeability forms an important parameter in the regulation of water and solute exchange between the circulation and tissues (9;24). It is important that 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 (3;21;22). 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 endothelial cells and the single line of passing red blood cells forms a relatively easy way of quantifying the in vivo thickness of the ESL, which was found to be ~0.5-0.7 μm (12;35;36). Most likely, all endothelial cells 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 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, mounted on θ-pipets to allow for rapid perfusate changes (19;20;23). Localization of the wall permeability barrier to FITC-dextrans of different sizes was performed using confocal microscopy. In addition, the effect of destruction of the ESL by light-dye-treatment (35) on the dye distribution was studied.

2.3 MATERIALS AND METHODS

2.3.1 Artery preparation

All experiments were performed according to the institutional guidelines. Male Wistar rats, (N=24, 200-250 g) were decapitated, the mesentery was excised and immediately put into cold (4°C) 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.

2.3.2 Myograph

The isolated arteries were cannulated at one end with a double-barreled cannula, a θ-pipet (World Precision Instruments Inc.), and at the other end with a regular glass cannula. Arteries were pressurized and perfused with MOPS-BSA at input and output pressures of respectively
65 and 55 mmHg. This pressure difference resulted in a flow through the arteries of 2.3 ± 0.4 μl·min⁻¹, as measured by a μFlow liquid mass flowmeter (Bronkhorst Hi-Tec Holland BV). The arteries could be perfused with a solution containing fluorescent tracers via the second barrel of the θ-pipet. Fluorescent perfusate was also pressurized to 65 mmHg. The superfusate, 37°C MOPS-PSS of which PO₂ was maintained at ambient value, was continuously recirculated with a rollerpump. PCO₂ was not controlled since pH was buffered by MOPS. Under these conditions these arteries were without tone and maintained a constant diameter during the protocol.

### 2.3.3 Solutions
The MOPS-buffered physiological saline solution (MOPS-PSS) contained: NaCl (145 mM), KCl (4.7 mM), MgSO₄ (1.17 mM), NaH₂PO₄ (1.2 mM), CaCl₂ (2 mM), MOPS (3-[N-Morpholino]propanesulfonic acid) (3 mM), glucose (5 mM) and pyruvate (2 mM). As perfusate MOPS-PSS was supplemented with 10 mg/ml bovine serum albumin (BSA). All chemicals were purchased from SIGMA. All solutions were adjusted to pH 7.35.

### 2.3.4 Fluorescent probes
Fluorescein-isothiocyanate-labeled dextrans (FITC-Δs) of different sizes (MW 4.4 kD (FITC-Δ4), 50.7 kD (FITC-Δ50) and 148 kD (FITC-Δ148)) were purchased from SIGMA; the lipophilic membrane tracer DiI (1,1'-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate) was purchased from Molecular Probes. FITC-Δs were applied in concentrations of 36.0 mg FITC-Δ4 / liter solution, 45.0 mg FITC-Δ50 / liter or 13.85 mg FITC-Δ148 / liter, resulting in a concentration of 1.0·10⁻⁶ M FITC in all three cases. DiI was applied at 1.0·10⁻⁵ M. Once incorporated in the endothelial membrane, DiI will stay there for the remainder of the experiment. DiI is able to spread along the membrane of an endothelial cell, but cannot migrate from one cell to another (14-16).

### 2.3.5 Confocal microscopy
Images of 512x512 pixels were recorded with a Leica Fluovert Confocal Laser Scanning Microscope (CLSM). Arteries were visualized from below through a cover glass that formed the bottom of the cannulation chamber. Excitation was obtained by an Ar-Kr laser using the 488 nm line. A 16x/0.5 objective resulted in a pixelsize of 611 nm × 611 nm in the plane of focus. Green (FITC-Δs) and red fluorescence (DiI) were detected using respectively a 525 nm band pass filter and a 610 nm long pass filter and measured with photomultipliers (PMTs). Crosstalk between
both fluorescence channels was negligible. The detection pinhole was 25 μm wide. Images were recorded at mid-plane of the arteries every 1.85 sec during the first 1.8 minutes 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 in order to prevent phototoxic damage (35) except in the light-dye-treatment experiments.

For the light-dye-treatment (LDT) experiments a newly obtained Leica DM-IRBE microscope equipped with a Leica TCS-SP2 confocal unit was used. Excitation was obtained by an Ar-ion laser using the 488 nm line. A 20x/0.70 objective with a zoom factor of 2 resulted in a pixelsize of 366 nm × 366 nm. Green (500-530 nm) and red (625-750 nm) fluorescence 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 minutes and images were recorded every 3 sec during the first 1.5 min of dye perfusion. Subsequently, images were recorded every minute. After 30 minutes of dye perfusion LDT was started for 10 minutes, using a bright mercury lamp whose light was guided through the FITC-filter block. During confocal image acquisition the arteries were not exposed to the light of the mercury lamp.

2.3.6 Image analysis
Profiles in radial direction, averaged along the length of the arteries, were made of all fluorescence images, recorded at mid-plane, with the image analysis software ImageJ (NIH, USA). Measurement of the arterial diameter was based on the position of the endothelium, which was determined from the peak in the Dil fluorescence profiles, after subtraction of luminal Dil fluorescence (i.e. the profile after 1.5 min). FITC-A fluorescence profiles, normalized to average mid-luminal 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 luminally of the endothelium.

2.3.7 Quantification of optical properties
Rayleigh's criterion 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 since 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 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 z-direction, i.e. the optical section thickness, was ~13 μm. Since DiI specifically labels endothelial cell membranes and 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 (31). This LSF (depicted in fig. 2.3D) 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 mid-plane; 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-A concentration distributions in radial direction results in predicted fluorescence profiles, that, after fitting to the measured fluorescence profiles, provide an estimate of the position of the FITC-A front near the arterial wall (see fig. 2.4). This method was validated for a glass tube with an inner diameter of 167 ± 0.13 μm (mean ± SD, n=8 pieces of ~1 cm broken from the same supply tube) and a wall thickness of ~65 μm. Tube diameter was accurately determined from scanning electron microscopic (EM) 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 0-pipet using silicon glue. 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-A, which varied only with a SD of 0.12 μm (n=512 profiles along the tube). Electron and confocal microscopes are well maintained by the institutional Center for Microscopical Research and calibrations 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 plane of focus, a calibration micrometer was studied in the two media at objective working distance position. Estimated pixelsizes were within 0.1% of each other and of the standard system value.

**2.3.8 Statistics**

Data are presented as means ± SEM. Parameters describing fluorescence profiles for the different FITC-As were compared using ANOVA and bonferroni post-hoc tests. A paired t-test was used to test the effect of LDT.
Figure 2.1: Behavior of FITC-Δ148 (green, left panels) and Dil (red, right panels) in a cannulated small artery (diameter 157 μm). Top panels show autofluorescence before switching to dye perfusion, middle panels show fluorescence after 2 and 30 minutes of dye perfusion. Bottom panels show the differences between 30 and 2 min, where blue pixels represent negative values.
Figure 2: Fluorescence profiles of arteries perfused with FITC-A48 (A), FITC-A50 (B) or FITC-A4 (C), and of the dummy perfused with FITC-A48 (D). The top panel shows the difference in fluorescence, the middle panel shows profiles after 2 and 30 minutes, and the bottom panel illustrates the results in fluorescence between 2 and 30 minutes of dye perfusion. Dotted lines indicate the D1 and D2 peaks (A-C) or the inner/outer wall (D). Note the difference made by the vertical axis of all panels.
2.4 RESULTS

Typical images of combined infusion of FITC-Δ148 (green) and Dil (red) are depicted in figure 2.1. The top panels show autofluorescence, the middle panels show fluorescence images after 2 and 30 minutes, and the bottom panels illustrate the increase in fluorescence between 2 and 30 min of dye perfusion. Dil accumulated in the endothelium, as is clear both in the 30 minutes image and, more pronounced, in the subtraction image. In contrast, no increase in FITC-Δ148 fluorescence was observed between 2 and 30 minutes. From images such as these, fluorescence profiles in radial direction at mid-plane of the arteries were determined. Typical profiles are shown in figure 2.2.

For both colors there is autofluorescence of the arterial wall (top panels, note the different vertical scales in the panels). Endothelial Dil accumulation is clear from the fluorescence profiles and further accentuated by the difference signals shown in the bottom panels, regardless of which FITC-Δ was used in combination with Dil. Peak Dil fluorescence increased steadily from 2 to 30 minutes with \(265 \pm 20\%\) (\(p<0.05\), 30 min vs. 2 min; \(n=24\) experiments; no differences between the different groups of arteries). The shape of the Dil-peaks, as expressed by their width (FWHM), remained constant within 5% over time.

The fluorescence profiles of the glass tube are also provided in figure 2.2. 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 the artery with FITC-Δ148, notwithstanding the difference in molecular size. Average data (7 experiments per FITC-Δ) on the development of the fluorescence distributions near the endothelium are provided in figure 2.3. All FITC-Δ curves are normalized to mid-luminal fluorescence, which was constant within 2% for all time points and all FITC-Δs. Also depicted in figure 2.3A-C are the average normalized fluorescence levels at mid-luminal position. The position of the endothelium as determined from peak Dil fluorescence, is taken as position 0 μm, and is indicated by the vertical dotted lines. Dil peak position remained constant over time; average standard deviation of variations in this position was 0.24 ± 0.01 μm, i.e. less than one pixel, allowing accurate localization of the endothelium. Figures 2.3A-C show that the smaller the dextran the more the fluorescence distribution extends over the endothelial position. For the large FITC-Δ148 there is no difference between 2 and 30 minutes indicating that an equilibrium state is reached after 2 minutes (fig. 2.3A), and practically all fluorescence falls inside of the Dil-peaks. For the smaller FITC-Δs the fluorescence profiles are still developing after 2 minutes, but outward migration of FITC-Δ4 between 2 and 30 min (fig. 2.3C) is more pronounced than...
Figure 2.3: Average normalized fluorescence after 2 and 30 min of perfusion with FITC-A148 (A), and after 2, 10 and 30 min of perfusion with FITC-A50 (B) or FITC-A4 (C). Fluorescence was normalized to mid-luminal fluorescence. The dotted lines indicate the endothelial position, determined from peak Dil fluorescence and therefore negative x-values indicate positions abluminally of the endothelium. Profiles are mean values ± SEM (n=7 experiments for each FITC-A). FITC-A fluorescence profiles after 30 minutes from panels A-C have been replotted in panel D, which also shows the fluorescence profile in the dummy after 30 minutes (dashed line), as well as the average fluorescence distribution of Dil, normalized to peak Dil fluorescence.

Figure 2.4: A: Example of fitting an arterial FITC-A148 fluorescence profile after 30 min of dye perfusion (triangles) with a predicted fluorescence profile (solid line), calculated from the convolution of an assumed square-shaped concentration profile (dashed line) and the Dil-derived LSF. Fitting resulted in a predicted dye front position relative to Dil peak position (dotted line), in this case characterized by X = 2.0 µm. B: Verification that fitting the measured glass tube profile (dots) with a predicted fluorescence profile (solid line), resulted in a dye front that coincided with the inner tube wall.
for FITC-Δ50 (fig. 2.3B). To directly compare the location of the different tracers, the FITC-Δ fluorescence profiles of the arteries after 30 minutes have been replotted in figure 2.3D. Also shown is the FITC-Δ fluorescence profile in the dummy after 30 minutes (dashed line), where tube wall position, known from the diameter measurement by scanning EM, is superimposed on endothelial position. The average fluorescence distribution of Dil bound to the endothelium, normalized to peak fluorescence, is shown as well. The FITC-Δ profiles after 30 minutes 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 towards the luminal side over ~2-3 μm, whereas the arterial FITC-Δ4 profile was shifted towards the abluminal side over ~1 μm.

The relation between an assumed dye concentration front, its fluorescence distribution in the image and the Dil-derived line-spread function (LSF), is demonstrated in figure 2.4A. 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 figure 2.4A (symbols), such that an optimal fit between data and predicted fluorescence profile is obtained, reveals that the FITC-Δ148 concentration front is a distance X, 'shifted' from the location of the Dil-peak (EC).

The same procedure applied to the measured glass tube profile (fig. 2.4B) revealed that the dye

![Figure 2.5: Average normalized fluorescence near the endothelium for arteries during the light-dye-treatment (LDT) protocol. Arteries were perfused with FITC-Δ148 for 30 minutes, after which LDT was started and dye perfusion and measurements were continued for another 10 minutes. Profiles are mean values ± SEM (n=3 experiments).](image-url)
front in the tube coincides exactly with the inner tube wall ($X = 0.001 \mu m$).
Averaged over all vessel experiments $X_i$ was $2.6 \pm 0.5 \mu m$ for FITC-$\Delta 148$ ($p<0.05$ vs. $0 \mu m$; $n=7$ experiments). The shapes of the fluorescence distributions after 30 minutes are quite similar for all dextrans (see fig. 2.3D) and therefore the same procedure was repeated for FITC-$\Delta 50$ and FITC-$\Delta 4$. For FITC-$\Delta 50$, $X_i$ was $0.1 \pm 0.9 \mu m$ ($p=NS$ vs $0 \mu m$; $p<0.05$ vs FITC-$\Delta 148$; $n=7$ experiments). For FITC-$\Delta 4$, $X_i$ was $-0.7 \pm 0.4 \mu m$ ($p<0.05$ vs. $0 \mu m$; $p<0.05$ vs. FITC-$\Delta 148$; $n=7$ experiments).

As shown in figure 2.5, light-dye-treatment (LDT), shifted the FITC-$\Delta 148$ fluorescence profile outward, which was quantified by a change in $X_i$ from $3.2 \pm 0.3 \mu m$ ($p=NS$ vs. the FITC-$\Delta 148$ group without LDT) to $0.1 \pm 0.1 \mu m$ after 10 minutes ($p<0.05$, 40 min vs. 30 min; $n=3$ experiments). EM of sections of the arteries made after the experiments revealed no damage to the endothelium by LDT (see fig. 2.6). Endothelial thickness was $0.19 \pm 0.01 \mu m$ ($n=66$ images) in general (left panel) and $0.64 \pm 0.03 \mu m$ ($n=14$ images) in regions of nuclei (middle panel).
2.5 DISCUSSION

We demonstrated by direct observation that FITC-Δ148 is excluded from a 2-3 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 light-dye-treatment (LDT).

2.5.1 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 (6). More direct evidence was obtained from intravital microscopy demonstrating a gap between red blood cells (RBCs) and the endothelium in the order of 0.5-1 μm (4;12;13;34-36). Moreover, it has been demonstrated that molecular penetration rates in the ESL depend on size and charge of the molecules (36).

The ESL thickness measured in the present study is larger than 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 (35). 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 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 (20), enzymes that degrade the ESL (6). 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 that used confocal microscopy (2). Model interpretation of these enzyme data resulted in an estimate of ESL thickness up to 7 μm (20), roughly a factor 3 larger than found presently from more direct observations of dye distributions. However, permeability studies are usually limited to a time period of ~2 minutes (2;18-20;23), while fluorescence profiles are still significantly changing after this time, as is clear from our experiments.
2.5.2 Criticism of the method

Due to the size of the arteries used, a long working distance objective with 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-A5s and the effect of LDT on the FITC-A148 fluorescence profiles are not affected by the correction of these curves for optical distortions, since these shifts follow directly from comparison of the measured profiles (fig. 2.3 and 2.5). However, estimation of the shift in concentration distributions with respect to the endothelium required fluorescent labeling of the endothelium with Dil and an estimation of the dye front with respect to the fluorescence profile.

Dil is able to spread along the membrane of an endothelial cell, but cannot migrate from one cell to another, leaving the smooth muscle cells unlabeled (14-16). In histological sections of arteries that had been perfused with Dil, this dye was only found luminally of the internal elastic lamina. EM pictures of the arteries used in the experiments demonstrated clearly that the endothelium was relatively thin (~0.2 µm) compared to 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 whether Dil concentrates more at the luminal side or at the abluminal side of the endothelium is therefore not relevant. Peak Dil fluorescence at the endothelium increased steadily over time, but the shape of these Dil-peaks as well as their position remained constant. Therefore, peak location was already clearly detectable after 2 minutes. Light-dye-treatment did not change position or shape of the Dil profiles, indicating that the generated free radicals are solely destroying the ESL and not the endothelial cells (see also below) and do not affect the determination of the endothelial position from the Dil measurements.

Under the assumption that the optical properties would be specific for the experimental setup we used the LSF that resulted from the averaged Dil 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 Dil-peaks, 6.4 ± 0.6 µm. The difference results from the 'bell'-shape of the peaks. The spread in FWHM of the individual Dil-peaks is most likely due to heterogeneity in Dil-labaling of the endothelial cells (ECs). If ECs at mid-plane (i.e. the plane of focus) are strongly labeled with Dil, the LSF will be narrow; if ECs out of the mid-plane are more strongly labeled, this will result in widening of the LSF and may in extreme cases result in a shift of the Dil-peak from the mid-plane 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 Dil-peak from the mid-plane EC position will be towards the luminal direction and will therefore result in an underestimation of the ESL thickness (see also Streekstra et al. (33)).

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 1/3 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 1/3 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. 2.3A), which makes it highly unlikely that the dye front had touched the endothelium.

Since the dye front will have reached the wall in 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 EM 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 in water and oil differed only 0.1%. Hence, in the worst case the uncertainty in inner wall position was about 0.2 μm and thus not larger than ~10% of the estimated ESL thickness.

Based on the glass tube measurements we are confident that the luminal dye filling of the arteries was completed well within the initial period of 1.8 minutes. Hence, subsequent differences in the way fluorescence profiles develop in arteries are dependent on molecular 'interactions' with the ESL or the arterial wall. The rather wide line-spread function for fluorescence observations has the consequence that fluorescence intensity may increase over a long period of time at a rather large distance from the endothelium, say 15 μm. Dye concentration may be constant at this...
distance of the wall, but measured fluorescence may still increase because of an increase in concentration close to the endothelium.

We did not systematically study wash-out of tracers since limitations of the system forced us to limit the number of measurements. From some observations we know that wash-out of FITC-Δs is within minutes, whereas Dil bound to the endothelium was not removed from the arteries upon wash-out.

We chose for LDT above a more physiological oxidative stress such as the application of oxidized low-density lipoproteins (ox-LDL) (4;5;34). The latter would require switching to yet an additional perfusion solution and a longer period for the oxidative stress to develop: 15-30 min for ox-LDL (4;5;34) versus a few minutes for LDT (35). 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 (4;34;35). 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 endothelial cells, 3) histology performed on the arteries after LDT, which showed normal coverage of the arterial wall with endothelial cells.

### 2.5.3 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 (10;18), agonists (19;23), temperature (8;25) and pH (8). However, the exact localization of the permeability barrier had not yet been determined satisfactorily.

Current models of vascular permeability state that the endothelial cell layer forms the main permeability barrier allowing exchange of water and solutes through pores and vesicles that are covered with a fibrous matrix (9;24). However, at least in capillaries it has been suggested that the ESL itself could form an additional barrier (35;36) 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 (4;5;34). 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 (30) and Nielsen (26)).
Possibly many concepts on endothelial function in vascular regulation have to be reconsidered knowing that endothelium can be covered by a layer many times thicker than the cells themselves.

2.5.4 Conclusion
We demonstrated the presence of a permeability barrier to solutes on the luminal side of the endothelium in isolated small arteries. This endothelial surface layer, with a thickness of 2-3 μm, is likely to form an essential domain for transvascular transport and blood-vascular wall interaction. Its damage during oxidative stress could form a key initial event in atherosclerosis.

2.6 REFERENCES
WALL PERMEABILITY OF ISOLATED SMALL ARTERIES

2. Localizing the permeability barrier