The permeability barrier of isolated rat mesenteric small arteries can be affected by the charge density of the endothelial surface layer (ESL). This study aimed to demonstrate that the modulation of solvent ionic strength influences the ESL charge density by varying solvent ionic composition.

**Materials and Methods**

**Artery preparation.** All experiments were performed according to institutional guidelines. Male Wistar rats (n = 21, 200–250 g) were euthanized by decapitation, and 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 189 ± 0.5 mm; no significant differences in diameter existed between the various groups of arteries in this study. Each rat provided one vessel. Other vessels or organs from the same rat were used in other experiments.

**Myograph.** The isolated artery was cannulated at one end with a double-barreled theta-cannula (World Precision Instruments) and at the other end with a regular cannula. Other vessels or organs from the same rat were used in other experiments.

**Solutions.**

- MOPS-buffered physiological salt solutions: MOPS-solutions were of normal ionic strength (162 mM, MOPS), low ionic strength (81 mM, LO-MOPS), or high ionic strength (323 mM, HI-MOPS), to modulate ESL charge density (normal, high, or low ESL charge, respectively). Osmolarity of MOPS, LO-MOPS, and HI-MOPS was kept constant at 297 mosmol/l, using additional glucose when necessary. Perfusion solutions were supplemented with 1% BSA. Arteries were cannulated with a double-barreled theta-pipet on the inlet side and a regular pipet on the outlet side.

- FITC-Labeled Dextran: Duling (26) in cremaster muscle capillaries by measuring the transport rate of uptake of fluorescently labeled dextrans in the ESL. In other experiments, the exclusion factor and tracer accumulation rate were predicted to depend on the volume density of fixed charges within the ESL and on the valence of solute charges, it was hypothesized that a modulation of the ESL charge density by varying solvent ionic composition should influence the ESL barrier properties.

**RESULTS**

- The present study was designed to demonstrate the effect of solvent ionic composition on ESL dimension and solute transport kinetics, applying our earlier developed technique to measure these properties using confocal laser scanning microscopy (CLSM). Solute transport through the ESL is characterized by convection and diffusion. Convection is dependent on the luminal solute concentration and the reflection coefficient of the arterial wall to the solute. Diffusion is dependent on the transvascular concentration gradient and the permeability of the arterial wall barrier to solute transport. MOPS-buffered physiological salt solutions (PSS) were used with either normal ionic strength, low ionic strength (LO-MOPS), or high ionic strength (HI-MOPS), while maintaining osmosality for all three solutions.

**DISCUSSION**

- Charge modification of the endothelial surface layer modulates the permeability barrier of isolated rat mesenteric small arteries. We hypothesized that modulation of the effective charge density of the endothelial surface layer (ESL) results in altered arterial barrier properties to transport of anionic solutes. Rat mesenteric small arteries (diameter ~190 μm) were isolated, cannulated, perfused, and superfused with MOPS-buffered physiological salt solutions. MOPS-solutions were of normal ionic strength (162 mM, MOPS), low ionic strength (81 mM, LO-MOPS), or high ionic strength (323 mM, HI-MOPS), to modulate ESL charge density (normal, high, or low ESL charge, respectively). Osmolarity of MOPS, LO-MOPS, and HI-MOPS was kept constant at 297 mosmol/l, using additional glucose when necessary. Perfusion solutions were supplemented with 1% BSA. Arteries were cannulated with a double-barreled theta-pipet on the inlet side and a regular pipet on the outlet side. After infusion of FITC-labeled dextran of 50 kDa (FITC-Δ50) and the endothelial membrane dye 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate, the dynamics of arterial dye filling were determined with confocal microscopy. ESL thickness, as determined from the initial exclusion zone for FITC-Δ50 permeated into the ESL with confocal microscopy. ESL thickness, as determined from the initial exclusion zone for FITC-Δ50 permeated into the ESL was measured using additional glucose when necessary. Perfusion solutions were supplemented with 1% BSA. Arteries were cannulated with a double-barreled theta-pipet on the inlet side and a regular pipet on the outlet side. After infusion of FITC-labeled dextran of 50 kDa (FITC-Δ50) and the endothelial membrane dye 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate, the dynamics of arterial dye filling were determined with confocal microscopy.
(Bronkhorst Hi-Tec Holland), resulting in an estimated wall shear stress of 1.0 ± 0.2 dyn/cm². The artery could be perfused with a solution containing fluorescent tracers via the second barrel of the 0-cannula. Fluorescent perfusate was also pressurized to 65 mmHg. The superfusate, 37°C MOPS-PSS, LO-MOPS-PSS, or HI-MOPS-PSS (see Solutions) of which PO₂ was maintained at ambient values, was continuously recirculated with a roller pump. The PCO₂ was not controlled because pH was buffered by MOPS. Under these conditions these arteries were without tone and maintained a constant diameter during the protocol. Viability of the vessels was tested by the administration of norepinephrine and ACh; all vessels reacted normally to these agents.

**Solutions.** The MOPS-PSS contained (in mM) 145 NaCl, 4.7 KCl, 1.17 MgSO₄, 1.2 NaH₂PO₄, 2 CaCl₂, 3 MOPS, 5 glucose, and 2 pyruvate.

LO-MOPS-PSS was based on MOPS-PSS, with NaCl reduced to 64.2 mM to reduce ionic strength, and glucose increased to 153.8 mM to increase ionic strength while maintaining isosmolarity. HI-MOPS-PSS was based on MOPS-PSS, with NaCl (38.2 mM) partially replaced by Na₂SO₄ (89.5 mM) to maintain isosmolarity.

Ionic strength of MOPS-PSS was 162 mM, of LO-MOPS-PSS 81 mM, and of HI-MOPS-PSS 323 mM, according to its definition: ionic strength $= \frac{1}{2}(c_1z_1^2 + c_2z_2^2)$, where $c_i$ is the concentration of ion $i$, and $z_i$ is its valence. Osmolarity ($\Omega$) for all three solutions was 297 mosmol/l, according to its definition $\Omega = \sum c_i z_i$, where $c_i$ is the osmotic coefficient of solute $i$, and $z_i$ is its number of particles formed on dissociation, and $c_i$ is its concentration. Perfusate MOPS-PSS, LO-MOPS-PSS, and HI-MOPS-PSS were supplemented with 10 mg/ml BSA (99% pure), freshly applied before use in the experiments. All chemicals were purchased from Sigma. All solutions were adjusted to pH 7.35.

**Fluorescent probes.** FITC-labeled dextran of 50.7 kDa (FITC-Δ50) was purchased from Sigma, and the lipophilic membrane tracer 1,1′-dioctadecyl-3,3,3′,3′-tetrachlorofluorescein (DiI) from Molecular Probes. FITC-Δ50 was applied in a concentration of 45.0 mg FITC-Δ50/l (9 × 10⁻⁶ M), resulting in a concentration of 1.0 × 10⁻⁹ M FITC. Labeling ratio for FITC-Δ50 was 0.004 FITC/glucose molecule, according to the distributor. Therefore, the net anionic charge on FITC-Δ50 is $\approx 1$ dextran. FITC-Δ50 was not filtered before use; thus there might be some free FITC present in the solutions. There might also be some polydispersity in the size of the dextran molecules. The possible contribution of free FITC and polydispersity will, however, be similar for all experiments in all groups of arteries. DiI was applied in a concentration of 1.0 × 10⁻⁶ 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 it cannot migrate from one cell to another (9, 11, 12).

**Confocal microscopy.** Images were recorded with a Leica DM IRBE microscope equipped with a Leica TCS SP2 confocal unit. Arteries were visualized from below through a cover glass that formed the bottom of the cannulation chamber. Excitation was obtained by an Ar ion laser using the 488 line. A ×20/0.70 objective in combination with a zoom factor of 2 resulted in a pixel size of $366 \times 366$ nm in the plane of focus. Green and red fluorescence were detected by using a prism and adjustable slits in front of two photomultipliers (PMTs). The wavelength of the detected light ranged from 500 to 530 nm (green) and from 625 to 750 nm (red), respectively. CROSSTALK between both fluorescence channels was negligible. The detection pinhole was 20 μm wide. Optical section thickness was about 13 μm. In this protocol, most CLSM settings, including laser power, wavelength settings, and pinhole size, were identical for all experiments. However, high-voltage settings for the PMTs were not identical for all experiments in order to obtain an optimal dynamic range of the fluorescence images recorded during the different experiments. Consequently, fluorescence intensity was not directly comparable for all the recorded images. Therefore, we normalized FITC-Δ50 fluorescence intensity to midluminal fluorescence before a comparison between different experiments. Images were recorded at midplane of the arteries every 3 s during the first 1.5 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 (27).

**Image analysis.** Profiles in radial direction were made of all fluorescence images, recorded at midplane, with the image analysis software ImageJ (National Institutes of Health). Measurement of the diameter of the arteries was based on the position of the endothelium, which was determined from the peak in the DiI fluorescence profiles, after subtraction of luminal DiI fluorescence (i.e., the profile after 1.5 min). FITC-Δ50 fluorescence profiles, normalized to midluminal fluorescence intensity, were quantified in a region spanning from 10 μm abuminally to 15 μm luminaly of the endothelium.

**Optical LSFs.** To quantify the effect of the optical transfer function of the microscope system on the measured dye distributions, we developed a correction procedure as previously described and verified position shift, the optical point spread function of the CLSM is characterized by a three-dimensional point-spread function (PSF) with a full-width half-maximum (FWHM) of $\approx 3$ μm in the image plane, which increases at increasing distances from the plane of focus and with a FWHM in z-direction of $\approx 13$ μm, i.e., the optical section thickness. The PSF results in a one-dimensional line-spread function (LSF), which we could estimate experimentally by direct observation of the DiI labeling of the endothelial membranes. Because the endothelium is only $\approx 0.2$ μm in thickness, this can be considered as a line; thus the average DiI fluorescence profile can be used as a LSF. This LSF has a FWHM of $\approx 5$ μm and is slightly asymmetric with respect to the endothelial position, because it takes the curvature of the endothelium along the arterial wall circumference into account. However, this does not impose any additional problem, because this asymmetry also reflects the curvature of the FITC-Δ50 core inside the vessels. Convolution of this LSF with square-shaped FITC-Δ50 arterial concentration profiles results in predicted FITC-Δ50 fluorescence profiles, which were fitted to the measured fluorescence profiles after 2–30 min of FITC-Δ50 perfusion to provide an estimate of the position of the FITC-Δ50 front near the arterial wall. The square-shaped concentration profiles are characterized by a shift $X_e$ from the endothelial position that represents the exclusion of FITC-Δ50 from the ESL. Although the concentration profiles can be considered discontinuous, the fluorescence profiles will always be continuous due to the convolution with the continuous LSF. The rather wide LSF has the consequence that FITC-Δ50 fluorescence intensity increases over a rather large distance from the endothelium, $\approx 15$ μm, even if the dye concentration would be perfectly constant at this distance from the wall. Nevertheless, this procedure allows us to localize the FITC-Δ50 concentration distribution with respect to the endothelial position with an accuracy of $<0.2$ μm and to quantify FITC-Δ50 transport kinetics (24). These kinetics are expressed in terms of the total ESL permeation time ($T_{ESL}$), the time needed for FITC-Δ50 to pass the ESL and to reach the endothelium, i.e., the time needed for $X_e$ to become $\approx 0$ μm.

**Statistics.** Data are means ± SE. The 95% confidence intervals were used to determine whether parameters were significantly different from 0 μm. Parameters describing fluorescence profiles for the different solutions were compared using ANOVA and Bonferroni post hoc tests. The dilution in response to ACh for the different groups of arteries were also compared using ANOVA and Bonferroni post hoc tests. Paired t-tests were used for the comparison of parameters at 2 min versus 30 min. A value of $P < 0.05$ was considered significant.

**RESULTS**

After being cannulated, arteries were equilibrated at 37°C for at least 30 min. Endothelial viability was tested by an administration of 10⁻⁶ M ACh to the superfusate of arteries,
preconstricted with $10^{-6}$ M norepinephrine (Nor). Ionic strength did not affect dilation in response to ACh [LO-MOPS, $58.9 \pm 9.1\%$; MOPS, $68.5 \pm 8.9\%$; and HI-MOPS, $53.8 \pm 11.2\%$; $P = P$ not significant (NS)]. After these viability tests, Nor and ACh were washed out, and further measurements were obtained at full dilation.

To determine the spatial distribution of FITC-$\Delta S$ over time, we measured fluorescence profiles from the recorded images during 30 min of dye perfusion for seven arteries in each group (LO-MOPS, MOPS, and HI-MOPS). Figure 1 provides the average fluorescence profiles in a region extending between 15 $\mu$m at the luminal side and 10 $\mu$m at the abluminal side of the endothelium, of cannulated arteries perfused and superfused with LO-MOPS (Fig. 1A), normal MOPS (Fig. 1B), or HI-MOPS (Fig. 1C). The position of the endothelium as determined from peak DiI fluorescence is taken as position 0 $\mu$m and is depicted with the vertical dotted lines. Figure 1 demonstrates that the development of the fluorescence profiles is slowest for LO-MOPS, faster for MOPS, and very fast for HI-MOPS, whereas after 30 min of dye perfusion, approximately identical fluorescence distributions are accomplished for the different groups of arteries. There were no significant differences in the fluorescence profiles after 30 min of dye perfusion for any of the three solutions.

Figure 2 shows the time dependence of $X_e$ for LO-MOPS, MOPS, and HI-MOPS, representing the time-dependent permeation of FITC-$\Delta S$ into the ESL for the different ionic strength conditions. The initial distance of FITC-$\Delta S$ from the endothelial position, i.e., the value of $X_e$ after 2 min of dye perfusion, represents the dimension of the ESL for the different ionic strength conditions. Initial $X_e$ for LO-MOPS ($6.3 \pm 1.4 \mu$m; $P < 0.05$ vs. 0 $\mu$m) was higher than for MOPS ($2.7 \pm 1.0 \mu$m; $P < 0.05$ vs. 0 $\mu$m) and significantly higher than for HI-MOPS ($1.1 \pm 1.3 \mu$m; $P = NS$ vs. 0 $\mu$m).

For LO-MOPS, $X_e$ decreased significantly during 30 min of dye perfusion from $6.3 \pm 1.4 \mu$m after 2 min to $-0.2 \pm 0.8 \mu$m ($P = NS$ vs. 0 $\mu$m) after 30 min, with a total $\tau_{ESL}$ of $\sim 26$ min. For MOPS, $X_e$ decreased significantly from $2.7 \pm 1.0 \mu$m ($P < 0.05$ vs. 0 $\mu$m) after 2 min to $-0.7 \pm 0.7 \mu$m ($P = NS$ vs. 0 $\mu$m) after 30 min, with $\tau_{ESL}$ of $\sim 20$ min. For HI-MOPS, no significant change in $X_e$ occurred (from $1.1 \pm 1.3 \mu$m after 2 min to $1.0 \pm 0.5 \mu$m after 30 min), indicating no detectable exclusion of FITC-$\Delta S$ from the ESL. There were no significant differences in $X_e$ after 30 min among the three groups of arteries.

**DISCUSSION**

We demonstrate that modulation of solvent ionic strength affects the dimension of the ESL and its kinetics for FITC-$\Delta S$ transport. Reduction of ionic strength increased ESL thickness and $\tau_{ESL}$ for FITC-$\Delta S$, whereas an increase in ionic strength minimized ESL dimension and its barrier properties.

**Criticism of method.** To visualize arteries of 150–200 $\mu$m, we needed a long working distance objective with relatively low numerical aperture. This results in blurring of the images, which complicates the localization of fluorescent tracers even when using a confocal microscope. To circumvent this complication, we previously developed and verified a correction procedure to estimate the concentration distribution of the tracers inside the cannulated arteries (24). By obtaining $X_e$, the shift of the FITC-$\Delta S$ concentration profile from the endothelial position, from 2 to 30 min of dye perfusion, we were able to study the kinetics of FITC-$\Delta S$ transport through the ESL and the dependence of these kinetics on solvent ionic strength. This led us to substantiate that the ESL dimension, as determined from the luminal exclusion zone to FITC-$\Delta S$ after 2 min of dye perfusion, as well as transport kinetics of FITC-$\Delta S$ into the ESL are dependent on ionic strength. We did not obtain $X_e$ for dye perfusion times < 2 min, because this would
Interpretation of results. Although a diminished transport of anionic solutes (2, 8, 15, 16) is in agreement with the presence of fixed negative charges on the luminal endothelial surface, the exact dimension of the endothelial structures carrying these negative charges has not yet been determined satisfactorily. Imaging of the fixed negative charges after staining with cationic probes (1, 3, 14, 18, 19, 23) shows that the charges are mainly located in membrane-bound structures (glycocalyx) of the ESL, such as glycoproteins and proteoglycans, but these techniques usually fail to obtain insight into the true in vivo dimensions of the charge-carrying structures.

ESL volume is determined by a dynamic equilibrium of water movement into the ESL due to interactions among the charge-carrying structures of the glycocalyx, free plasma ions, and other charged molecules present in the perfusate and the hydrostatic pressure of the perfusate (6). On modification of one of these factors, the ESL will adapt its structural organization to a new dynamic equilibrium. Therefore, modulation of perfusate and superfusate ionic strength is likely to result in dimensional changes of the ESL as well as changes in ESL charge distribution, both affecting ESL permeability properties. In the present study, the dimension of the exclusion zone increased by a factor ~2.3 as ionic strength was decreased twofold (LO-MOPS compared with normal MOPS), whereas ESL thickness decreased by a factor ~2.5 as ionic strength was doubled (HI-MOPS compared with normal MOPS). There might be a very small influence zone of the ESL outside the thickness of the layer itself. Nevertheless, the so-called Debye-Hückel length of this zone is generally in the order of nanometers (5), which is negligible compared with the thickness of the ESL. Furthermore, it is not likely that the very low concentration of FITC-Δ50 (9 × 10⁻⁷ M) and its low anionic charge of ~1.1 dextrans has altered the ESL dimension or promotes aggregation of FITC-Δ50 molecules at the different ionic strengths.

Our findings support the predictions from the electrochemical model of Damiano and Stace (6, 22). Their model predicts the initial exclusion of anionic tracers from the ESL and the transport of these tracers into the ESL over time. Both issues are dependent on the charge density of the ESL and on the valence of the tracers. According to this model, cations present in blood partially counterbalance fixed negative charges in the ESL. These authors proposed to use variations in perfusate ionic composition to modulate ESL charge density and thereby influence the ESL transport of anionic tracers. It was predicted that a twofold decrease in ionic strength would result in a twofold increase in ESL charge density and a twofold increase in the voltage differential over the luminal blood-ESL interface that forms the actual physical barrier to transport of anionic molecules (6, 22). Furthermore, an increase in ionic strength was predicted to decrease ESL charge density and to attenuate exclusion of anionic molecules from the ESL. In the present study, a twofold decrease in ionic strength increased the ESL permeation time for FITC-Δ50 by only 30%, but this may be due to the co-occurring increase in ESL dimension, which may partially attenuate the predicted increase in ESL charge density. A twofold increase in ionic strength resulted in a decreased ESL dimension and a loss of its barrier properties, which is probably due to a collapse of matrix components and a loss of structural organization.

Sörensson and coworkers (20, 21) have studied the influence of perfusate ionic strength on the charge selectivity of the glomerular capillary wall. These authors demonstrated that lowering ionic strength 4.5-fold reduced the fractional clearance of several anionic tracers ~1.5-fold, which was attributed to a reduction in the radius of the small pores responsible for...
the exchange and to a reduction in the charge density of the glomerular barrier. A 1.9-fold increase in ionic strength resulted in a slightly increased fractional clearance of anionic tracers. The present study suggests, however, that these clearance effects are due to volume and charge density alterations of the charged gel that covers endothelial cells. Furthermore, Granger and coworkers (8) demonstrated that neutralization of the negative fixed charges on the intestinal capillary wall by infusion of polycations induces an approximately sixfold increase in permeability to fluid and approximately a fourfold increase in protein clearance.

A single study on direct conformational changes in red blood cell glycocalyx due to variations in ionic strength has been reported by Wolf and Gingell (28). These authors demonstrated that the red blood cell glycocalyx swells roughly by a factor ~2.2 as ionic strength falls by a factor 4. The swelling effects at low ionic strength might explain the large exclusion zone to FITC-D50 when using LO-MOPS observed in the present study.

**Implications of the study.** Because vascular permeability is fundamentally important for normal solute transport as well as for early alterations in a variety of pathologies, including inflammation or the development of atherosclerosis, a proper understanding of the physical properties of the vascular wall barrier is required. The present study demonstrates that charge distribution within the ESL plays a key role in the transport of large molecules. This study was designed as a proof of principle, to demonstrate an effect of ionic strength modulations on ESL dimension and its kinetics for solute transport. Although changes in ionic strength as applied in the current study do not normally occur, effects on ESL dimension and charge density may also result from oxidative stress (4, 25–27), and the resulting alterations in transport properties may form a very early event in vascular pathologies.

**REFERENCES**