A Three-Pore Model Describes Transport Properties of Bovine Retinal Endothelial Cells in Normal and Elevated Glucose

Sandra V. Lopez-Quintero, Xin-Ying Ji, David A. Antonetti, and John M. Tarbell

PURPOSE. Changes in blood vessel barrier properties contribute to retinal edema in diabetic retinopathy (DR). However, limited data are available to describe the routes of transport for fluids and solutes across the inner blood–retinal barrier (iBRB). In this study, a three-pore model was developed to characterize such routes in normal and elevated glucose levels.

METHODS. Diffusive and apparent permeabilities to TAMRA (467 Da), dextran (70 kDa), and LDL (2000 kDa), as well as hydraulic conductivity, were measured across bovine retinal endothelial cell (BREC) monolayers after exposure to normal- and high-glucose media for 6 days. The data were used to develop a model of transport dynamics. Claudin 5 and eNOS Western blot analysis were used to measure changes in expression and phosphorylation. Immunolocalization of ZO-1 and VE-cadherin demonstrated organization of the junctional complex. Apoptosis was measured by TUNEL assay.

RESULTS. A three-pore model describes the fractional transport of water and molecular tracers across the retinal endothelial barrier. No change in permeability or hydraulic conductivity was observed after exposure to high glucose, whereas VEGF increased permeability in both normal- and high-glucose environments. The transport results were consistent with ZO-1 and VE-cadherin immunocytochemistry and expression of claudin-5, which were all unaltered by high glucose.

CONCLUSIONS. The data describe, for the first time, a model for transport of various size solutes and fluids across endothelial cells of the iBRB. Further, the results support the existence of an indirect pathway by which iBRB permeability is increased through the upregulation of retinal VEGF in response to hyperglycemia. (Invest Ophthal Vis Sci. 2011;52:1171–1180) DOI:10.1167/iovs.10-5971

Diabetic retinopathy (DR) is the leading cause of blindness among working-age adults, but the mechanisms by which diabetes leads to retinal microvascular complications is still not completely understood. The initial stage of DR is marked by an elevated permeability of the blood vessels of the retina and subsequent plasma leakage to the interstitial space. Elevated glucose is believed to contribute to various vascular dysfunctions—among them, loss of microvascular barrier integrity. However, an increase in VEGF that occurs even before morphologic abnormalities are observed has been described in patients with DR. The relative contribution of the direct effect of glucose compared with changes in cytokine expression on the inner blood–retinal barrier (iBRB) remains under investigation.

Expression and organization of a well-developed tight junction (TJ) complex in the inner retinal capillaries contributes to the formation of the iBRB. Immunohistochemical staining for albumin performed in patients at different stages of DR has shown that these vessels are the primary site of the vascular leakage that results in retinal edema. However, while changes in lipid, albumin, and fluorescein accumulation in the retina are routinely observed as an indication of altered endothelial permeability in patients with diabetic retinopathy, little has been done to characterize the various routes of transport that may affect vascular permeability. Transport of molecules across the vascular endothelium may occur by transcellular pathways, including specific transporters or by paracellular transport, which includes transport across the junctional complex, across a broken junctional complex, or across a large gap caused by cell death or cell division.

The two principal mechanisms driving paracellular transport of molecules and fluid across the endothelium are diffusion, which is movement from regions of higher concentration to regions of lower concentration, and convection, a mechanism of transport that results from the bulk motion of fluid typically driven by a pressure gradient. Molecular motion may differ from local fluid motion, because the solute molecules also diffuse simultaneously. Thus, onocytic pressure and hydraulic pressure drive both fluid and solute transport, but the availability of various routes of transport controls the rate of flux across the endothelium.

Information specific to the transport pathways of the iBRB is essential to understanding vascular permeability in DR; however, this information is difficult to obtain with in vivo models. Previous in vitro results suggested that hyperglycemia increases BREC diffusive permeability to small solutes, but more recent data on human retinal endothelial cells (HRECs) have suggested that upregulation of several cytokines causes endothelial dysfunction and not hyperglycemia, per se. Moreover, experiments in human retinal pigment epithelial cells (HRPECs) have demonstrated that hyperglycemia reduces the diffusive flux of both 40- and 70-kDa dextran, with no changes in TJs. In animal studies, increased permeability is normally observed anywhere from 1 to 6 months after streptozotocin-induced hyperglycemia, but this may be secondary to an increase in cytokines, such as IL-1 and TNF-α, and growth factors, such as VEGF.

In the present study, we examined an in vitro model system of retinal endothelial cells to quantify fluid and solute transport...
after both elevated glucose and VEGF treatments. Results from this analysis allowed the development of a three-pore model—namely, transcellular transport through vesicles, broken TJs, and large pores, also termed leaky junctions. A determination of the fractional transport through each pore for various size solutes was calculated. Hyperglycemia was shown to have no effect on water or solute transport across the endothelium, whereas VEGF increased permeability in both low- and high-glucose concentrations. The data suggest that 6 days of high glucose have little direct effect on endothelial permeability and suggest that hyperglycemia in diabetes alters permeability indirectly through cytokine production.

**Materials and Methods**

Bovine serum albumin (BSA, 30% solution), trypsin, penicillin-streptomycin solution, sodium bicarbonate, fibronectin, fetal bovine serum (FBS), l-glutamine, and d-glucose were obtained from Sigma (St. Louis, MO). MCDB-131 (phenol red free), t-cysteine, and l-methionine were obtained from U.S. Biological (Swampscott, MA). An antibiotic-antimycotic mixture was obtained from Invitrogen-Gibco (Rockville, MD) and heparin and Dulbecco’s PBS (1X without Ca²⁺ and Mg²⁺) from Fisher Scientific (Houston, TX). MCDB-131 complete (with phenol red, serum, and a cocktail of growth factors) was obtained from VEC Technologies (Rensselaer, NY). Porous polyester inserts (0.4 μm pore size, 12-mm diameter; Transwell) were obtained from Corning Costar (Acton, MA). VE-cadherin polyclonal primary antibody was obtained from Cayman Chemical (Ann Arbor, MI). ZO-1 primary antibody was obtained from Zymed (San Francisco, CA). Claudin-5 primary antibody was obtained from Invitrogen (Carlsbad, CA). The fluorescent solutes carboxyfluorescein-dextran (CF-Dex, 70-kDa), DiI-LDL (22 nm, 2,000 kDa), and DiI-dextran (80 kDa) were purchased from Invitrogen (Carlsbad, CA).

**Cell Culture**

Primary BREC monolayers were harvested from bovine eyes, as described by Barber, and then the frozen vials were thawed as needed and grown on fibronectin-coated (2 μg/cm²) T-75 flasks with MCDB-131 (US Biological) medium prepared as indicated by the vendor by addition of l-glutamine, t-cysteine, l-methionine, and heparin and supplemented with 10% FBS, 10 ng/mL EGF, 0.2 mg/mL growth factor (ENDOGRO; VEC Technologies, Inc., Rensselaer, NY) and antibiotic-antimycotic at 37°C and 5% CO₂. For transport experiments, porous migration assay inserts were seeded at a density of 6.0 × 10⁴ cells/cm². The BREC monolayers were grown with 100 mM hydrocortisone, to enhance monolayer formation and barrier properties. The normal glucose (NG) level of the culture media was 5 mM and the high glucose (HG) level of 25 mM was reached by addition of d-glucose. The monolayers were cultured for 4 to 6 days (until they reached confluence). Transport experiments were performed using phenol red-free MCDB-131 with 1% BSA, penicillin-streptomycin solution, and no other supplements. These experiments lasted no more than 4 hours. Cells were used between passages 3 and 8. In experiments where cells were treated with VEGF and 70-kDa dextran permeability was measured, HG was 10 mM.

**Measurement of Water and Solute Flux**

The simultaneous measurement of water and solute flux was performed with an apparatus described previously. Briefly, the BREC-seeded porous filters were placed inside each of eight chambers, which were sealed so that they formed a luminal (top) and an abluminal (bottom) compartment. All the chambers were connected to a reservoir that could be lowered 10 cm in relation to the media level in the luminal compartment, thereby imposing a hydrostatic pressure differential to drive fluid flow across each monolayer. In two of the chambers, this fluid flow moved a bubble that was previously inserted in a borosilicate glass tube that connected the chamber to the reservoir to allow measurement of water flow. The movement of the bubble was tracked using a spectrophotometer and accompanying software (Bt-Millenia, both from C&L Instruments, Hershey, PA). The displacement versus time data were used to calculate the water flow per unit area across the monolayer (Fig. 1). At steady state, the slope of the curve was constant and could be obtained by applying a linear fit to the data. The water flow per unit area was calculated with the relationship

\[
J_w = \frac{\Delta d}{\Delta t} \frac{F}{A}
\]

where \(\Delta d/\Delta t\) is the slope of the linear fit of the displacement versus time curve, \(F\) is a volumetric factor for the glass tube (volume per unit length), and \(A\) is the cross-sectional area of the filter.

Three fluorescently tagged molecules of different sizes were used, to measure solute transport across the BREC monolayer, (Table 1). Each tagged molecule was initially added to the luminal compartment in separate experiments and was allowed to pass through the BREC monolayer to the abluminal compartment. During the first hour of the experiment, the abluminal reservoir was allowed to equilibrate with the height of the media in the luminal compartment to eliminate fluid flow. Then, a 10-cm H₂O hydrostatic pressure differential was applied and maintained for 2 hours to drive convective flow across the monolayer. Finally, convective flow was stopped by eliminating the pressure differential, and the fluorescent molecule was allowed to cross the monolayer by diffusion for 1 hour. Fluorescence in the abluminal compartment was acquired as a function of time (FluoMeasure software; C&L Instruments). The fluorescence counts generated were converted to units of concentration by using a calibration curve. The

<table>
<thead>
<tr>
<th>Table 1. Fluorescent Molecules Used to Characterize Solute Transport Properties of BREC Monolayers</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Molecule</strong></td>
</tr>
<tr>
<td>-----------------------------------------------</td>
</tr>
<tr>
<td>TAMRA</td>
</tr>
<tr>
<td>Dextran</td>
</tr>
<tr>
<td>DiI-DLD</td>
</tr>
</tbody>
</table>

The Stokes diameter, molecular weight, and concentration used at the luminal side of the insert are listed.
slopes of the concentration versus time curve \((\Delta C/\Delta t)\) were used to calculate the permeability to the fluorescent molecule as

\[
P_e/P_a = \frac{(\Delta C/\Delta t)}{C_i} \times \frac{V_s}{A} \tag{2}
\]

where \(P_e/P_a\) is either the apparent permeability, \(P_e\), which is calculated from the slope in the time interval where there is a convective component, or \(P_a\), which is calculated using the slope during the time interval when only diffusion is taking place (Fig. 2). \(V_s\) is the volume of the abluminal compartment, \(C_i\) is the solute concentration on the luminal side, and \(A\) is the area of the monolayer. The concentration on the luminal side is very high compared with that on the abluminal side and is assumed to be constant during the course of the experiment. Before the experiments, the monolayers were rinsed twice with phenol red–free MCDB-151 medium supplemented with 1% BSA. During the experiments, the medium was maintained at 37°C and equilibrated with 5% CO2-95% balanced air, to regulate the pH at 7.4.

**Pore Model**

The endothelium has been modeled as a membrane with pores or pathways of different sizes that impose sieving restrictions on the molecules being transported but do not interact with each other. The model used herein to describe transport across BREC (bovine aortic endothelial cell) monolayers has been used before to describe transport across BAEC (bovine aortic endothelial cell) monolayers. For a single pathway and solute, the apparent permeability \(P_e\) results from diffusive and convective contributions, as described by

\[
P_e = P_o Z + J_v(1 - \sigma). \tag{3}
\]

The \(Z\) term is given by

\[
Z = \frac{N_{pe}}{e^\sigma - 1} \tag{4}
\]

where \(N_{pe}\) is the Péclet number defined as

\[
N_{pe} = \frac{J_e}{P_o}. \tag{5}
\]

and \(J_e\) is the water flux across the pathway, \(P_o\) is the diffusive permeability, and \(\sigma\) is the reflection coefficient of the solute, which lies between 0 and 1.

The three pathways used to model the endothelium are pathway 1, transcellular/vesicular; pathway 2, breaks in the TJs (average size, 20 nm), and pathway 3, large-pore leaky junctions (average size, 600 nm). The model assumes that water is transported only through pathways 2 and 3, because the hydraulic resistance of the very narrow TJs effectively blocks water flow, and vesicles are not believed to conduct significant water. Therefore, the total water flow \((J_v)\) across the endothelium is the sum of the water flow across pathways 2 and 3

\[
J_e = J_{v2} + J_{v3} \tag{6}
\]

It seems reasonable to assume that, due to its size (2–4 nm), the TJ pathway excludes 70-kDa dextran and LDL. Also, due to its size, LDL is unlikely to go through pathway 2, breaks in the TJs. Assuming that LDL transport occurs through pathways 1 and 3 (vesicles and leaky junctions), and that \(\sigma_o\), the reflection coefficient for LDL in pathway 3, is equal to 0, since leaky-junction dimensions are much greater than LDL diameter, then equation 5 for this molecule reduces to equation 7. It is worth mentioning that there is no convection through pathway 1 vesicles, since there is assumed to be no water flux.

\[
P_e = P_{o1LDL} + P_{o2DEX} Z_{2DEX} + J_v \tag{7}
\]

However, since \(N_{pe}\) is large (\(Z\) tends toward 0) for LDL, the total permeability to LDL tends to be equal to the water flow through the leaky junctions \((J_v)\), which is much larger than the diffusive transport through vesicles \((P_{o1LDL})\). The LDL transport through pathway 1 has been estimated to be less than 10% in BAECs.

Similarly, if 70-kDa dextran is assumed to be transported through pathways 2 and 3 (2DEX or 3DEX) only, since previous experiments in BAECs show that there is no vesicle contribution to the transport of this molecule, and if \(\sigma_{2DEX} = 0\) (dextran is much smaller than the large-pore leaky junction) and \(\sigma_{3DEX}\) is different from 0, then equation 3 for 70-kDa dextran can be written as

\[
P_e = P_{o2DEX} Z_{2DEX} + J_v(1 - \sigma_{2DEX}) + P_{o3DEX} Z_{3DEX} + J_v \tag{8}
\]

To solve the system of equations 6 to 8, it is first recognized that equations 6 and 7 can be solved for the two unknowns: \(J_{v2}\) and \(J_{v3}\). The other variables in the equations are measured experimentally. For instance, by fixing the monolayers to block vesicular transport, one can determine the values for \(P_{o2DEX}\) subsequently, \(P_{o3DEX}\) can be determined from equation 7a. Then, with those values for \(J_{v2}\) and \(J_{v3}\), equation 8 is solved together with equation 8a for \(P_{o3DEX}\) and \(J_v\). The solution to these equations will exist only for a certain range of \(\sigma_{2DEX}\) between 0 and 1.

**Junction Protein Immunostaining**

To visualize the TJ protein ZO-1 and the adherens junction protein VE-cadherin, we fixed BREC so filters with 1% paraformaldehyde for 10 minutes, washed them with PBS, permeabilized them with 0.2% Triton X-100 in PBS for 10 minutes, and then blocked them with 10% BSA and 0.1% Triton X-100 in PBS (blocking solution) for 1 hour. After the filters were washed with PBS, either rabbit anti-ZO-1 or VE-cadherin polyclonal primary antibody was diluted in blocking solution.
(1.25 μg/mL) and added to them. The next day, the cells were washed five times with PBS and incubated for 1 hour with Alexa Fluor 488 donkey anti-rabbit secondary antibody (Invitrogen) diluted in blocking solution (4 μg/mL). Once again, the cells were washed four times with PBS and placed under an inverted microscope (Eclipse TE2000-E; Nikon, Tokyo, Japan) for observation. Two random fields were chosen per filter, and fluorescent images were captured (Photometrics Cascade 650 camera; Roper Scientific, Tucson, AZ) and the images acquired on computer (MetaVue 6.2r2; Universal Imaging, Downingtown, PA). The same fixation was used for immunostaining or to block vesicular transport and determine the contribution of active to total transport for LDL and dextran.

Determination of Apoptotic Cells
A terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) assay protocol was used to determine the percentage of apoptotic cells. The monolayers were fixed in 4% paraformaldehyde for 1 hour at room temperature, rinsed with PBS, incubated with 50 μL blocking solution (0.1% Triton X-100 in 0.1% sodium citrate) for 10 minutes at room temperature, and rinsed again with PBS. The TUNEL reaction mixture was prepared and added to each filter (50 μL). The filters then were incubated in the dark for 1 hour at 37°C, rinsed three times with PBS, and observed by microscope (Eclipse TE2000-E; Nikon).

Incubation with VEGF
In some experiments, the monolayers were incubated for either 2 or 4 hours with 100 ng/mL VEGF in culture medium and then harvested for Western blot analysis. In a separate set of experiments, BREC monolayers were incubated with VEGF for 4 hours before water and solute flux were determined.

Western Blot Analysis
BRECs were grown in MCDB-131 complete (VEC Technologies), and the monolayers where scraped from the filters in the presence of RIPA lysis buffer (NaCl 0.6 M, NP-40 4%, Tris 0.2 M, Brij 35 0.4%, and EDTA 4 mM) supplemented with a protease and phosphatase inhibitor cocktail, Na3VO4 and PMSF. Protein concentration was determined with the standard techniques, loading 20 μg of protein into the gel wells and incubating overnight with the appropriate antibodies phospho-eNOS (Serine 1177) or claudin-5 and the regulatory protein β-actin, followed by reaction with matched secondary anti-rabbit antibody. The blots were scanned in a light-tight enclosure (Universal Hood II; Bio-Rad) and visualized (Quantity One ver. 4.5; Bio-Rad).

PCR Analysis
PCR was performed with a cDNA synthesis kit (Cells-to-cDNA II Kit; Applied Biosystems-Ambion, Austin TX). After RNA was converted to cDNA, the products were incubated with Taq master mix (New England Biolabs, Beverly, MA), and PCR primers. The PCR program was: 95°C for 5 minutes; 30 cycles of 94°C, 52°C, and 72°C for 30 seconds for each step; and a final extension of 72°C for 10 minutes. The primer sequences (Integrated DNA, Coralville, IA) for the target genes were claudin-5, forward: ACGGGCGCATATGACAAAGA and reverse: AGGTCATCATCTTCGACC (with 227 bp of PCR product); and for the internal standard GAPDH were forward: AGGGTCATCATCTTCGACC and reverse: CCATCCCCAGTCTCTGGGT (with a 218 bp of PCR product). The PCR products generated were determined by 2.5% agarose gel (Invitrogen) electrophoresis in the presence of ethidium bromide, scanned in a light-tight enclosure (Universal Hood II; Bio-Rad), and analyzed with imaging software (Quantity One; Bio-Rad).

Statistical Analysis
Water flux and solute permeability are presented as the mean ± SE. Differences were assessed by using the unpaired Student’s t-test with P < 0.05 considered significant.

RESULTS
Water Flow and Solute Permeability
Table 2 summarizes the water flow and permeability data for LDL, 70-kDa dextran, and TAMRA in BREC monolayers incubated with 5 mM or NG and 25 mM or HG for 6 days. Jw/A represents the water flow per unit area, Pw represents the permeability under diffusive conditions, and Pe represents the steady state apparent permeability.

A typical bubble displacement versus time curve is shown in Figure 1, where the slope of the linear range was used to calculate the water flux values at steady state by using equation 1 (the Methods section). The water flux across the BREC monolayers in both NG and HG showed a typical transient decrease that has been observed before in vitro and in vivo and has been termed the sealing effect or adaptive response.18,22,23

Table 2. Water Flow per Unit Area (Jw/A) and Solute Permeability to LDL, 70-kDa Dextran, and TAMRA of BREC Monolayers Incubated with NG and HG

<table>
<thead>
<tr>
<th>Molecule</th>
<th>n</th>
<th>Jw/A × 10⁻⁶ (cm/s)</th>
<th>Pw × 10⁻⁶ (cm/s)</th>
<th>Pe × 10⁻⁶ (cm/s)</th>
<th>Mean Pw/Pe</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water</td>
<td>12</td>
<td>NG 1.95 ± 0.34</td>
<td>HG 2.74 ± 0.35</td>
<td>P = 0.11</td>
<td></td>
</tr>
<tr>
<td>LDL</td>
<td>12</td>
<td>NG 0.20 ± 0.07</td>
<td>HG 1.39 ± 0.33</td>
<td>6.95</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>HG 0.23 ± 0.05</td>
<td>HG 1.43 ± 0.63</td>
<td>6.22</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>P = 0.42</td>
<td>P = 0.78</td>
<td></td>
<td></td>
</tr>
<tr>
<td>70-kDa dextran</td>
<td>8</td>
<td>NG 1.24 ± 0.21</td>
<td>HG 2.43 ± 0.45</td>
<td>1.70</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>HG 1.85 ± 0.25</td>
<td>HG 2.81 ± 0.49</td>
<td>1.52</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>P = 0.23</td>
<td>P = 0.59</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TAMRA</td>
<td>6</td>
<td>NG 0.64 ± 0.33</td>
<td>HG 11.4 ± 0.35</td>
<td>1.18</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>HG 0.90 ± 0.67</td>
<td>HG 10.5 ± 0.76</td>
<td>1.16</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>P = 0.66</td>
<td>P = 0.25</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The high-glucose level corresponds to 25 mM. After the monolayers reached confluence (4–6 days), they were placed in the transport apparatus to determine water flux (Jw/A) as well as solute permeability.
### LDL Permeability

On application of a 10-cm H₂O hydrostatic pressure differential, the concentration versus time curve for LDL showed a sharp increase in slope (Fig. 2), suggesting that the flux of this macromolecule is coupled to water flow by a solvent drag mechanism. This behavior has been observed before in BAEC monolayers and in intact hamster venules. The pronounced effect of solvent drag can be seen in the mean ratio of the apparent (Pₒ) to the diffusive (Pₒ,diff) permeability, which was 6.95 for NG and 6.22 for HG.

### 70-kDa Dextran Permeability

The increased transport associated with elevated water pressure, which had been observed previously, indicates that the flux of 70-kDa dextran is coupled to water flow as well. The solvent drag effect was highest at the moment of application of the hydrostatic pressure and then decreased as sealing occurred. Again, permeability values for NG and HG were not significantly different. The mean ratio of the apparent to the diffusive permeability calculated at the steady state region was 1.7 for NG and 1.52 for HG.

### TAMRA Permeability

On application of a 10-cm H₂O hydrostatic pressure differential, the concentration versus time curve for this molecule did not show the sharp increase seen with the two larger molecules. The convective component did not have a significant contribution to transport; therefore, TAMRA is readily transported by diffusion across BREC monolayers. A similar behavior had been observed in early studies of muscle endothelium, where small solute transport was little affected by convection. The ratio of the apparent to the diffusive permeability was 1.18 for NG and 1.16 for HG. The permeability values obtained for NG versus HG were not significantly different.

### Cell Fixation Experiments

Table 3 shows the diffusive permeability for control (Pₒ) and fixed (Pₒ, fixed) monolayers, as well as the ratio of the two permeabilities, calculated from the mean values. On fixation, the diffusive permeability for LDL was significantly reduced to one third of its original value (P = 0.002), whereas the diffusive permeability for dextran was not significantly different (P = 0.62). These results suggest that BREC monolayers show a significant vesicle contribution for LDL transport but not for 70-kDa dextran. These results were used in the three-pore analysis to assign a value to the LDL vesicle transport.

### Three-Pore Model Analysis

The results for the three-pore model are shown in Table 4. For this analysis, it was assumed that the endothelium can be modeled as a membrane containing three pores that do not interact with each other. The interaction between pores in heteroporous membranes, which has been observed to be relevant for low filtration rates in the presence of a significant osmotic gradient, has been neglected, because we used 1% BSA-MEM medium for both the luminal and abluminal compartments; therefore, the osmotic gradient across the monolayer is basically 0. The retinal TJ gap, which has been described as the place where the membranes touch, has not been included as a pore. The three pores are: (1) transcellular/vesicle, (2) breaks in the TJs, and (3) large-pore leaky junctions. We did not include a water-only channel (aquaporin), as in the previous three-pore models used to describe transport during peritoneal dialysis, because it has been observed that the aquaporin level is low or nonexistent in retinal endothelium, and as stated before, there are no large osmotic gradients across the endothelial cells. The experimental values obtained in NG were used to develop this model because no significant difference was observed in HG conditions. A comparison of solute transport under normal and fixation conditions (Table 3) demonstrated that LDL, but not dextran, is transported transcellularly, and the model was constructed after this observation. Also, given the sizes of the molecules, it was assumed that LDL, dextran, and water are transported through the large-pore leaky junctions, but only dextran and water are transported through breaks in the TJ that have been estimated to open to the width of the adherent junction (20 nm). Finally, it was also assumed that the reflection coefficient at the large-pore leaky junctions was 0 for both dextran and LDL, but the reflection coefficient at the breaks in the TJs for dextran was a value between 0 and 1. Only a limited range of this coefficient yielded real solutions when applied to the transport equations. For NG, the equations had real solutions for σ2DEX ranging from 0.4 to 0.55, this range is in good agreement with the range obtained for BAEC monolayers that had been grown in NG.

The solution to the model shows that the large-pore leaky junction is a dominant transport pathway for both 70-kDa dextran and LDL, whereas water tends to be more evenly distributed through the breaks in the TJs and the large-pore leaky junctions. Renkin made a similar prediction with a two-pore model in which most of the albumin flux and all the larger proteins are carried by convection through the large pores. In both NG and HG monolayers, vesicle transport accounts for less than 11% of the LDL transport. These results suggest that alterations in pathways 2 and 3 would significantly affect solute and water transport.

### Table 3. Effect of Fixation on Transport of LDL and 70-kDa Dextran

<table>
<thead>
<tr>
<th>Molecule</th>
<th>n</th>
<th>Pₒ × 10⁻⁶ (cm/s)</th>
<th>Pₒ, fixed × 10⁻⁶ (cm/s)</th>
<th>P for Significance</th>
<th>Mean Pₒ/Pₒ, fixed</th>
</tr>
</thead>
<tbody>
<tr>
<td>LDL</td>
<td>6</td>
<td>0.29 ± 0.04</td>
<td>0.09 ± 0.03</td>
<td>0.002</td>
<td>3.00</td>
</tr>
<tr>
<td>70-kDa dextran</td>
<td>6</td>
<td>1.24 ± 0.23</td>
<td>1.52 ± 0.32</td>
<td>0.620</td>
<td>0.81</td>
</tr>
</tbody>
</table>

Fixation of the cells with 1% paraformaldehyde significantly decreased the transport of LDL but not of 70-kDa dextran.

### Table 4. Molecules and Pathways Analyzed with a Three-Pore Model

<table>
<thead>
<tr>
<th>Component</th>
<th>Pathway 1: Vesicles</th>
<th>Pathway 2: Breaks in the TJ</th>
<th>Pathway 3: Leaky Junctions</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water</td>
<td>34.75</td>
<td>65.25</td>
<td>90.58</td>
<td>100</td>
</tr>
<tr>
<td>LDL</td>
<td>9.42</td>
<td>90.58</td>
<td>80.07</td>
<td>100</td>
</tr>
<tr>
<td>Dextran</td>
<td>19.93</td>
<td>80.07</td>
<td>80.07</td>
<td>100</td>
</tr>
</tbody>
</table>

The percentages shown correspond to σ2DEX = 0.55. The model was constructed based on the assumptions and equations established in the Methods section. The percentage of solute and water being transported through each pathway were estimated from the solutions to equations 6 to 8, using the experimental permeability values.
For a random fiber arrangement, the solute, and where (1 - ε) represents the fractional fiber volume when the random arrangement is used, and Vf represents the fractional fiber volume when the ordered arrangement is used. Using 5.5 nm for the solute radius and 0.6 nm for the fibers, equations 10 and 11 predict volume fraction values that are in agreement with values estimated for capillaries.31

The partition coefficient ϕ is calculated for a random or an ordered fiber arrangement. (1 - ε) represents the fractional fiber volume when the random arrangement is used, and Vf represents the fractional fiber volume when the ordered arrangement is used. Using 5.5 nm for the Stokes radius of dextran and 0.6 nm for the fiber radius,9 equation 10 predicts when cells were cultured in HG compared to NG (Fig. 4).

It is generally believed that the reflection coefficient for the breaks in the TJ, σ2, is determined by the fiber matrix (glycocalyx) that fills the apical aspect of this pore. With the σ2DEX values obtained, between 0.4 and 0.55, and the following equations, it is possible to estimate the glycocalyx fractional fiber volume Vf by modeling the glycocalyx as a fiber matrix,9 where the reflection coefficient σ is given by

\[
σ = (1 - ϕ)^2. \tag{9}
\]

For a random fiber arrangement, ϕ, the partition coefficient, is given by

\[
ϕ = \exp[-(1 - ϕ)(2a/r_f + a^2/r_f^2)] \tag{10}
\]

where (1 - ε) is the fractional fiber volume, a is the radius of the solute, and r_f is the fiber radius.

For an ordered fiber arrangement, the partition coefficient is given by

\[
ϕ = 1 - V_f(1 + a/r_f)^2 \tag{11}
\]

where V_f represents the fractional fiber volume. The results are shown in Table 5. With 5.5 nm used for the Stokes radius of dextran and 0.6 nm for the fiber radius,9 equation 10 predicts a volume fraction (1 - ε) that ranges from 0.98% to 1.32% for a random fiber arrangement, and equation 11 predicts a volume fraction V_f that ranges from 0.61% to 0.72% for an ordered fiber arrangement with NG treatment. These predicted fiber volume fractions are low but are in agreement with values estimated for capillaries (<5%).31 Our maximum fiber volume was 1.32%. Our minimum spacing between the fibers as a function of fiber volume (V_f) and fiber radius (r_f) as defined by:

\[
Δ = [(π/V_f)^{1/2}] - 2 \times r_f
\]

was 11.3 nm, which is larger than the value of 7 nm previously established.32 However, it should be noted that our in vitro model differs from the in vivo models on which those observations were based and that permeabilities are higher in vitro.

With the σ values for which our pore model has solutions (0.45 < σ < 0.55), using a pore analysis, we estimate the radius of the cylindrical pore with the relationship31

\[
ϕ = (1 - γ)^2 \tag{12}
\]

and equation 9, where γ is the ratio of the pore to the solute radius (γ = p/a); for 70-kDa dextran, a is equal to 5.5 nm. For our range of σ, the pore diameter is estimated to be between 22.36 and 25.8 nm, which is in the range of observations for capillaries where the average size of the breaks in the TJs has been measured to be 20 nm.18

**Junction Proteins under HG**

Organization of the TJs requires ZO family members, and ZO-1 organization is an excellent marker of TJ formation, whereas the transmembrane protein VE-cadherin is a marker of adherens junction organization. Representative images of for ZO-1 and VE-cadherin immunostaining are shown in Figure 3. Qualitatively, no differences in the intensity or continuity of these junctional proteins were observed between the NG and HG monolayers. The transmembrane TJ protein claudin-5 is expressed in endothelial cells, is essential for formation of the blood–brain barrier,33 and is also present in the retina.14 Western blot analysis and RT-PCR demonstrated that neither claudin-5 protein nor gene expression was significantly different when cells were cultured in HG compared to NG (Fig. 4). Image analysis showed a 13% ± 0.45% decrease in protein

---

**TABLE 5. Fractional Fiber Volume for Different Dextran Reflection Coefficients through Pathway 2**

<table>
<thead>
<tr>
<th>σ2DEX</th>
<th>% Pore 2</th>
<th>ϕ</th>
<th>(1 - ε)</th>
<th>Vf</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.40</td>
<td>32.00</td>
<td>0.37</td>
<td>0.0098</td>
<td>0.0061</td>
</tr>
<tr>
<td>0.50</td>
<td>22.20</td>
<td>0.29</td>
<td>0.0120</td>
<td>0.0068</td>
</tr>
<tr>
<td>0.55</td>
<td>19.93</td>
<td>0.26</td>
<td>0.0132</td>
<td>0.0072</td>
</tr>
</tbody>
</table>

**FIGURE 3.** Representative micrographs of the TJ protein ZO-1 and the adherens junction protein VE-cadherin. On exposure to HG (25 mM) for 6 days, there was no significant change in the intensity or continuity of either ZO-1 or VE-cadherin compared with the NG control.
expression and an 11% ± 0.34% increase in gene expression, but neither was statistically significant.

**Apoptotic Cells**

Representative pictures of the TUNEL assay staining are presented in Figure 5. Cells were cultured with 25 mM glucose medium for 6 days and compared to control cells. In an analysis of six random fields, the percentage of apoptotic cells was 2.1% ± 0.56% and 2.9% ± 0.46% for NG and HG, respectively. Again, this change was not statistically significant (P = 0.62).

**Effect of VEGF on 70-kDa Dextran’s Apparent Permeability and Water Flux across BREC Monolayers**

It has been shown that BREC monolayers respond to VEGF with an elevated $J_v/A$ that is attenuated by an e-NOS inhibitor; therefore, BREC monolayers were incubated with 100 ng/mL VEGF to determine the relative amount of phosphorylated eNOS compared with control monolayers. We observed that the expression of phosphorylated eNOS increased with time and was significantly different from the control, 4 hours after initial incubation (Fig. 6). Therefore, the effect of VEGF on BREC monolayer permeability was measured 4 hours after addition of VEGF in both NG (5 mM) and HG (10 mM) environments. The results of our measurements are shown in Table 6. Comparing NG without VEGF and NG with VEGF, there was a significant increase in water flux (P = 0.0062). Comparing HG without VEGF and HG with VEGF, again a significant increase in water flux (P = 0.0067) was observed. Similarly, there was a significant increase in 70-kDa dextran permeability when the cells grown in NG were incubated with VEGF (P = 0.017), and there was a significant increase when the cells grown in HG were incubated with VEGF (P = 0.0071). Even though there was a tendency for glucose to sensitize the monolayer to VEGF, there was no significant difference between the NG and HG monolayers incubated with VEGF (P = 0.1).

**DISCUSSION**

In this study, an in vitro model of the iBRB was used to accurately assess water and solute flux across the endothelium simultaneously. A three-pore model was developed to describe the diffusive and convective flux of small, intermediate, and large molecules and to determine their routes of transport. To our knowledge, no other study addresses the transport of molecules over a broad range of sizes across BREC monolayers under convective conditions and estimates the distribution of molecular transport through the principal endothelial pathways. Therefore, this set of data is an important characterization of BREC monolayers in vitro and provides a novel understanding of the various routes of transport across the BRB.

The results shown herein for retinal endothelial cells reflect similarities to those previously reported for aortic endothelium but also demonstrate important differences. Results for LDL apparent permeability (Table 2) are very similar to those found for BAEC monolayers, where $P_e$ was measured to be

**FIGURE 4.** Western blots and PCR showing the expression of the TJ protein claudin-5 in monolayers incubated for 6 days in either NG (5 mM) or HG (25 mM). After the images were analyzed, it was observed that the claudin-5 protein expression of monolayers in HG decreased by 13% ± 0.45% compared to NG (n = 3), whereas the gene expression increased by 11% ± 0.34% (both changes not significant; n = 3).

**FIGURE 5.** TUNEL assay for apoptotic cells. Staining for cells grown in both NG (5 mM) and HG (25 mM) glucose is shown. The mean percentage of stained cells was slightly elevated in the cells grown in HG. The values were 2.1% ± 0.56% and 2.9% ± 0.46% for normal and HG, respectively; however, they were not significantly different (P = 0.62, n = 6).
1.31E–6 ± 0.36 cm/s. However, water flux was measured to be 5.39E–6 ± 0.7 cm/s in BAECs16; whereas, BREC water flux is about half the value in BAECs. The difference may be due to the specialized junctional complex in the BREC that form the iBBB. Indeed, the mean water flux through the large-pore leaky junction for BREC monolayers was 1.26E–6 cm/s, which is very similar to the value of 1.20E–6 in BAEC monolayers,16; however, water flux through the break in the TJs is much lower in BREC than in BAECs: 6.17E–7 compared with 4.19E–7, respectively. The similarity in LDL’s apparent permeability can be explained by the similarity in water flux through the large-pore leaky junctions, whereas the difference in total water flux can be explained by reduced water transport through junctional breaks.

The predictions of our three-pore model, summarized in Table 4, also indicate that the large-pore leaky junctions are a dominant pathway for water, 70-kDa dextran, and LDL transport. The estimated LDL transport is consistent with a previous report. The estimated LDL transport is consistent with a previous

<table>
<thead>
<tr>
<th>TABLE 6. Effect of VEGF on BREC Permeability</th>
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<tbody>
<tr>
<td>n</td>
</tr>
<tr>
<td>---</td>
</tr>
<tr>
<td>Jp/A × 10^-6 (cm/s)</td>
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<tr>
<td></td>
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<tr>
<td></td>
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<tr>
<td>70-kDa dextran</td>
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<tr>
<td>Pp × 10^-6 (cm/s)</td>
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Jp/A and 70-kDa dextran apparent permeability, Pp, were determined for monolayers cultured for 6 days in either NG or HG (10 mM). Half of the monolayers were incubated with 100 ng/mL VEGF for 4 hours before the measurement of Jp/A and Pp. VEGF significantly increased Lp and Pe in both NG and HG monolayers.
dextran diffusion, whereas occludin and ZO-1 mRNA levels remained constant and claudin-1 levels increased. The ability of VEGF to induce BREC permeability and the lack of effect of 25 mM glucose on any permeability or apoptosis suggests that, at least within the duration of glucose challenge in these experiments, endothelial permeability is not altered by hyperglycemia directly but rather in response to local cytokines or growth factors. In vitro exposure to HG has been shown to rapidly increase VEGF expression in various cell types and tissues, which may account for the elevated VEGF in the retina of humans and animals with diabetes. VEGF mRNA levels in human vascular smooth muscle cells have been observed to increase after just 5 hours of incubation with HG, and VEGF peptide production increases after 24 hours. Finally, our group has recently found that hyperglycemia increases expression of VEGF in retinal Müller cells in vitro and in vivo through translational control.

Another study of BREC monolayers demonstrated that VEGF (100 ng/mL) increases hydraulic conductivity dependent on NO synthase. VEGF increases hydraulic conductivity as well as diffusive and apparent permeability of BREC monolayers to 70kDa dextran (Table 6) associated with increased eNOS phosphorylation, and this change in permeability was significantly attenuated with the nitric oxide synthase inhibitor, Nω-monomethyl-arginine. In addition to the requirement for NO production, it has been demonstrated that VEGF alters phosphorylation and ubiquitination of occludin necessary for the VEGF-induced increase in permeability, and alterations in ZO-1 may also affect paracellular permeability. These studies clearly demonstrate that VEGF increases the breaks in the junction increasing both water flux and large solutes that are carried through these pores by convective flux. Interestingly, small molecules such as TAMRA were shown to be unaffected by convective forces.

Taken together, these data demonstrate that short-term (6-day) hyperglycemia does not alter IRRB endothelial permeability and suggests that a direct effect of hyperglycemia on the endothelial cells transport barrier is not the cause of increased permeability in the early stages of DR. Hyperglycemia may indirectly affect vascular permeability through upregulation of VEGF and cytokines that alter the junctional complexes or increase large pore formation through endothelial cell division or cell death.

Acknowledgments

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References


