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

Sandra V. Lopez-Quintero,¹ Xin-Ying Ji,¹ David A. Antonetti,^{2,3} and John M. Tarbell¹

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 Ophthalmol Vis Sci.* 2011;52:1171–1180) DOI:10.1167/iov.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 retina¹ and subsequent plasma leakage to the interstitial space. Elevated

glucose is believed to contribute to various vascular dysfunctions^{2,3}—among them, loss of microvascular barrier integrity.^{4,5} However, an increase in VEGF that occurs even before morphologic abnormalities are observed has been described in patients with DR.^{6,7} 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.¹ 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.⁸

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.⁹ Thus, oncotic pressure and hydrostatic 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,^{10,11} 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.¹² 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,¹³ 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

From the ¹Department of Biomedical Engineering, The City College of The City University of New York, New York, New York; and the Departments of ²Cellular and Molecular Physiology and ³Ophthalmology, Penn State College of Medicine, Hershey, Pennsylvania.

Supported by National Institutes of Health Grants R01 HL56093 (JMT) and R01 EY012021 (DAA).

Submitted for publication May 28, 2010; revised September 13, 2010; accepted October 4, 2010.

Disclosure: S.V. Lopez-Quintero, None; X.-Y. Ji, None; D.A. Antonetti, None; J.M. Tarbell, None

Corresponding author: John M. Tarbell, Department of Biomedical Engineering, The City College of The City University of New York, Steinman Hall Room T403, 140th Street and Convent Avenue, New York, NY 10031; tarbell@ccny.cuny.edu.

FIGURE 1. Bubble displacement versus time. As water moved from the luminal to the abluminal side of the chamber, the displacement of a bubble inserted in the borosilicate glass tube was measured. The water flux at equilibrium was then calculated from the slope in that range, according to equation 1.

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), L-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 ($1\times$ without Ca^{2+} and Mg^{2+}) 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 carboxytetramethylrhodamine (TAMRA) and 70-kDa TRITC-dextran were obtained from Molecular Probes (Carlsbad, CA) and DiI-LDL from Biomedical Technologies (Stoughton, MA). The TUNEL assay kit was obtained from Roche (Indianapolis, IN), and the Western blot kit, Bradford assay, and PVDF membrane (Immun-Blot) were from Bio-Rad (Hercules, CA). The phospho-eNOS (Ser1177) and α -actin antibodies were obtained from Cell Signaling Technologies (Beverly, MA) and the VEGF from R&D Systems (Minneapolis, MN).

Cell Culture

Primary BRECs were harvested from bovine eyes, as described by Barber,¹⁴ and then the frozen vials were thawed as needed and grown on fibronectin-coated (2 $\mu\text{g}/\text{cm}^2$) T-75 flasks with MCDB-131 (US Biological) medium prepared as indicated by the vendor by addition of L-glutamine, L-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% CO_2 . For transport experiments, porous migration assay inserts were seeded at a density of 6.0×10^4 cells/ cm^2 . The BRECs were treated with 100 nM 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.^{15,16} 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

$$J_v = \frac{\Delta d}{\Delta t} \times \frac{F}{A} \quad (1)$$

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 H_2O 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 (FluorMeasure software; C&L Instruments). The fluorescence counts generated were converted to units of concentration by using a calibration curve. The

TABLE 1. Fluorescent Molecules Used to Characterize Solute Transport Properties of BREC Monolayers

Molecule	Stokes Diameter	Molecular Weight	Luminal Concentration
TAMRA	1.3 nm	460 Da	300 ng/mL
Dextran	11 nm	70 kDa	1 μM
DiI-LDL	22 nm	2,000 kDa	10 $\mu\text{g}/\text{mL}$

The Stokes diameter, molecular weight, and concentration used at the luminal side of the insert are listed.

slope of the concentration versus time curve ($\Delta C/\Delta t$) was used to calculate the permeability to the fluorescent molecule as

$$P_c/P_o = \frac{(\Delta C/\Delta t) \times V_a}{C_l \times A} \quad (2)$$

where P_c/P_o is either the apparent permeability, P_c , which is calculated from the slope in the time interval where there is a convective component, or P_o , which is calculated using the slope during the time interval when only diffusion is taking place (Fig. 2). V_a is the volume of the abluminal compartment, C_l 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-131 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 BRECs monolayers has been used before to describe transport across BAEC (bovine aortic endothelial cell) monolayers.¹⁶ For a single pathway and solute, the apparent permeability P_c results from diffusive and convective contributions, as described by¹⁷

$$P_c = P_o Z + J_v(1 - \sigma). \quad (3)$$

The Z term is given by

$$Z = \frac{N_{pe}}{e^{N_{pe}} - 1} \quad (4)$$

where N_{pe} is the Péclet number defined as

FIGURE 2. Concentration of tagged molecule in the abluminal compartment as a function of time. In this case, a typical DiLDL concentration versus time curve is shown. The apparatus was calibrated to convert units of fluorescence counts into units of concentration for each tagged molecule. During the first 60 minutes, the system was left to equilibrate, after which 10-cm H₂O hydrostatic pressure was applied for 2 hours, and P_c was calculated by using the slope of that interval and equation 2. The pressure then was removed, and the diffusive permeability P_o was calculated from the new slope of that interval.

$$N_{pe} = \frac{J_v(1 - \sigma)}{P_o}. \quad (5)$$

and J_v is the water flux across the pathway, P_o is the diffusive permeability, and σ 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/vesicle; pathway 2, breaks in the TJs (average size, 20 nm),¹⁸ and pathway 3, large-pore leaky junctions (average size, 600 nm).¹⁹ 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,²⁰ and vesicles are not believed to conduct significant water.¹⁶ Therefore, the total water flow (J_v) across the endothelium is the sum of the water flow across pathways 2 and 3

$$J_v = J_{v2} + J_{v3} \quad (6)$$

It seems reasonable to assume that, due to its size (2–4 nm),^{18,21} 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),¹⁶ and that σ_3 , the reflection coefficient for LDL in pathway 3, is equal to 0, since leaky-junction dimensions are much greater than LDL diameter,¹⁹ then equation 3 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.

$$P_{cLDL} = P_{oLDL} + P_{o3LDL}Z_{3LDL} + J_{v3} \quad (7)$$

$$P_{oLDL} = P_{o1LDL} + P_{o3LDL}. \quad (7a)$$

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_{v3}), 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.¹⁶

Similarly, if 70-kDa dextran is assumed to be transported through pathways 2 and 3 (2DEX or 3DEX) only, since previous experiments in BAECs²² and our own results in BRECs show that there is no vesicle contribution to the transport of this molecule, and if $\sigma_{3DEX} = 0$ (dextran is much smaller than the large-pore leaky junction) and σ_{2DEX} is different from 0, then equation 3 for 70-kDa dextran can be written as

$$P_{cDEX} = P_{o2DEX}Z_{2DEX} + J_{v2}(1 - \sigma_{2DEX}) + P_{o3DEX}Z_{3DEX} + J_{v3} \quad (8)$$

$$P_{oDEX} = P_{o2DEX} + P_{o3DEX}. \quad (8a)$$

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_{o3LDL} subsequently, P_{o1LDL} 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_{o2DEX} and P_{o3DEX} . The solution to these equations will exist only for a certain range of σ_{2DEX} between 0 and 1.

Junction Protein Immunostaining

To visualize the TJ protein ZO-1 and the adherens junction protein VE-cadherin, we fixed BRECs on 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 $\mu\text{g/mL}$) 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 $\mu\text{g/mL}$). 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 were 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, Na_3VO_4 and PMSF. Protein concentration was determined with the Bradford protein assay kit (Bio-Rad). Western blot was performed by 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; BioRad) 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: ACCGGCGACTATGACAAGAA and reverse: AGGGTCATCATCTCTGCACC (with 227 bp of PCR product); and for the internal standard GAPDH were forward: AGGGTCATCATCTCTGCACC and reverse: CCATCCACAGTCTTCTGGGT (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 $\pm$ 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. J_v/A represents the water flow per unit area, P_o represents the permeability under diffusive conditions, and P_e 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} L_p values for NG and HG were 1.93 and 2.74×10^{-7} cm/s cm H_2O , respectively, representing an increase of 40% in HG. However the difference was not statistically significant.

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

Molecule	<i>n</i>	$J_v/A \times 10^{-6}$ (cm/s)	$P_o \times 10^{-6}$ (cm/s)	$P_e \times 10^{-6}$ (cm/s)	Mean P_e/P_o
Water	12	NG 1.93 ± 0.34 HG 2.74 ± 0.35 $P = 0.11$			
LDL	12		NG 0.20 ± 0.07 HG 0.23 ± 0.05 $P = 0.42$	NG 1.39 ± 0.33 HG 1.43 ± 0.63 $P = 0.78$	6.95 6.22
70-kDa dextran	8		NG 1.24 ± 0.21 HG 1.85 ± 0.25 $P = 0.23$	NG 2.43 ± 0.45 HG 2.81 ± 0.49 $P = 0.59$	1.70 1.52
TAMRA	6		NG 9.64 ± 0.33 HG 9.02 ± 0.67 $P = 0.66$	NG 11.4 ± 0.35 HG 10.5 ± 0.76 $P = 0.25$	1.18 1.16

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 (J_v/A) as well as solute permeability.

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

Molecule	<i>n</i>	$P_o \times 10^{-6}$ (cm/s)	$P_{o, \text{fixed}} \times 10^{-6}$ (cm/s)	<i>P</i> for Significance	Mean $P_o/P_{o, \text{fixed}}$
LDL	6	0.29 ± 0.03	0.09 ± 0.03	0.002	3.00
70-kDa dextran	6	1.24 ± 0.23	1.52 ± 0.32	0.620	0.81

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

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 monolayers¹⁶ and in intact hamster venules.²⁴ The pronounced effect of solvent drag can be seen in the mean ratio of the apparent (P_o) to the diffusive (P_o) 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,²² 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.²⁵ 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 (P_o) and fixed ($P_{o, \text{fixed}}$) 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 BRECs present 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,²⁶ 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,²⁷ because it has been observed that the aquaporin level is low or nonexistent in retinal endothelium,^{28,29} 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).¹⁸ 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 $\sigma_{2\text{DEX}}$ 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.^{16,22}

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. Renkin³⁰ 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 4. Molecules and Pathways Analyzed with a Three-Pore Model

Component	Pathway 1: Vesicles	Pathway 2: Breaks in the TJ	Pathway 3: Leaky Junctions	Total
Water		34.75	65.25	100
LDL	9.42		90.58	100
Dextran		19.93	80.07	100

The percentages shown correspond to $\sigma_{2\text{DEX}} = 0.55$. 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.

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

σ_{2DEX}	% Pore 2	φ	$(1 - \varepsilon)$	V_f
0.40	32.00	0.37	0.0098	0.0061
0.50	22.20	0.29	0.0120	0.0068
0.55	19.93	0.26	0.0132	0.0072

The partition coefficient φ is calculated for a random or an ordered fiber arrangement. $(1 - \varepsilon)$ represents the fractional fiber volume when the random arrangement is used, and V_f 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

It is generally believed that the reflection coefficient for the breaks in the TJ, σ_2 , is determined by the fiber matrix (glycocalyx) that fills the apical aspect of this pore. With the σ_{2DEX} values obtained, between 0.4 and 0.55, and the following equations, it is possible to estimate the glycocalyx fractional fiber volume V_f by modeling the glycocalyx as a fiber matrix,⁹ where the reflection coefficient σ is given by

$$\sigma = (1 - \varphi)^2. \quad (9)$$

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

$$\varphi = \exp[-(1 - \varepsilon)(2a/r_f + a^2/r_f^2)] \quad (10)$$

where $(1 - \varepsilon)$ 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

$$\varphi = 1 - V_f(1 + a/r_f)^2 \quad (11)$$

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 $(1 - \varepsilon)$ 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: $\Delta = [(\pi/V_f)^{1/2} - 2] \times r_f$ 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 ($0.45 < \sigma < 0.55$), using a pore analysis, we estimate the radius of the cylindrical pore with the relationship³¹

$$\varphi = (1 - \gamma)^2 \quad (12)$$

and equation 9, where γ is the ratio of the pore to the solute radius ($\gamma = p/a$); 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 $13\% \pm 0.45\%$ decrease in protein

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.

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 $13\% \pm 0.45\%$ compared to NG ($n = 3$), whereas the gene expression increased by $11\% \pm 0.34\%$ (both changes not significant; $n = 3$).

expression and an $11\% \pm 0.34\%$ 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 $2.1\% \pm 0.56\%$ and $2.9\% \pm 0.46\%$ 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 inhibitor³⁴; therefore, BREC monolayers were incubated with 100 ng/mL VEGF to determine the relative amount of phosphorylated e-NOS compared with control monolayers. We observed that the expression of phosphorylated e-NOS 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 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 $2.1\% \pm 0.56\%$ and $2.9\% \pm 0.46\%$ for normal and HG, respectively; however, they were not significantly different ($P = 0.62$, $n = 6$).

FIGURE 6. Western blot for phospho-eNOS. After the BREC monolayers were incubated with VEGF (100 ng/mL) for 2 and 4 hours, Western blot analysis was performed to observe changes in the phosphorylation of eNOS. After 4 hours of incubation with VEGF, eNOS phosphorylation increased significantly ($P < 0.005$). The dark lower band corresponds to β -actin, which was used as the control.

$1.31\text{E}-6 \pm 0.36$ cm/s. However, water flux was measured to be $5.39\text{E}-6 \pm 0.7$ 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 iBRB. Indeed, the mean water flux through the large-pore leaky junction for BREC monolayers was $1.26\text{E}-6$ cm/s, which is very similar to the value of $1.20\text{E}-6$ in BAEC monolayers¹⁶; however, water flux through the break in the TJs is much lower in BREC than in BAECs: $6.17\text{E}-7$ compared with $4.19\text{E}-6$, 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 in vitro model for BAEC monolayers¹⁶ and observations that regions with increased uptake of albumin predicted regions of enhanced LDL uptake.³⁵ The low percentage of LDL transported in vesicles is also consistent with observations by Wiklund et al.,³⁶ who used methylated LDL to block its recognition by the LDL receptor in rabbit aortas.

The effect of HG on solute and water permeability for the three solutes used showed that overall, 6 days of culture in HG did not significantly alter the diffusive permeability, P_o , or the apparent permeability, P_e , of TAMRA, 70-kDa dextran, or LDL. The water flux across the monolayers did not significantly change with HG either, although a 40% increase was observed (see Table 2). Further, on application of hydrostatic pressure, a significant increase in P_e was observed for 70-kDa dextran and, in particular, LDL, indicating that convective flux contributed to the overall transport of the larger solutes, although no effect of HG was observed. Monolayers cultured in HG for 6 days did not significantly change the distribution or intensity of TJ protein ZO-1 or the AJ protein VE-cadherin and did not demonstrate a change in expression of claudin 5. These observations are consistent with previous high-power electron mi-

crographs of BREC monolayers grown for 30 days in HG, which revealed no significant cell damage or change in the intercellular junctions.³⁷ Further, Shivers³⁸ demonstrated that the edge and groove of the TJ revealed no alteration in freeze-fracture images of brain capillary endothelium of hyperglycemic lizards and Vorbrodt et al.³⁹ were also unable to detect any changes in the ZO-1 content of brain microvessels in diabetic rats when using a quantitative immunogold procedure. While decreased expression and relocation of ZO-1 has been observed in rat glomerular epithelial cells exposed to HG,⁴⁰ no changes have been observed in BREC monolayers.⁴¹ Taken together, these data suggest that in BREC, the permeability and junctional complex do not change in response to 25 mM glucose treatment for 6 days.

Several studies have observed increased apoptosis or altered permeability of endothelium with HG. For instance, 7 to 9 days of HG treatment decreased monolayer electrical resistance by 20% and increased diffusive transport of C-inulin 5% (Stokes diameter, 3 nm) across BREC monolayers.¹⁰ However, the same cells cultured onto microcarriers did not show any increase in sodium fluorescein (Stokes diameter, 0.9 nm) and cyanocobalamin (Stokes diameter, 1.3 nm) permeability during the first 21 days of incubation in 25 mM glucose.¹¹ Differences in cell type and duration or degree of hyperglycemia may account for the observed differences of the effect of hyperglycemia on permeability.

Alternatively, glucose may not have a direct effect on endothelial permeability. Busik et al.,¹² demonstrated that human retinal endothelial cell survival or inflammatory activation were not affected by 4 days of HG, but in contrast, exposure of these cells to the proinflammatory cytokines IL-1 or TNF- α increased mitochondrial superoxide production, ERK and JNK phosphorylation, tyrosine phosphorylation, NF- κ B activation, and caspase activation, suggesting that hyperglycemia may have little direct effect on vascular endothelium but rather may indirectly induce the release of cytokines and growth factors. Moreover, human retinal pigment epithelial cells exposed to 25 mM glucose for 21 days showed significantly higher trans-epithelial electrical resistance (TER) and lower 40- or 70-kDa

TABLE 6. Effect of VEGF on BREC Permeability

	<i>n</i>	Without VEGF	With VEGF (100 ng/mL)	<i>P</i>
$J_v/A \times 10^{-6}$ (cm/s)	4	NG 2.42 ± 0.69	NG 4.27 ± 0.57	$P = 0.0062$
		HG 3.56 ± 0.56	HG 5.23 ± 0.60	$P = 0.0067$
		$P = 0.25$	$P = 0.37$	
70-kDa dextran $P_e \times 10^{-6}$ (cm/s)	4	NG 2.04 ± 0.46	NG 3.03 ± 0.52	$P = 0.017$
		HG 2.31 ± 0.22	HG 4.15 ± 0.28	$P = 0.007$
		$P = 0.64$	$P = 0.10$	

J_v/A and 70-kDa dextran apparent permeability, P_e , 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 J_v/A and P_e . VEGF significantly increased L_p and P_e in both NG and HG monolayers.

dextran diffusion, whereas occludin and ZO-1 mRNA levels remained constant and claudin-1 levels increased.⁴² 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,⁴³ which may account for the elevated VEGF in the retina of humans and animals with diabetes.^{44,45} VEGF mRNA levels in human vascular smooth muscle cells have been observed to increase after just 3 hours of incubation with HG, and VEGF peptide production increases after 24 hours.⁴⁶ Finally, our group has recently found that hyperglycemia increases expression of VEGF in retinal Müller cells in vitro and in vivo through translational control.⁴⁷

Another study of BREC monolayers demonstrated that VEGF (100 ng/mL) increases hydraulic conductivity dependent on NO synthase.³⁴ VEGF increases hydraulic conductivity as well as diffusive and apparent permeability of BREC monolayers to 70-kDa dextran (Table 6) associated with increased eNOS phosphorylation, and this change in permeability was significantly attenuated with the nitric oxide synthase inhibitor, *N*^G-monomethyl-L-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,^{47,48} and alterations in ZO-1⁴⁹ 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 iBRB 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

The authors thank Ellen B. Wolpert for lending excellent technical expertise in producing the primary BRECs.

References

- Engler CB, Sander B, Larsen M, Koefoed P, Parving HH, Lund-Andersen H. Probenecid inhibition of the outward transport of fluorescein across the human blood-retina barrier. *Acta Ophthalmol (Copenh)*. 1994;72:663–667.
- Fujita H. Vascular endothelial cell dysfunction in diabetes mellitus (in Japanese). *Nippon Rinsho*. 1999;57:573–577.
- Brausewetter F, Jehle PM, Jung MF, et al. Microvascular permeability is increased in both types of diabetes and correlates differentially with serum levels of insulin-like growth factor I (IGF-I) and vascular endothelial growth factor (VEGF). *Horm Metab Res*. 2001;33:713–720.
- Frank RN. Diabetic retinopathy. *N Engl J Med*. 2004;350:48–58.
- Frank RN. On the pathogenesis of diabetic retinopathy. *Ophthalmology*. 1984;91:626–634.
- Mondal LK, Baidya KP, Bhaduri G, Bandyopadhyay R, Bhattacharya B. Alteration of timing of secretion of vascular endothelial growth factors is responsible for progression of diabetic retinopathy. *J Indian Med Assoc*. 2008;106:508, 510, 515.
- Qaum T, Xu Q, Jousen AM, et al. VEGF-initiated blood-retinal barrier breakdown in early diabetes. *Invest Ophthalmol Vis Sci*. 2001;42:2408–2413.
- Gardner TW, Antonetti DA, Barber AJ, Lieth E, Tarbell JA. The molecular structure and function of the inner blood-retinal barrier. Penn State Retina Research Group. *Doc Ophthalmol*. 1999;97:229–237.
- Curry FE, Michel CC. A fiber matrix model of capillary permeability. *Microvasc Res*. 1980;20:96–99.
- Gillies MC, Su T, Naidoo D. Electrical resistance and macromolecular permeability of retinal capillary endothelial cells in vitro. *Curr Eye Res*. 1995;14:435–442.
- Haselton FR, Dworska EJ, Hoffman LH. Glucose-induced increase in paracellular permeability and disruption of beta-receptor signaling in retinal endothelium. *Invest Ophthalmol Vis Sci*. 1998;39:1676–1684.
- Busik JV, Mohr S, Grant MB. Hyperglycemia-induced reactive oxygen species toxicity to endothelial cells is dependent on paracrine mediators. *Diabetes*. 2008;57:1952–1965.
- Antonetti DA, Barber AJ, Khin S, Lieth E, Tarbell JM, Gardner TW. Vascular permeability in experimental diabetes is associated with reduced endothelial occludin content: vascular endothelial growth factor decreases occludin in retinal endothelial cells. Penn State Retina Research Group. *Diabetes*. 1998;47:1953–1959.
- Barber AJ, Antonetti DA. Mapping the blood vessels with paracellular permeability in the retinas of diabetic rats. *Invest Ophthalmol Vis Sci*. 2003;44:5410–5416.
- Sill HW, Chang YS, Artman JR, Frangos JA, Hollis TM, Tarbell JM. Shear stress increases hydraulic conductivity of cultured endothelial monolayers. *Am J Physiol*. 1995;268:H535–H543.
- Cancel LM, Fitting A, Tarbell JM. In vitro study of LDL transport under pressurized (convective) conditions. *Am J Physiol Heart Circ Physiol*. 2007;293:H126–H132.
- Curry FE. Mechanics and thermodynamics of transcapillary exchange. Microcirculation, part 1. *The Cardiovascular System*. Vol. 4. 1984:309–374. *Handbook of Physiology*; section 2.
- Adamson RH, Michel CC. Pathways through the intercellular clefts of frog mesenteric capillaries. *J Physiol*. 1993;466:303–327.
- Chen YL, Jan KM, Lin HS, Chien S. Ultrastructural studies on macromolecular permeability in relation to endothelial cell turnover. *Atherosclerosis*. 1995;118:89–104.
- Fu BM, Weinbaum S, Tsay RY, Curry FE. A junction-orifice-fiber entrance layer model for capillary permeability: application to frog mesenteric capillaries. *J Biomech Eng*. 1994;116:502–513.
- Ward BJ, Bauman KF, Firth JA. Interendothelial junctions of cardiac capillaries in rats: their structure and permeability properties. *Cell Tissue Res*. 1988;252:57–66.
- DeMaio L, Tarbell JM, Scaduto RC Jr, Gardner TW, Antonetti DA. A transmural pressure gradient induces mechanical and biological adaptive responses in endothelial cells. *Am J Physiol Heart Circ Physiol*. 2004;286:H731–H741.
- Kim MH, Harris NR, Tarbell JM. Regulation of capillary hydraulic conductivity in response to an acute change in shear. *Am J Physiol Heart Circ Physiol*. 2005;289:H2126–H2135.
- Rutledge JC, Curry FE, Blanche P, Krauss RM. Solvent drag of LDL across mammalian endothelial barriers with increased permeability. *Am J Physiol*. 1995;268:H1982–H1991.
- Rippe B, Kamiya A, Folkow B. Transcapillary passage of albumin, effects of tissue cooling and of increases in filtration and plasma colloid osmotic pressure. *Acta Physiol Scand*. 1979;105:171–187.
- Rippe B, Haraldsson B. Transport of macromolecules across microvascular walls: the two-pore theory. *Physiol Rev*. 1994;74:163–219.
- Rippe B, Stelin G. Simulations of peritoneal solute transport during CAPD: application of two-pore formalism. *Kidney Int*. 1989;35:1234–1244.
- Hamann S, Zeuthen T, La Cour M, et al. Aquaporins in complex tissues: distribution of aquaporins 1–5 in human and rat eye. *Am J Physiol*. 1998;274:C1332–C1345.
- Motulsky E, Koch P, Janssens S, et al. Aquaporin expression in blood-retinal barrier cells during experimental autoimmune uveitis. *Mol Vis*. 2010;16:602–610.

30. Renkin EM. Capillary transport of macromolecules: pores and other endothelial pathways. *J Appl Physiol*. 1985;58:315-325.
31. Michel CC, Curry FE. Microvascular permeability. *Physiol Rev*. 1999;79:703-761.
32. Weinbaum S, Tsay R, Curry FE. A three-dimensional junction-pore-matrix model for capillary permeability. *Microvasc Res*. 1992;44:85-111.
33. Nitta T, Hata M, Gotoh S, et al. Size-selective loosening of the blood-brain barrier in claudin-5-deficient mice. *J Cell Biol*. 2003;161:653-660.
34. Lakshminarayanan S, Antonetti DA, Gardner TW, Tarbell JM. Effect of VEGF on retinal microvascular endothelial hydraulic conductivity: the role of NO. *Invest Ophthalmol Vis Sci*. 2000;41:4256-4261.
35. Schwartz CJ, Valente AJ, Sprague EA, Kelley JL, Nerem RM. The pathogenesis of atherosclerosis: an overview. *Clin Cardiol*. 1991;14:11-16.
36. Wiklund O, Carew TE, Steinberg D. Role of the low density lipoprotein receptor in penetration of low density lipoprotein into rabbit aortic wall. *Arteriosclerosis*. 1985;5:135-141.
37. Leto G, Pricci F, Amadio L, et al. Increased retinal endothelial cell monolayer permeability induced by the diabetic milieu: role of advanced non-enzymatic glycation and polyol pathway activation. *Diabetes Metab Res Rev*. 2001;17:448-458.
38. Shivers RR. The effect of hyperglycemia on brain capillary permeability in the lizard, *Anolis carolinensis*: a freeze-fracture analysis of blood-brain barrier pathology. *Brain Res*. 1979;170:509-522.
39. Vorbrodt AW, Dobrogowska DH, Tarnawski M, Meeker HC, Carp RI. Immunogold study of altered expression of some interendothelial junctional molecules in the brain blood microvessels of diabetic scrapie-infected mice. *J Mol Histol*. 2006;37:27-35.
40. Rincon-Choles H, Vasylyeva TL, Pergola PE, et al. ZO-1 expression and phosphorylation in diabetic nephropathy. *Diabetes*. 2006;55:894-900.
41. Grammas P, Riden M. Retinal endothelial cells are more susceptible to oxidative stress and increased permeability than brain-derived endothelial cells. *Microvasc Res*. 2003;65:18-23.
42. Villarreal M, Garcia-Ramirez M, Corraliza L, Hernandez C, Simo R. High glucose concentration leads to differential expression of tight junction proteins in human retinal pigment epithelial cells. *Endocrinol Nutr*. 2009;56:53-58.
43. Natarajan R, Bai W, Lanting L, Gonzales N, Nadler J. Effects of high glucose on vascular endothelial growth factor expression in vascular smooth muscle cells. *Am J Physiol*. 1997;273:H2224-H2231.
44. Mathews MK, Merges C, McLeod DS, Luty GA. Vascular endothelial growth factor and vascular permeability changes in human diabetic retinopathy. *Invest Ophthalmol Vis Sci*. 1997;38:2729-2741.
45. Gilbert RE, Vranes D, Berka JL, et al. Vascular endothelial growth factor and its receptors in control and diabetic rat eyes. *Lab Invest*. 1998;78:1017-1027.
46. Williams B, Gallacher B, Patel H, Orme C. Glucose-induced protein kinase C activation regulates vascular permeability factor mRNA expression and peptide production by human vascular smooth muscle cells in vitro. *Diabetes*. 1997;46:1497-1503.
47. Schrufer TL, Antonetti DA, Sonenberg N, Kimball SR, Gardner TW, Jefferson LS. Ablation of 4E-BP1/2 prevents hyperglycemia-mediated induction of VEGF expression in the rodent retina and in Muller cells in culture. *Diabetes*. 2010;59:2107-2116.
48. Harhaj NS, Felinski EA, Wolpert EB, Sundstrom JM, Gardner TW, Antonetti DA. VEGF activation of protein kinase C stimulates occludin phosphorylation and contributes to endothelial permeability. *Invest Ophthalmol Vis Sci*. 2006;47:5106-5115.
49. Murakami T, Felinski EA, Antonetti DA. Occludin phosphorylation and ubiquitination regulate tight junction trafficking and vascular endothelial growth factor-induced permeability. *J Biol Chem*. 2009;284:21036-21046.
50. Antonetti DA, Barber AJ, Hollinger LA, Wolpert EB, Gardner TW. Vascular endothelial growth factor induces rapid phosphorylation of TJ proteins occludin and zonula occluden, 1: a potential mechanism for vascular permeability in diabetic retinopathy and tumors. *J Biol Chem*. 1999;274:23463-23467.