TNFα-Induced Disruption of the Blood–Retinal Barrier In Vitro Is Regulated by Intracellular 3′,5′-Cyclic Adenosine Monophosphate Levels

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PURPOSE. Proinflammatory cytokines such as tumor necrosis factor (TNFα) may have a causative role in blood–retinal barrier (BRB) disruption, which is an essential step in the development of diabetic macular edema. The purpose of our study was to determine whether TNFα increases permeability in an in vitro model of the BRB and to explore the mechanisms involved.

METHODS. Primary bovine retinal endothelial cells (BRECs) were grown on Transwell inserts and cells were stimulated with TNFα or a combination of TNFα, IL1β, and VEGF. Molecular integrity of the BRB was determined by gene and protein expression of BRB-specific components, and barrier function was assessed using permeability assays.

RESULTS. TNFα reduced the expression of tight and adherens junctions in BRECs. Permeability for a 376 Da molecular tracer was increased after TNFα stimulation, but not for larger tracers. We found that 3′,5′-cyclic adenosine monophosphate (cAMP) stabilized the barrier properties of BRECs, and that TNFα significantly decreased intracellular cAMP levels. When BRECs were preincubated with a membrane-permeable cAMP analog, the effects of TNFα on claudin-5 expression and permeability were mitigated. The effects of TNFα on barrier function in BRECs were largely independent of the small Rho guanosine triphosphate (GTP)ases RhoA and Rac1, which is in contrast to TNFα effects on the nonbarrier endothelium. The combination of TNFα, IL1β, and VEGF increased permeability for a 70 kDa-FITC tracer, also mediated by cAMP.

CONCLUSIONS. TNFα alone, or in combination with IL1β and VEGF, induces permeability of the BRB in vitro for differently sized molecular tracers mediated by cAMP, but independently of Rho/Rac signaling.

Keywords: blood-retinal barrier, cyclic AMP, vascular permeability, tumor necrosis factor alpha, tight junction

More than one third of diabetic persons have some form of diabetic retinopathy (DR), and approximately 5% to 10% develop vision-threatening complications such as proliferative DR and macular edema1; the latter is the leading cause of vision loss in DR.2 Although disruption of the blood–retinal barrier (BRB) is an essential step in the development of diabetic macular edema, its mechanisms are poorly understood. This lack of understanding precludes the development of novel effective treatment strategies.

Focal and diffuse BRB disruption is caused by retinal ischemia as a result of capillary nonperfusion3 and eventually leads to vascular leakage and the development of diabetic macular edema. Although it is widely accepted that vascular endothelial growth factor (VEGF) is one of the main drivers of BRB disruption, a subset of patients does not benefit from therapeutics targeting VEGF.4 Furthermore, these invasive treatments are burdensome for the patient, requiring monthly to bimonthly intravitreal injections5 for several years.

In addition to VEGF, it has been suggested that proinflammatory cytokines such as tumor necrosis factor (TNFα) have a causative role in BRB disruption.6–8 Indeed, there are studies that show elevated TNFα levels in the vitreous of patients with active DR,9–12 but also studies that do not.13 Furthermore, (small) clinical trials using anti-TNFα antibodies or soluble TNFα receptors in patients with diabetic macular edema or other ocular diseases have had limited success to date.6,14–17 Therefore, whether and how proinflammatory cytokines contribute to the development of BRB disruption and subsequent macular edema is still controversial.

In nonbarrier type endothelium of peripheral origin, TNFα is known to induce vascular leakage via the second messenger molecule 3′,5′-cyclic adenosine monophosphate (cAMP) and the Rho family of small GTPases, including Rac1 and RhoA. Upon receptor binding, TNFα can lower intracellular cAMP levels and inactivate Rac18,19 or activate RhoA20–22. This leads to the disruption of adherens junctions and stress fiber formation and eventually increased permeability. Although these actions of TNFα have been shown to greatly affect peripheral (micro)vascular permeability, little is known about...
these mechanisms in the context of barrier-forming endothelia such as that of the BRB. The purpose of the present study was to address the following questions: In an in vitro model of the BRB, (a) does TNFα induce endothelial permeability and, if so, (b) is TNFα-induced endothelial barrier permeability mediated by cAMP and/or small Rho GTPases?

MATERIALS AND METHODS

Cell Cultures

Bovine retinal endothelial cells (BRECs) were isolated from freshly enucleated cow eyes obtained from the slaughterhouse and cultured as described previously.25 First passage BRECs were used in all experiments, and the cells were kept in complete Dulbecco’s modified Eagle’s medium containing 5 mM HEPES and 4.5 g/l glucose (Lonza, Breda, The Netherlands), supplemented with 10% fetal calf serum, 1× MEM nonessential amino acids (Thermo Fisher Scientific, Landsmeer, The Netherlands), Fungizone Antimycotic (Gibco, Landsmeer, The Netherlands), 1% penicillin-streptomycin-glutamine (PenStrep; Invitrogen, Landsmeer, The Netherlands), 2 mM L-glutamine (Thermo Fisher Scientific), and 10 μg/ml hydrocortisone (Sigma-Aldrich, Zwijndrecht, The Netherlands) in 10% CO2 at 37°C. Human umbilical vein endothelial cells (HUVECs; passage 3) were isolated as described previously.24 HUVECs were cultured in 2% gelatin-coated plates and kept in M199 basal medium (Gibco) supplemented with 1% PenStrep, 10% fetal calf serum, and 10% human serum at 5% CO2 at 37°C. The experiments were repeated with HUVECs of at least three donors.

In Vitro BRB Model

An in vitro model of the BRB was developed by us and was used in this study with minor adaptations. When cells were confluent, they were transferred to 24-well Transwell inserts (0.33 cm2, pore size 0.4 μm; Greiner Bio-One, Alphen a/d Rijn, The Netherlands) coated with collagen type IV (Sigma-Aldrich). The next day, fresh medium was given to the cells and in some experiments supplemented with 312.5 μM 8-(4-Chlorophenylthiophen-CPT)-cAMP (Sigma-Aldrich) and 17.5 μM RO-20-1724 (a specific phosphodiesterase [PDE] inhibitor, to prevent cAMP breakdown); Calbiochem, Darmstadt, Germany). Based on a literature search, off-target effects of RO-20-1724 on endothelial cells are unlikely. After 3 or 4 days (depending on the duration of stimulation), rhTNFα (10 ng/ml; ProSpec, Ness Ziona, Israel) or a combination of rhTNFα (10 ng/ml), rhIL1β (10 ng/ml; ProSpec), and rhVEGF-FITC (25 ng/ml; R&D Systems, Abingdon, UK) was added in 20 μl to the apical side of the insert.

Permeability Analysis

Permeability assays were performed at 2, 6, and 24 hours after TNFα or triple cytokine (TNFα, IL1β, and VEGF) stimulation. Fluorescent tracers of different sizes (376 Da fluorescein sodium salt, 250 μg/ml [Invitrogen]; 766 Da Cy3, 50 μg/ml [GE Healthcare, Eindhoven, The Netherlands]), 7 kDa FITC-dextran, 250 μg/ml [Invitrogen], or BSA-FITC, 250 μg/ml [Invitrogen]) were added to the apical side of the Transwell insert, and samples were collected from the upper and lower compartments after 4 hours. Concentrations of the tracer molecules were measured with a fluorescence plate reader (BMG POLARstar; MX Lab Systems, Bradenton, FL, USA), and tracer passage to the lower compartment was calculated on the basis of the initial concentration in the upper compartment. The permeability of stimulated cells was calculated relative to that of unstimulated cells.

Immunofluorescence Staining

BRECs were grown to confluence on collagen type IV- and fibronectin-coated plastic coverslips (Nunc, Thermo Fisher Scientific). Preincubation with 8-(4-CPT)-cAMP and RO-20-1724 was performed during the night before stimulation with TNFα by changing the medium to medium supplemented with 312.5 μM 8-(4-CPT)-cAMP and 17.5 μM RO-20-1724. The cells were fixed with ice-cold 75% ethanol for 15 minutes at −20°C (anti-claudin-5 antibody), or with 4% paraformaldehyde for 15 minutes at 4°C (other antibodies) and stained as described previously.25 The following primary antibodies were used: rabbit anti-claudin-5 (cat. #4-1600, 1:100; Invitrogen), rabbit-anti-ZO1 (cat. 61-7300; 1:250, Invitrogen), rabbit-anti VE-cadherin (cat. #ab35168; 1:400, Abcam, Cambridge, UK) or a phallolidin probe conjugated with Texas Red (cat. #T7471; 1:200, Thermo Fisher Scientific). The secondary antibodies directed against the relevant species were conjugated with Alexa Fluor-488 (1:100; Thermo Fisher Scientific) or Cy3 (1:100; Jackson ImmunoResearch, Suffolk, UK). Cells were mounted on glass slides with Vectashield containing 4’,6-diamidino-2-phenylindole (Vector Laboratories, Burlingame, CA, USA) for nuclear staining and embedding. Images were recorded using a confocal laser scanning microscope SP8 (Leica Microsystems, Wetzlar, Germany) at the van Leeuwenhoek Centre for Advanced Microscopy, AMC. Specificity of the staining reaction was checked by the absence of fluorescence signal in samples when the primary antibody was omitted.

RNA Isolation and mRNA Quantification

BRECs were grown to confluence in a 12-well plate and harvested in 500-μl TRIzol reagent (Invitrogen). Total RNA was isolated according to the manufacturer’s protocol and dissolved in RNAse-free water. RNA yield was measured spectrophotometrically using NanoDrop (Thermo Scientific, Wilmington, DE, USA), and 1 μg RNA was treated with DNase-I (Invitrogen) and reverse transcribed into first-strand cDNA with a Maxima First Strand cDNA Synthesis Kit (ThermoFisher). Real-time quantitative PCR was performed on 20x diluted cDNA samples using a CFX96 system (Bio-Rad, Hercules, CA, USA) as described previously.27 Primer details are published in Klaassen et al.27 Data were normalized to the geometric mean of the most stable reference genes as determined with NormFinder.26

Measurement of Intracellular cAMP Levels

Intracellular levels of cAMP in BRECs were measured using the acetylated format of the Direct cyclic AMP ELISA kit (Enzo Life Sciences, Raamsdonkveer, The Netherlands), according to the manufacturer’s protocol. Absorbance was measured at 405 nm using a microplate spectrophotometer (VERSmax, Molecular Devices, Sunnyvale, CA, USA), and data were analyzed with SoftMax Pro software (v5.4.1, Molecular Devices). Protein concentration of the cell lysate was measured with Precision Red Advanced Protein Assay (Cytoskeleton, Inc., Denver, CO, USA). Concentrations of intracellular cAMP (pmol/ml) of unstimulated cells were set to one and compared to cAMP levels of treated cells.
RhoA and Rac1 Activation Assays

Activated RhoA and Rac1 in BREC lysates was measured with the absorbance-based RhoA or Rac1 Activation Assay G-LISA (Cytoskeleton, Inc.), according to the manufacturer’s protocol. Absorbance was measured at 490 nm using a microplate spectrophotometer (VERSAmax, Molecular Devices).

Statistics

Data are depicted as mean ± standard deviation. Experimental conditions were carried out in triplicate, and at least three independent experiments were performed. The differences between groups were determined using an unpaired Student’s t-test or analysis of variance followed by Dunnett’s test for multiple comparisons. Differences were considered statistically significant when P ≤ 0.05. Statistical analyses and graphing were performed using GraphPad Prism 6 (GraphPad Software, Inc., La Jolla, CA, USA) software.

RESULTS

TNFα Reduces mRNA and Protein Levels of Tight and Adherens Junctions

Stimulation of BREC with TNFα (10 ng/ml) for 24 hours significantly reduced mRNA levels of the tight junction components claudin-5 and ZO-1, and the adherens junction components VE-cadherin and β-catenin. On the protein level, expression of claudin-5 appeared to be similar to that in control cells after 24 hours of stimulation with TNFα except that claudin-5 was expressed in a more ruffled manner around cell membranes (Fig. 1B). Protein expression of ZO-1 and VE-cadherin was reduced after 24-hour TNFα stimulation compared to control cells (Fig. 1B). In contrast with what has been reported for peripheral endothelium (dermal, pulmonary, umbilical vein)18,20,29,30 and with what we observed in HUVECs (Supplementary Fig. S1), TNFα did not induce stress fiber formation in BREC as shown by immunofluorescence staining with a phalloidin probe to label filamentous actin (F-actin; red). (D) Quantification of F-actin content showed similar results in control cells and TNFα-stimulated cells. *P < 0.01, ***P < 0.001. Data are depicted as mean ± standard deviation. Red scale bar is 50 μm. White scale bar is 10 μm.

TNFα Increases Permeability Only for Small Molecular Tracers

To test the effect of TNFα-induced downregulation of endothelial junctions on barrier function, we performed permeability assays for molecular tracers of different sizes. TNFα increased the permeability of BREC monolayers for a small molecular tracer, sodium fluorescein (376 Da) after 6-hour and 24-hour stimulation (Fig. 2A) to 131% and 125%, respectively (P < 0.05). Permeability for two larger tracers (766 Da and 70 kDa) was not altered after TNFα treatment (Figs. 2B, 2C). These data suggest that TNFα has a selective effect on the paracellular transport pathway, likely as a consequence of disrupted tight junctions.

cAMP Has a Stabilizing Effect on Barrier Properties

Although cAMP signaling in barrier-forming endothelium in pathologic conditions has not been thoroughly investigated, the stabilizing effects of cAMP on barrier-endothelium properties in vitro are well known23,31. To confirm that cAMP stabilizes the endothelial barrier in our BRB model, we used 8-
(4-CPT)-cAMP (a membrane-permeable cAMP analog) and RO-20-1724 (a PDE IV inhibitor) to increase intracellular cAMP levels. We found a marked increase in barrier function, reflected by low permeability for both small (376 Da, 766 Da) and large (70 kDa) tracers with addition of the two compounds (Figs. 3A–C). A concentration-response experiment for RO-20-1724 revealed that 17.5 μM RO-20-1724 (a concentration regularly used in BRB and BBB cell models) resulted in the lowest permeability (data not shown). The addition of 17.5 μM RO-20-1724 alone signifi-

**Figure 2.** Effect of TNFα on permeability for differently-sized fluorescent tracers. (A) TNFα (10 ng/ml) significantly increased permeability for sodium fluorescein (376 Da) after 6 hours and 24 hours of stimulation. (B) Permeability for a 766 Da-Cy3 tracer and (C) 70 kDa-FITC tracer was not changed after TNFα stimulation. *P < 0.05. Data are depicted as mean ± standard deviation.

**Figure 3.** Effects of various concentrations of 8-(4-CPT)-cAMP (cAMP) and RO-20-1724 on BREC. Permeability for sodium fluorescein (A), 766 Da-Cy3 tracer (B), and 70 kDa-FITC tracer (C) decreased with increasing concentrations of cAMP and was lowest when 17.5 μM RO-20-1724 and 312.5 μM cAMP were added simultaneously. (D) Preincubation of BREC with 312.5 μM cAMP and 17.5 μM RO-20-1724 resulted in decreased transverse actin stress fibers and increased cortical F-actin bundles as shown by immunofluorescence using a phalloidin probe (red). (E) Quantification of F-actin content indicated a significantly decreased F-actin signal when compared with control cells. (F) Morphology of BREC changed after addition of cAMP and RO-20-1724 as shown with VE-cadherin staining (green). Control cells are elongated, whereas addition of cAMP and RO-20-1724 to the BREC resulted in a more cobblestone-like morphology. Nuclear staining is shown in blue. *P < 0.05, **P < 0.01, ***P < 0.001. Data are depicted as mean ± standard deviation. Green scale bar is 50 μm.
cantly decreased permeability to sodium fluorescein and the 766 Da-Cy3 tracer, but this effect was reduced when simultaneously low levels (3.1 μM or 31.2 μM) of 8-(4-CPT)-cAMP were added to the cells. For all 3 tracers, permeability was lowest and significantly decreased when compared with control when 312.5 μM 8-(4-CPT)-cAMP in combination with 17.5 μM RO-20-1724 was added. Based on these findings, subsequent experiments were performed using 312.5 μM 8-(4-CPT)-cAMP and 17.5 μM RO-20-1724.

In addition, cAMP induced reorganization of the actin cytoskeleton (Figs. 3D, 3E). After preincubation of the cells with 8-(4-CPT)-cAMP and RO-20-1724, the F-actin content was significantly reduced and transverse actin stress fibers were decreased in numbers, whereas the amount of cortical F-actin bundles was increased. These actin rearrangements resulted in an altered morphology of the BRECs (i.e., less elongated; Fig. 3F) and may underlie in part the mechanisms of strengthening of the endothelial barrier by cAMP.

TNFα Decreases Intracellular cAMP Levels

Similar to the peripheral endothelium,18,30,35 intracellular cAMP levels in BRECs were decreased after TNFα stimulation. Intracellular cAMP was significantly reduced up to at least 24 hours after the addition of TNFα (Fig. 4A), suggesting that TNFα-induced barrier disruption is mediated by reduced intracellular cAMP levels. Stimulating the cells with 8-(4-CPT)-cAMP and RO-20-1724 led to an approximately 10-fold increase in intracellular cAMP levels after 24 hours (Fig. 4B), and preincubation of the cells with 8-(4-CPT)-cAMP and RO-20-1724 prevented the TNFα-induced reduction in intracellular cAMP levels (Fig. 4C).

The Effects of TNFα on Barrier Function Are Largely Independent of the Small Rho GTPases RhoA and Rac1

To test the activation state of Rac1 and RhoA, we measured GTP-bound (i.e., activated) RhoA and Rac1 in BREC lysates after TNFα stimulation. Because small Rho GTPases are generally activated very rapidly and transiently upon stimulation, we measured activation at 5, 30, and 120 minutes after TNFα stimulation. We observed a small increase in both RhoA and Rac1 activation as compared to unstimulated cells after TNFα stimulation. The effects on RhoA activation were not statistically significant (Fig. 5A). Thirty minutes after TNFα addition, Rac1 activation showed a modest increase when compared with unstimulated cells (1.4-fold, \( P < 0.05 \), Fig. 5B). However, taking the small effects of TNFα on RhoA activation into consideration, the balance of Rac1/RhoA activation is not altered by TNFα. Therefore, the effects of TNFα on BRECs appear to be largely independent of RhoA and Rac1.

**FIGURE 4.** Intracellular cAMP levels after TNFα stimulation. (A) TNFα (10 ng/ml) significantly reduced intracellular cAMP levels when compared with control in BRECs up to at least 24 hours after stimulation. (B) Preincubation of BRECs for 24 hours with 312.5 μM 8-(4-CPT)-cAMP (cAMP) and 17.5 μM RO-20-1724 led to significantly increased intracellular cAMP levels. (C) TNFα had no effect on intracellular cAMP levels when cells were preincubated with cAMP and RO-20-1724. **\( P < 0.01 \). Data are depicted as mean ± standard deviation.

**FIGURE 5.** Effect of TNFα on activation of the small GTPases Rac1 and RhoA. (A) RhoA activation was slightly elevated after TNFα (10 ng/ml) stimulation (not significant), both in control cells in the presence of control medium (circles) and cells preincubated with 312.5 μM 8-(4-CPT)-cAMP (cAMP) and 17.5 μM RO-20-1724 (squares). (B) TNFα significantly increased Rac1 activation after 30 minutes when compared with unstimulated cells in the control medium (circles). TNFα-induced Rac1 activation did not occur when cells were preincubated with 312.5 μM cAMP and 17.5 μM RO-20-1724 (squares). *\( P < 0.05 \) versus unstimulated cells in the control medium. Data are depicted as mean ± standard deviation.
Preincubation With 8-(4-CPT)-cAMP and RO-20-1724 Prevents Claudin-5 Downregulation and TNFα-Induced Increase in Permeability for Sodium Fluorescein

Finally, we determined whether the TNFα-induced decrease in intracellular cAMP levels was responsible for the disruption of tight junctions and increased permeability. We found that preincubation of the cells with 8-(4-CPT)-cAMP and RO-20-1724 effectively prevented the downregulation of claudin-5 mRNA levels (Fig. 6). Whereas TNFα stimulation reduced claudin-5 mRNA levels to 69% after 24 hours ($P < 0.001$), this effect was not observed when cells were preincubated with 8-(4-CPT)-cAMP and RO-20-1724 (Fig. 6A). On the protein level, claudin-5 expression as shown by immunofluorescence staining (red) was continuous around the cell membrane in TNFα-stimulated cells that were preincubated with cAMP and RO-20-1724. Nuclear staining is shown in blue. TNFα did not induce permeability for sodium fluorescein (G), 766 Da-Cy3 (D), and 70 kDa-FITC (E) when cells were preincubated with cAMP and RO-20-1724. $*** P < 0.001$. Data are depicted as mean ± standard deviation. Red scale bar is 50 μm.

Preincubation With 8-(4-CPT)-cAMP and RO-20-1724 Prevents Claudin-5 Downregulation and TNFα-Induced Increase in Permeability for Sodium Fluorescein

Simultaneous Stimulation With TNFα, IL1β, and VEGF Increases Permeability for Large Molecular Tracers, and This Is Reduced by Preincubation With 8-(4-CPT)-cAMP and RO-20-1724

Because in (low grade) inflammatory states, such as DR, there is usually an interplay of multiple cytokines in the local microenvironment, we tested the effects of simultaneous stimulation with TNFα (10 ng/ml), IL1β (10 ng/ml), and VEGF (25 ng/ml) on permeability status of BRECs. Stimulating the cells with this triple cytokine combination of 6 hours caused no change in permeability for sodium fluorescein, both without and with preincubation with 8-(4-CPT)-cAMP and RO-20-1724 (Fig. 7A). A nonsignificant trend of increased permeability for a 766 Da tracer (165% of control) was observed, which was less when cells were preincubated with 8-(4-CPT)-cAMP and RO-20-1724 (118% of control, Fig. 7B). However, permeability for a large 70 kDa molecular tracer was significantly increased to 400% when compared with unstimulated cells after triple cytokine stimulation ($P < 0.05$). This effect was largely prevented by preincubation of the cells with 8-(4-CPT)-cAMP and RO-20-1724 (136% of control, Fig. 7C). Because these results are indicative of selective transport for large tracers, we also tested permeability for FITC-conjugated BSA (66 kDa) because albumin is known to be predominantly translocated via the transcellular route.36 Permeability was increased significantly after 6-hour stimulation with the triple cytokine combination (121% of control, $P < 0.05$), and this was not the case when cells were preincubated with 8-(4-CPT)-cAMP and RO-20-1724 (Fig. 7D). These data suggest that cAMP may be a key signaling molecule in cytokine-induced perme-
increased permeability for large tracers. Furthermore, our data suggest that both TNFα and VEGF-induced permeability in BRECs is mediated by cAMP and that barrier integrity can be rescued by increased intracellular cAMP levels. The increased permeability of our cells after TNFα stimulation alone was small and specific for the small molecular tracer of 376 Da. These results do not match the findings of a previous study where an almost 4-fold increase in permeability for sodium fluorescein (376 Da), both without and with preincubation of cells with cAMP and RO-20-1724. (B) Permeability for a 766 Da-Cy3 tracer was slightly but nonsignificantly increased after triple cytokine stimulation and to a lesser extent when cells were preincubated with cAMP and RO-20-1724. (C) Permeability for a 70 kDa-FITC tracer was significantly increased to 400% of control after triple cytokine stimulation and this increase was largely reduced by preincubating the cells with cAMP and RO-20-1724. (D) Permeability for BSA-FITC was significantly increased after triple cytokine stimulation, and this was not the case when cells were preincubated with cAMP and RO-20-1724. *P < 0.05. Data are depicted as mean ± standard deviation.

ability in BRECs and that modulation of intracellular cAMP levels may prevent cytokine-induced increases in permeability for large proteins, which is a prerequisite for the formation of macular edema.

**DISCUSSION**

Our in vitro study shows that TNFα reduces the expression of tight and adherens junctions in BRECs and thereby increases permeability for small molecules in our BRB model. In contrast, a combination of TNFα with IL1β and VEGF causes selectively increased permeability for large tracers. Furthermore, our data suggest that both TNFα and VEGF-induced permeability in BRECs is mediated by cAMP and that barrier integrity can be rescued by increased intracellular cAMP levels. The increased permeability of our cells after TNFα stimulation alone was small and specific for the small molecular tracer of 376 Da. These results do not match the findings of a previous study where an almost 4-fold increase in permeability was reported for a 70 kDa tracer by TNFα in BRECs. However, in agreement with this study, we found that mRNA expression of pivotal tight and adherens junction molecules was decreased after TNFα treatment, and this was confirmed on the protein level. In addition, we did not observe any changes in the cytoskeletal arrangements and hence no gaps between cells after TNFα stimulation. Therefore, it seems unlikely that large molecular tracers with the size of small proteins such as albumin can pass through the cellular monolayer. We cannot readily explain the contradictory results of tracer permeability of our study and the report of Aveleira et al. other than by differences in medium supplements and cell isolation method. On the basis of our results, however, we suggest that TNFα in the absence of IL1β and VEGF selectively affects the paracellular pathway in retinal endothelial cells, resulting in increased permeability for small solutes and water.

We found that, similar to the situation in peripheral endothelial cells, TNFα caused a significant reduction in intracellular cAMP levels. In bovine aortic endothelial cells and HUVECs, it has been shown that hydrolysis of cAMP was consistently increased in the cytosol after TNFα treatment, most likely because of the activation of PDE II and IV. We did not investigate the mechanism underlying the TNFα-mediated reduction in intracellular cAMP levels in this study. However, it may well be at least partly attributable to PDE IV activation because the addition of RO-20-1724 and 8-(4-CPT)-cAMP to BRECs selectively prevented TNFα-induced changes. The mechanism of the TNFα-induced destabilization of barrier function appears to be different in retinal (barrier forming) endothelium than in endothelial cells of peripheral (nonbarrier forming) origin, as in our effort to identify the signaling pathways involved we found some interesting differences with endothelial cells of peripheral origin. Whereas multiple studies on nonbarrier endothelium have shown that TNFα induces permeability via RhoA or Rac1 GTPase activation or inhibition, respectively, we did not find evidence for such roles of these GTPases in our BRB endothelium. Rac1 activation was induced to a certain extent by TNFα, but the ratio of activated RhoA/Rac1 remained unchanged. In addition, the activation of Rap1 (another family member of the small Rho GTPases) was also unchanged after TNFα stimulation in BRECs (unpublished results). Therefore, it can be assumed that, although in these cells cAMP plays a central regulatory role in endothelial permeability, as is the case in the peripheral endothelium, the downstream pathway activated by cAMP is not the same. This may reflect differences between the barrier and nonbarrier endothelia, and may be in line with the need for the barrier endothelium to be more robust in preventing molecules entering the neural tissues from the bloodstream. However, endothelial cell responses to TNFα may be species dependent, and thus care should be taken in interpreting and extrapolating these data to the human situation.
We were able to prevent TNFα-induced permeability for sodium fluorescein by elevating the intracellular cAMP levels using exogenous cAMP and by preventing cAMP degradation. The protective effect of cAMP on TNFα-induced BRB disruption in BREC was dependent on claudin-5, as we found that preincubation of the cells with 8-(4-CPT)-cAMP and RO-20-1724 did not rescue the expression of ZO-1 and VE-cadherin after TNFα stimulation. In contrast, the rescuing effect of cAMP on claudin-5 expression was sufficient to prevent the TNFα-induced increased permeability for sodium fluorescein. This is in line with findings in claudin-5-knockout mice, which show only increased permeability of tracers <800 Da in the brain. In fact, the intermediate-sized tracer of 766 Da that we have used may be just on the borderline of the molecules that can pass or not pass through the disrupted junctions.

In addition to direct barrier-promoting effects of increased levels of cAMP, as shown in the present study, 8-(4-CPT)-cAMP and RO-20-1724 may have additional effects that may be beneficial for the diabetic retina. We observed a downregulation of vascular adhesion molecule-1, intracellular adhesion molecule-1, and E-selectin after addition of 17.5 μM RO-20-1724 alone, whereas intracellular cAMP levels were similar as in unstimulated cells after 24 hours (data not shown). This suggests that RO-20-1724 may have anti-inflammatory effects as well as barrier-promoting effects, as was also shown in other cell types and animal models. Furthermore, 8-(4-CPT)-cAMP is a highly lipophilic analog with good membrane permeability, and once in the cell, can activate downstream targets with anti-inflammatory potential, such as protein kinase A.

With the effects observed in this study, it seems unlikely that TNFα alone can cause an influx of proteins and lipids into the neural retina and thereby disrupt Starling's forces with consequent macular edema. However, it may be possible that TNFα makes the retinal endothelial cells more prone to the effects of VEGF and other cytokines. Indeed, we found that simultaneous stimulation of the cells with TNFα, IL1β, and VEGF three cytokines that are known to be increased in the vitreous of DR patients, caused a significant increase in permeability for a 70 kDa-FITC tracer and a BSA-FITC tracer, but not for the two smaller tracers. Increased transport of macromolecules such as albumin induced by this combination of cytokines can lead to an increased tissue oncotic pressure and subsequent edema in vivo. This possibility is further supported by a study in which BRB function was assessed at different time points in a streptozotocin-induced diabetic mouse model and a mutant (Ins2Akita) diabetic mouse model, both lacking Tnfα. It was concluded that neither TNFα nor inflammation were necessary for early BRB breakdown, but were essential for BRB breakdown at later time points as the disease progressed. We suggest that the interplay between different cytokines, rather than one cytokine alone, leads to the development of vision-threatening edema, prompting the need for multitargeted therapy.

The prominent difference in permeability after triple cytokine stimulation of BSA-FITC (121% of control) and the 70 kDa-FITC tracer (400% of control) may be a result of different modes of transportation. Both molecules are hydrophilic, have a comparable molecular weight and have a diameter that does not allow them to pass via paracellular junctions (BSA-FITC approximately 7.2 nm, 70 kDa-FITC approximately 12 nm). Indeed, albumin was observed to translocate predominantly through receptor-mediated transcytosis. Therefore, the uptake of BSA-FITC by the cells requires free binding sites, which can be a rate-limiting factor. In contrast, dextrans do not adsorb to the cell surface and are internalized via nonspecific fluid-phase endocytosis. More pronounced effects of cytokines on fluid-phase permeability, which has been shown to occur in the BBB, than on receptor-mediated transcytosis can explain the difference in transcellular transport for these two similarly sized fluorescent tracers. Alternatively, it is possible that a larger portion of albumin is targeted for degradation or recycling in the cell instead of being transported to the extracellular compartment. Although it was rather surprising that the triple cytokine stimulation did not induce increased permeability for sodium fluorescein after 6 hours, this is not uncommon in patients with diabetic macular edema. Several studies reported that patients presenting with cystoid abnormalities on optical coherence tomography had no signs of leakage on fluorescein angiography or vice versa. Hence, leakage of small solutes and larger proteins do not always occur in concert in the retina. Regardless, our findings are limited to an in vitro model of the BRB and a monoculture of endothelial cells, and it is of high interest to validate our findings on the BRB in an in vivo retinal environment.

We conclude that TNFα alone induces small-molecule permeability of the BRB in vitro, whereas the combination of TNFα, IL1β, and VEGF induces permeability to large molecules and provide evidence that this permeability is mediated via cAMP. This suggests that TNFα can contribute to the development of retinal vascular leakage and consequently diabetic macular edema, although likely as part of a multifaceted pathogenesis.

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