OBJECTIVE—Tumor necrosis factor-α (TNF-α) and interleukin-1 beta (IL-1β) are elevated in the vitreous of diabetic patients and in retinas of diabetic rats associated with increased retinal vascular permeability. However, the molecular mechanisms underlying retinal vascular permeability induced by these cytokines are poorly understood. In this study, the effects of IL-1β and TNF-α on retinal endothelial cell permeability were compared and the molecular mechanisms by which TNF-α increases cell permeability were elucidated.

RESEARCH DESIGN AND METHODS—Cytokine-induced retinal vascular permeability was measured in bovine retinal endothelial cells (BRECs) and rat retinas. Western blotting, quantitative real-time PCR, and immunocytochemistry were performed to determine tight junction protein expression and localization.

RESULTS—IL-1β and TNF-α increased BRECs permeability, and TNF-α was more potent. TNF-α decreased the protein and mRNA content of the tight junction proteins ZO-1 and claudin-5 and altered the cellular localization of these tight junction proteins. Dexamethasone prevented TNF-α-induced cell permeability through glucocorticoid receptor transactivation and nuclear factor-kappaB (NF-κB) transrepression. Preventing NF-κB activation with an inhibitor κB kinase (IKK) chemical inhibitor or adenoviral overexpression of inhibitor κB alpha (IκBα) reduced TNF-α-stimulated permeability. Finally, inhibiting protein kinase C zeta (PKCζ) using both a peptide and a novel chemical inhibitor reduced NF-κB activation and completely prevented the alterations in the tight junction complex and cell permeability induced by TNF-α in cell culture and rat retinas.

CONCLUSIONS—These results suggest that PKCζ may provide a specific therapeutic target for the prevention of vascular permeability in retinal diseases characterized by elevated TNF-α, including diabetic retinopathy. Diabetes 59:2872–2882, 2010

The cause of vision loss in diabetic retinopathy is complex and remains incompletely understood; however, changes in blood vessel permeability and macular edema are associated with loss of visual acuity (1–5), with center point thickness and fluorescein leakage combined with age accounting for 33% of the variation in visual acuity (5). A growing body of evidence suggests that diabetic retinopathy includes a neuroinflammatory component, with increased expression of cytokines, microglia activation, leukostasis, and vascular permeability (6–9). Increased levels of interleukin-1 beta (IL-1β) and tumor necrosis factor-α (TNF-α) have been detected in the vitreous of diabetic patients with proliferative diabetic retinopathy (10,11) and in diabetic rat retinas (6,7,12). Moreover, intravitreal administration of IL-1β increases vascular permeability, associated with nuclear factor-kappaB (NF-κB) activation, increased leukocyte adhesion, and retinal capillary cell death (13,14). TNF-α also increases leukocyte adhesion to retinal endothelium (15) and blood–retinal barrier (BRB) permeability (16). The inhibition of TNF-α with etanercept, a soluble TNF-α receptor, inhibits NF-κB activation, leukostasis, and BRB breakdown in diabetic rat retinas (12). Altogether, these findings indicate that proinflammatory cytokines contribute to vascular permeability in diabetic retinopathy.

Changes in retinal vascular permeability may result from alterations of the tight junction complex. Tight junctions are composed of a combination of more than 40 proteins including the transmembrane proteins occludin, the claudin family, and the junction adhesion molecule (JAM) family, several peripheral membrane-associated proteins, including members of the zonula occludens (ZO) family, and several regulatory proteins (17). To date, evidence has been provided for the presence of occludin, claudin-5, ZO-1, and JAM-A in retinal vascular endothelium (18–21). Changes in occludin content, localization, and phosphorylation occur in response to vascular endothelial growth factor (VEGF) regulating endothelial permeability (18,19,22–24). Gene deletion studies have shown claudin-5 to be essential for blood–brain barrier function (25), and likely for the BRB as well. Recent studies demonstrated that ZO proteins are essential for the formation and organization of tight junction complex assembly (26,27). Therefore, changes in occludin, claudin-5, or ZO-1 likely contribute to alterations in endothelial permeability, in response to inflammatory cytokines.

Although several studies support the involvement of proinflammatory cytokines in BRB breakdown in diabetes, little attention has been given to the molecular mecha-
Materials and Methods. According to manufacturer’s instructions, as described in the supplementary materials and methods. Invitrogen, Carlsbad, CA) was used for the assessment of cell viability. Retinas were harvested 4 h after injection, immunolabeled for tight junction proteins, and evaluated by immunohistochemistry in whole retinas, as described previously (33). The retinas were incubated with monoclonal anti-occludin (1:50) and polyclonal anti-ZO-1 (1:50) antibodies for three days at 4°C. Primary antibodies were detected with Alexa Fluor 647-conjugated anti-rat IgG (Molecular Probes, Invitrogen) or Cy3-conjugated anti-rabbit IgG secondary antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA). Coverslips were mounted onto slides using Aqua PolyMount (AquaMount; Polysciences, Warrington, PA) and analyzed in a Leica TCS SP2 AOBS confocal microscope. Ten confocal Z-stacks were collected over a depth of 2.56 μm and projected onto one image. ZO-1 and occludin localization in retinal vessels were assessed by immunohistochemistry in whole retinas, as described previously (33).

RESEARCH DESIGN AND METHODS

Materials used to carry out the experiments are described in the supplementary materials and methods. In ophthalmic and vision research, and were approved and monitored by the Institutional Animal Care and Use Committee (IACUC) at the Penn State College of Medicine. Under anesthesia (66.7 mg/kg ketamine and 6.7 mg/kg xylazine), the animal received an intravitreal injection of TNF-α or vehicle (2.5 μl/eye) with a 5 μl Hamilton syringe (Hamilton Company, Reno, NV) through a puncture created by a 32-gauge needle. The animals were assessed for retinal permeability with Evans blue assay, 2 h after receiving the intravitreal injection of either PBS with 1% BSA, plus PKCζ, plus PKCζ/1. In a separate study, retinas were harvested 4 h after injection, and the expression of tight junction proteins, and analyzed by confocal microscopy.

Evans blue assay. Accumulation of the albumin binding dye Evans blue was used to assess changes in retinal vascular permeability. Evans blue dye accumulation in the retina was quantified using a published protocol (20), with normalization to blood plasma after 2-h circulation and expression as microliters of plasma per gram of retina (dry weight) per hour of circulation. Assessment of cell viability. The LIVE viability assay (Molecular Probes, Invitrogen, Carlsbad, CA) was used for the assessment of cell viability according to manufacturer’s instructions, as described in the supplementary materials and methods.

CaspaTag-3/7 activity assay. CaspaTag-3/7 activity was measured by the ApoONE Homogenous caspase-3/7 assay (Promega, Madison, WI), according to the manufacturer’s instructions, as briefly described in the supplementary materials and methods.

Western blotting. Western blotting of cellular lysates was performed as described in the supplementary materials and methods. RNA extraction and reverse transcription. RNA extraction and reverse transcription were performed as described in the supplementary materials and methods.

Quantitative real-time PCR. Quantitative real-time PCR (qPCR) analysis was performed using the 7,900 HT Sequence Detection System in 384-well optical plates using TaqMan Universal PCR Master Mix Assay-on-Demand and Assay-by-Design primers and probes (Applied Biosystems) as previously described (30). Primer/probes used were as follows: claudin-5 Bt03288088_s1, occludin Bt02355225_m1, IL-8 Bt0211907_g1, and bovine ZO-1 specific primers 5’-AGAAAGCTTGTTACGCGCTAGTGTG-3’ (forward), 5’-ATCCCTCCTCATATTTAAGAAGGTGCTGA-3’ (reverse), and 6-carboxy-fluorescein (FAM) 5’-ACCACATGGATTCT-3’ minor groove binding (MGB) probe. For each sample, qPCR were performed in triplicate and relative quantities were calculated using ABI SDS 2.0 RQ software and the 2-ΔΔCT analysis method.
lying TNF-α–induced retinal endothelial cell permeability were further investigated.

**TNF-α alters tight junction protein content and localization.** To determine the effect of TNF-α on expression of specific tight junction proteins, BRECs were exposed to 5 ng/ml TNF-α for 0.5 and 6 h and the protein contents of ZO-1, claudin-5, and occludin were determined by Western blotting. TNF-α significantly decreased ZO-1 content (58.2 ± 6.5% of control) after 6 h of exposure (Fig. 2A). Claudin-5 content was rapidly reduced after 0.5 h of treatment (70.8 ± 7.0% of control) and 6 h of TNF-α exposure further downregulated claudin-5 content (57.6 ± 8.7% of control). In contrast, TNF-α increased occludin content (130.6 ± 6.6% of control; Fig. 2C). To determine whether alterations in protein content were due to changes in mRNA expression, total mRNA content was evaluated by qPCR 6 h after TNF-α exposure. TNF-α significantly decreased ZO-1 (74.6 ± 3.3% of control; Fig. 2D) and claudin-5 mRNA content (80.9 ± 5.7% of control; Fig. 2E) but induced a twofold increase in occludin mRNA (Fig. 2F). To investigate whether TNF-α alters the tight junction complex at the cell membrane, the cellular localization of the tight junction proteins were evaluated by immunocytochemistry and confocal microscopy. In con-
Control conditions, ZO-1, claudin-5, and occludin immunoreactivity appeared as a near continuous staining at the cell border (Fig. 2G and supplementary Fig. 1, available in an online appendix). Upon TNF-α treatment, a loss of both ZO-1 and claudin-5 immunostaining was observed, leading to a fragmented border staining, although the effect on ZO-1 was more pronounced. Also, a diffuse cytoplasmic distribution of claudin-5 and occludin was observed in TNF-α-treated cells. After TNF-α treatment, occludin staining increased, and it was irregularly distributed at the cell border (Fig. 2G and supplementary Fig. 1).
**PKCζ REGulates Retinal Vascular Permeability**

Dexamethasone prevents TNF-α–induced BREC permeability. The effect of the glucocorticoid dexamethasone on TNF-α–induced endothelial permeability was determined. BREC were treated with 50 ng/ml dexamethasone 18 h before TNF-α treatment. The increase in cell permeability induced by TNF-α was completely prevented by dexamethasone (Fig. 3A). To investigate if the effect of dexamethasone was dependent on transcriptional activation by the glucocorticoid receptor, BREC were pretreated with 5 μmol/l RU486, a glucocorticoid receptor antagonist that inhibits the transactivation function of this receptor (35). A 1-h pretreatment of RU486 before the addition of dexamethasone significantly reduced the protective effect of dexamethasone on TNF-α–induced cell permeability (Fig. 3B). RU486 alone had no effect on permeability and did not alter the TNF-α response but did prevent the dexamethasone reduction in permeability as previously reported (36). These data show that dexamethasone’s protective effect is partially due to glucocorticoid receptor transactivation but also suggest that transrepression of TNF-α–responsive transcription factors also contributes to the inhibition of TNF-α–induced cell permeability.

**Dexamethasone prevents TNF-α–induced alterations in tight junction proteins.** The effect of dexamethasone on TNF-α–induced changes in tight junction protein content and cellular localization was also evaluated. Dexamethasone alone significantly increased ZO-1, claudin-5, and occludin protein content (Fig. 4). TNF-α decreased ZO-1 and claudin-5 protein content and these effects were prevented by dexamethasone pretreatment (Fig. 4A and B), whereas dexamethasone and TNF-α treatment yielded an additive threefold increase in occludin protein content (Fig. 4C). Dexamethasone increased ZO-1, claudin-5, and occludin staining at the cell border and prevented the TNF-α–induced fragmentation of these tight junction proteins (Fig. 4D and supplementary Fig. 1).

**NF-κB inhibition reduces TNF-α–induced cell permeability.** The involvement of NF-κB activation in TNF-α–induced endothelial permeability was investigated. BREC were exposed to 1 μmol/l IKK VII, an IkB kinase complex inhibitor, 30 min before TNF-α addition. As a control for inhibitor IKK VII effectiveness, TNF-α–induced IkBα phosphorylation by IKK was evaluated. Figure 5A shows that IkBα phosphorylation was blocked by IKK VII. The TNF-α–induced increase in permeability (275.0 ± 46.7% of control) was significantly reduced by IKK VII (160.5 ± 21.7% of control; Fig. 5B). The effect of adenovirus-mediated overexpression of IkBα on cell permeability was also evaluated. Western blotting was used to confirm the adenovirus-mediated expression of GFP and IkBα 30 h after adenoviral infection. GFP expression was similar in both AdEmpty- and AdIkBα-transduced cells, while IkBα was heavily expressed in AdIkBα-transduced cells compared with AdEmpty-transduced cells (Fig. 5C). The ability of IkBα overexpression to inhibit NF-κB activation was evaluated by examining the TNF-α–induced expression of IL-8 mRNA, a transcriptional target of NF-κB. In both nontransduced and AdEmpty-transduced cells, IL-8 mRNA levels were significantly increased (by 18- and 16-fold, respectively) after 2 h of TNF-α stimulation (Fig. 5D). This increase in IL-8 expression was significantly reduced (by 41%) in AdIkBα-transduced cells, demonstrating that adenovirus-mediated overexpression of IkBα effectively blocked NF-κB activation after TNF-α treatment. Subsequently, the effect of IkBα overexpression on TNF-α–induced cell permeability was determined. In AdEmpty-transduced cells, TNF-α induced a significant increase in cell permeability (273.6 ± 71.0% of control; Fig. 5E), similar to nontransduced cells (Fig. 5F). IkBα overexpression reduced TNF-α–induced permeability to 157.5 ± 5.7% of control. These data suggest that NF-κB activation is necessary for a substantial part of the TNF-α–induced changes in BREC permeability.

**PKCζ-1 inhibits NF-κB activation induced by TNF-α**. PKCζ has a critical role in the activation of the NF-κB pathway (37). To determine whether PKCζ modulates NF-κB activation in retinal endothelial cells induced by TNF-α, the effect of PKCζ-1, a novel PKCζ inhibitor with little or no inhibitory activity on PKCβ or PKCδ (manuscript in preparation), on TNF-α–induced IL-8 mRNA expression, was evaluated. BREC were treated with 10 μmol/l PKCζ-1 30 min before treatment of cells with TNF-α for 2 h. TNF-α induced a significant 14-fold increase in IL-8 transcripts, which was significantly reduced to 6-fold by PKCζ-1 (Fig. 6A). The effect of PKCζ-1 on TNF-α–induced NF-κB reporter activity was investigated in a 293/NF-κB/luc stable reporter cell line. Cells were

---

**FIG. 3.** Dexamethasone prevents TNF-α–induced cell permeability through transactivation of the glucocorticoid receptor. A: BREC were grown to confluence on transwell filters and treated with 50 ng/ml dexamethasone (Dex) 18 h before TNF-α treatment (5 ng/ml, 6 h). B: Cells were treated with 5 μmol/l RU486 1 h before Dex treatment. The monolayer permeability to 70 kDa dextran was measured as described in **RESEARCH DESIGN AND METHODS**. The results represent the mean ± SEM of at least seven independent experiments and are expressed relative to control (Ctrl). *P < 0.05, **P < 0.001, significantly different as determined by ANOVA followed by Bonferroni post hoc test.
permeability was investigated. BRECs were treated with 2 mol/l H9262 in the presence of LY294002 (Fig. 7A). These observations suggest that pharmacological inhibition of PKCζ reduces NF-κB activation in response to TNF-α.

**TNF-α-induced cell permeability requires PKCζ.** PKCζ activation can occur downstream of the PI3K pathway (38), which has been shown to be activated by TNF-α in vascular endothelial cells (39). Therefore, the effect of PI3K/PKCζ pathway inhibition on retinal endothelial cell permeability was investigated. BRECs were treated with 2 μmol/l LY294002, a PI3K inhibitor, 30 min before the treatment with TNF-α for 6 h. The increase in cell permeability induced by TNF-α was significantly inhibited by 50% in the presence of LY294002 (Fig. 7A). Next, BRECs were exposed to 10 μmol/l PKCζ-1 or the myristoylated pseudosubstrate inhibitor of PKCζ (PKCζP; 250 nmol/l) 30 min prior to TNF-α addition. Both PKCζ-1 and PKCζP completely suppressed the increase in cell permeability induced by TNF-α (Fig. 7B and C). Because conventional PKC isoforms contribute to the VEGF-induced permeability in retinal endothelial cells (23), the contributions of conventional and novel PKC isoforms for TNF-α-induced cell permeability were also evaluated. BRECs were treated with 5 μmol/l BIM I (inhibitor of conventional and novel PKC isoforms) or with 30 nmol/l PKCβ inhibitor 30 min before TNF-α treatment. These inhibitors had no effect on TNF-α-induced cell permeability (supplementary Fig. 2A and B, available in an online appendix). These results suggest that PKCζ, but not conventional and novel PKC isoforms, mediate TNF-α-induced retinal endothelial cell permeability. The effect of PKCζ inhibition on tight junction protein content in TNF-α–treated BRECs was also evaluated. The protein content of ZO-1, claudin-5, and occludin was not affected by treatment with PKCζ-1. However, the decrease in ZO-1 and claudin-5 protein levels induced by TNF-α was effectively prevented by PKCζ-1 (Fig. 7E and F). Surprisingly, in the presence of PKCζ-1, TNF-α still caused an increase in occludin protein content, which actually was greater than the effect of TNF-α alone (Fig. 7G).

FIG. 4. Dexamethasone prevents TNF-α–induced alterations in the tight junction complex. Confluent BRECs were treated with 50 ng/ml Dex 18 h before TNF-α treatment (5 ng/ml, 6 h). Whole-cell extracts were assayed for (A) ZO-1, (B) claudin-5, and (C) occludin immunoreactivity by Western blotting as described in RESEARCH DESIGN AND METHODS. Representative Western blotting for each tight junction protein and β-actin (loading control) are presented above each respective graph. The results are normalized to β-actin and represent the mean ± SE of at least five independent experiments and are expressed as the relative amount compared with control. *P < 0.05, **P < 0.01, ***P < 0.001, significantly different from control; #P < 0.05, ##P < 0.01, ###P < 0.001, significantly different from TNF-α, as determined by ANOVA followed by Bonferroni post hoc test. D: Cells were immunolabeled for ZO-1, claudin-5, and occludin and 10 confocal Z-stacks were taken through 2.56 μm and projected into one image. These results are representative of four independent experiments. Scale bar, 25 μm.

treated with 10 or 50 μmol/l PKCζ-1 30 min before the addition of TNF-α for 6 h, and luciferase activity was determined in whole-cell lysates. The results show that PKCζ-1 significantly decreased TNF-α–induced NF-κB-responsive luciferase expression in these cells (Fig. 6B). These observations suggest that pharmacological inhibition of PKCζ reduces NF-κB activation in response to TNF-α.
PKCζ REGULATES RETINAL VASCULAR PERMEABILITY

PKCζ-1 prevents TNF-α–induced BRB permeability in vivo. The effect of PKCζ-1 on TNF-α–induced BRB permeability was evaluated in vivo by the Evans blue assay. The injection of TNF-α in the vitreous induced the accumulation of Evans blue (20.30 ± 2.46 μl plasma/g/h) as compared with PBS-injected animals (10.25 ± 2.48 μl plasma/g/h; Fig. 8A). PKCζ-1 alone had no effect on the accumulation of Evans blue but completely prevented TNF-α–induced accumulation of the dye. To determine the effect of TNF-α injection and PKCζ inhibition on retinal vascular tight junction organization, retina whole flat mounts were immunolabeled for ZO-1 and occludin proteins after injection of the cytokine or cytokine with PKCζ-1. In PBS-injected rat eyes, ZO-1 and occludin immunoreactivities were intense and localized at the junctions of the cell membranes of endothelial cells in retinal vessels. In TNF-α–injected eyes, changes in ZO-1 immunostaining were particularly evident, which became markedly reduced and intermittently absent from the cell borders. These alterations were prevented by cotreatment with PKCζ-1 (Fig. 8J). Consistent with the results obtained with BRECs in culture, the occludin content was not reduced with TNF-α but displayed regions with disorganized cell-border labeling that were reversed by PKCζ-1 treatment.

DISCUSSION

Elevated levels of proinflammatory cytokines IL-1β and TNF-α have been detected in the vitreous of diabetic patients with retinopathy (10,11) and in diabetic rat retinas correlated with increased vascular permeability (12,40). However, the mechanisms underlying the effects of IL-1β and TNF-α on retinal microvascular barrier have not been addressed. The results presented herein demonstrate that IL-1β and TNF-α increase retinal endothelial cell permeability and that TNF-α acts through PKCζ/NF-κB to reduce the expression and alter the distribution of the tight...
expression of TNF-α-dependent retinal endothelial cell permeability is associated with changes in the expression or distribution of the tight junction proteins. The content of occludin and ZO-1 decreases in the retinas of diabetic animals (18,33,41). In
**FIG. 8. PKCζ regulates retinal vascular permeability**

**A**  

PKCζ prevents TNF-α-induced retinal vascular permeability in vivo. Animals’ eyes were injected with PBS with 0.1% BSA, TNF-α (10 ng); PKCζ-1 (280 ng); or with both PKCζ-1 and TNF-α. A: Evans blue leakage was evaluated 24 h after intravitreal injections. The results represent the mean ± SEM (n = 7–8 animals per group) and are expressed relative to control (Ctrl; PBS-injected eyes). **P < 0.01, significantly different from control; ##P < 0.01, significantly different from TNF-α, as determined by ANOVA followed by Bonferroni post hoc test. B: PKCζ-1 prevents the alterations in tight junction proteins induced by TNF-α in vivo. Whole retinas were immunolabeled for ZO-1 and occludin 4 h after injection. Images were obtained on a Leica TCS SP2 AOBS confocal microscope and are presented as a maximum projection. Arrows indicate loss and/or discontinuous cell border staining. Scale bar, 25 μm. (A high-quality digital representation of this figure is available in the online issue.)

BRECs, TNF-α downregulated ZO-1 and claudin-5 expression and decreased the junctional localization of both proteins, which was associated with an increase in cell permeability. These studies are consistent with previous publications demonstrating reduced claudin-5 gene expression after TNF-α treatment in brain capillary endothelial cells (42,43). However, in these same studies, a decrease in occludin was observed after TNF-α treatment, which contrast the increase in occludin observed in the present study. Further, TNF-α reduced occludin promoter activity in a human intestinal cell line (44). The cause for this difference is unclear but may relate to the use of cell lines versus primary culture or differences in epithelial and endothelial cell types. Regardless, TNF-α consistently reduces both claudin-5 and ZO-1 expression, two tight junction proteins essential for barrier properties.

The effect of TNF-α and VEGF on retinal endothelial permeability is distinct. Occludin becomes phosphorylated on multiple sites after VEGF treatment in a conventional PKC-dependent manner (23) and Ser490 has recently been identified as a phosphorylation site necessary for VEGF-induced permeability (24,45). TNF-α did not lead to an increase in occludin phosphorylation on Ser490 (data not shown) but rather led to an increase in occludin expression. Further, inhibition of conventional or novel PKC isoforms did not prevent TNF-α–induced permeability. These data demonstrate that VEGF and TNF-α alter retinal endothelial barrier properties by distinct molecular mechanisms.

The data presented here suggest that glucocorticoid receptor activation inhibits TNF-α–induced cell permeability through both transactivation and transrepression mechanisms. Inhibition of IKK and adenovirus-mediated overexpression of IκBα blocked the increase in BREC permeability induced by TNF-α treatment. Together, these data demonstrate that TNF-α–induced retinal endothelial cell permeability is mediated, at least in part, by NF-κB. The mechanisms by which NF-κB may regulate cell permeability and the tight junction complex are largely unknown. NF-κB putative binding sites and several E-box sequences were identified within the claudin-5 promoter sequence (43). Recent studies demonstrated that NF-κB induces the expression of Snail and Slug transcription factors (46,47), which repress E-cadherin, occludin, and claudin family members gene expression by binding to specific E-box sequences during epithelial-mesenchymal transition (48–50). These reports suggest the possibility that a similar regulation by transcriptional repressors might also play a role in claudin-5 and ZO-1 expression in retinal endothelial cells.

PKCζ has been shown to contribute a critical and selective role in the regulation of NF-κB. In PKCζ-deficient mice, NF-κB transcriptional activity as well as the phosphorylation of p65 in response to TNF-α is severely impaired (37). In endothelial cells, the transcriptional activity of NF-κB is dependent on the phosphorylation of the p65 subunit by PKCζ (51). The observations that TNF-α–induced increase in IL-8 expression and NF-κB–dependent luciferase reporter expression are inhibited by PKCζ-1 further support the hypothesis that PKCζ is important for NF-κB transcriptional activity.

In endothelial cells, PKCζ has been shown to be activated by TNF-α (51–53). The mechanism of activation of PKCζ remains to be fully clarified, but it has been shown to be an important downstream target of PI3K (38). TNF-α stimulates PI3K in endothelial cells through endothelial/epithelial tyrosine kinase induced activation of VEGFR2 (39). Interestingly, this is only a partial activation of VEGFR2 that fails to activate the classical PKC isoforms necessary for VEGF-stimulated permeability (23,54). PI3K inhibition and inhibition of its downstream target PKCζ (38) blocked TNF-α–induced cell permeability, whereas...
targeting PKCζ reduced NF-κB activation and most likely inhibited additional signaling pathways that contribute to the regulation of the tight junction complex and endothelial permeability induced by TNF-α. PKCζ-1 is also effective at blocking VEGF-induced permeability (manuscript in preparation). Then, although VEGF and TNF-α alter the tight junction complex by distinct mechanisms, inhibition of PKCζ is a common target for blocking both VEGF- and TNF-α-induced cell permeability. Therefore, targeting PKCζ may provide a specific therapeutic option for the prevention of vascular permeability in retinal diseases with elevated TNF-α and VEGF, such as diabetic retinopathy.

ACKNOWLEDGMENTS

This study was supported by the Foundation for Science and Technology, Portugal (SFRH/BD/18827/2004), FEDER, National Institutes of Health Grants EY-016413 and EY-012021 (D.A.A.), and the Juvenile Diabetes Research Foundation International (D.A.A.). No potential conflicts of interest relevant to this article were reported. C.A.A. and A.F.A. researched data, contributed to discussion, wrote the manuscript, and reviewed/edited the manuscript. C.-M.L., S.F.A., and D.A.A. researched data, contributed to discussion, and reviewed/edited the manuscript. The authors thank Ellen Wolpert and Kristin Gonsar of the Department of Cellular and Molecular Physiology, Penn State College of Medicine, and Rob Brucklacher and Georgina Bixler of the Functional Genomics Core Facility of the Section of Research Resources, Penn State Hershey College of Medicine, for technical assistance.

REFERENCES

1. Antonetti DA, Barber AJ, Bronson SK, Freeman WM, Gardner TW, Jeffer-son LS, Kester M, Kimball SR, Krady JK, LaNoe KF, Norbury CC, Quinn PG, Sandrasegarane L, Simpson IA, JDRF Diabetic Retinopathy Center Group. Diabetic retinopathy: seeing beyond glucose-induced microvascu-lardisease. Diabetes2006;55:2401–2411.
2. Gardner TW, Larsen M, Girach A, Zhi X, on behalf of the Protein Kinase C Diabetic Retinopathy Study (PCK-DRS2) Study Group. Diabetic macular oedema and visual loss: relationship to location, severity and duration. Acta Ophthalmol 2009;87:709–713.
3. Moss SE, Klein R, Klein BE. The 14-year incidence of visual loss in a diabetic population. Ophthalmology1998;105:998–1003.
4. Sander B, Thornit DN, Colmorn L, Strøm C, Girach A, Hubbard LD, Lund-Andersen H, Larsen M. Progression of diabetic macular edema: correlation with blood retinal barrier permeability, retinal thickness, and retinal vessel diameter. Invest Ophthalmol Vis Sci 2007;48:3083–3087.
5. Diabetic Retinopathy Clinical Research Network, Browning DJ, Glassman AR, Aiello LP, Beck RW, Brown DM, Pong DS, Bressler NM, Dania RP, Kanyoum IL, Nguyen QD, Bhavsar AR, Gottlieb J, Pieramici DJ, Reiter ME, Apte RS, Lim JJ, Miskala PH. Relationship between optical coherence tomography-measured central retinal thickness and visual acuity in diabetic macular edema. Ophthalmology 2007;114:525–536.
6. Carvo A, Cunha-Vaz JG, Carvalho AP, Lopes MC. L-arginine transport in retinas from streptozotocin diabetic rats: correlation with the level of IL-1 beta and NO synthase activity. Vision Res 1999;39:3817–3823.
7. Krady JK, Basu A, Allen CM, Xu Y, LaNoe KF, Gardner TW, Levison SW. Minocycline reduces proinflammatory cytokine expression, microglial activation, and caspase-3 activation in a rodent model of diabetic retinopathy. Diabetes 2005;54:1559–1565.
8. Miyamoto K, Khosrof S, Bursell SE, Rohan R, Murata T, Clevert AC, Aiello LP, Ogura Y, Adams AP. Prevention of leukostasis and vascular leakage in streptozotocin-induced diabetic retinopathy via intercellular adhesion molecule-1 inhibition. Proc Natl Acad Sci U S A 1999;96:10836–10841.
9. Rungger-Brindle E, Doossa AA, Leuenberger PM. Glial reactivity, an early feature of diabetic retinopathy. Invest Ophthalmol Vis Sci 2000;41:1971–1980.
10. Abu el Asrar AM, Maimone D, Morse PH, Gregory S, Reder AT. Cytokinies in the vitreous of patients with proliferative diabetic retinopathy. Am J Ophthalmol 1992;114:731–736.
11. Demircan N, Safran BD, Soyu M, Ozcan AA, Sizmaz S. Determination of vitreous interleukin-1 (IL-1) and tumor necrosis factor (TNF) levels in proliferative diabetic retinopathy. Eye 2006;20:1366–1369.
12. Joussen AM, Poulaik V, Mitsiades N, Kirchhof B, Koizumi K, Dohmen S, Adams AP. Nonsteroidal anti-inflammatory drugs prevent early diabetic retinopathy via TNF-alpha suppression. FASEB J 2002;16:438–440.
13. Bamforth SD, Lightman SL, Greenwood J. Ultrastructural analysis of interleukin-1 beta-induced leukocyte recruitment to the rat retina. Invest Ophthalmol Vis Sci 1997;38:29–38.
14. Kowluru RA, Odendah S. Role of interleukin-1beta in the development of retinopathy in rats: effect of antioxidants. Invest Ophthalmol Vis Sci 2004;45:4161–4166.
15. Ben-Mahmud BM, Mann GE, Datti A, Orlacchio A, Kohner EM, Chibber R. Tumor necrosis factor-alpha in diabetic plasma increases the activity of core 2 GlcNAc-T and adherence of human leukocytes to retinal endothelial cells: significance of core 2 GlcNAc-T in diabetic retinopathy. Diabetes 2004;53:2968–2976.
16. Saishin Y, Saishin Y, Takahashi K, Melia M, Vinores SA, Campochoiar PH. Inhibition of protein kinase C decreases prostaglandin-induced breakdown of the blood-retinal barrier. J Cell Physiol 2003;195:210–219.
17. Matter K, Balda MS. Signalling to and from tight junctions. Nat Rev Mol Cell Biol 2003;4:225–236.
18. 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.
19. 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.
20. Gardner TW. Histamine, ZO-1 and increased blood-retinal barrier perme-ability in diabetic retinopathy. Trans Am Ophthalmol Soc 1995;93:583–621.
21. Tani M, Hosoya K. Application of magnetically isolated rat retinal vascular endothelial cells for the determination of transporter gene expression levels at the inner blood-retinal barrier. J Neurochem 2004;91:1244–1248.
22. Antonetti DA, Barber AJ, Hollinger LA, Wolpert EB, Gardner TW. Vascular endothelial growth factor inducer rapid phosphorylation of tight junction proteins occludin and zonula occluden 1. A potential mechanism for vascular permeability in diabetic retinopathy and tumors. J Biol Chem 1999;274:23463–23467.
23. Harhaj NS, Felinski EA, Wolpert EB, Sundstrom JM, Gardner TW, Antonet-ti DA. VEGF activation of protein kinase C stimulates occludin phosphorylation and decreases occludin levels and endothelial permeability. Invest Ophthal-mol Vis Sci 2006;47:5106–5115.
24. 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.
25. Nitta T, Hata M, Gotot S, SEO T, Sasaki H, Hashimoto N, Furuse M, Tsukita S. Three-dimensional composition of the blood-brain barrier in claudin-5-deficient mice. J Cell Biol 2003;161:553–600.
26. Umeda K, Ikouchou J, Kashiura-Tayama S, Furuse K, Sasaki H, Nakayama M, Matsu T, Tsukita S, Furuse M, Tsukita S. ZO-1 and ZO-2 independently determine where claudins are polymerized in tight-junction strand forma-tion. Cell 2006;126:741–754.
27. Katsuno T, Umeda K, Matsui T, Hata M, Tamura A, Itoh M, Takeuchi K, Fujimori T, Naeshihama Y, Noda T, Tsukita S, Tsukita S. Deficiency of zonula occludens-1 causes embryonic lethal phenotype associated with detected yolk sac angiogenesis and apoptosis of embryonic cells. Mol Biol Cell 2008;19:2465–2475.
28. Antonetti DA, Barber AJ. Diabetic Retinopathy. In: Barret J, editor. Molecular and cellular biology of the eye. 2nd ed. New York: McGraw-Hill; 2002. p. 290–408.
29. Harhaj NS, Barber AJ, Antonetti DA. Platelet-derived growth factor medi-ates tight junction redistribution and increases permeability in MDCK cells. J Cell Physiol 2003;193:349–364.
30. Barber AJ, Antonetti DA, Gardner TW. Altered expression of retinal occludin and glial fibrillary acidic protein in experimental diabetes. The
34. Bobrovnikova-Marjon EV, Marjon PL, Barbash O, Vander Jagt DL, Abecker SF. Expression of angiogenic factors vascular endothelial growth factor and interleukin-8/CCCL8 is highly responsive to ambient glutamine availability: role of nuclear factor-kappaB and activating protein-1. Cancer Res 2004;64:4858–4869

35. Mahajan DK, London SN. Mifepristone (RU486): a review. Fertil Steril 1997;68:967–976

36. Felinski EA, Cox AE, Phillips BE, Antonetti DA. Glucocorticoids induce transactivation of tight junction genes occludin and claudin-5 in retinal endothelial cells via a novel cis-element. Exp Eye Res 2008;86:867–878

37. Leitges M, Sanz L, Martin P, Duran A, Braun U, Garcia JF, Camacho F, Diaz-Meco MT, Rennert PD, Moscat J. Targeted disruption of the zetaPKC gene results in the impairment of the NF-kappaB pathway. Mol Cell 2001;8:771–780

38. Chou MM, Hou W, Johnson J, Graham LK, Lee MH, Chen CS, Newton AC, Schaffhausen BS, Toker A. Regulation of protein kinase C zeta by PI 3-kinase and PDK-1. Curr Biol 1998;8:1069–1077

39. Zhang R, Xu Y, Ekman N, Wu Z, Wu J, Altalak K, Min W. Etk/Bmx transactivates vascular endothelial growth factor 2 and recruits phosphatidylinositol 3-kinase to mediate the tumor necrosis factor-induced angiogenic pathway. J Biol Chem 2003;278:31267–31276

40. Carmo A, Cunha-Vaz JG, Carvalho AP, Lopes MC. Effect of cyclosporin-A on the blood–retinal barrier permeability in streptozotocin-induced diabetes. Mediators Inflamm 2000;9:243–248

41. Leal EC, Manivannan A, Hosoya K, Terasaki T, Cunha-Vaz J, Ambrosio AF, Forrester JV. Inducible nitric oxide synthase isoforms is a key mediator of leukostasis and blood-retinal barrier breakdown in diabetic retinopathy. Invest Ophthalmol Vis Sci 2007;48:5257–5265

42. Forster C, Burek M, Romero IA, Weksler B, Couraud PO, Drenckhahn D. Differential effects of hydrocortisone and TNFalpha on tight junction proteins in an in vitro model of the human blood-brain barrier. J Physiol 2008;586:1937–1949

43. Burek M, Forster CY. Cloning and characterization of the murine claudin-5 promoter. Mol Cell Endocrinol 2009;298:19–24

44. Mankertz J, Tavalali S, Schmitz H, Mankertz A, Riecken EO, Fromm M, Schulzke JD. Expression from the human occludin promoter is affected by tumor necrosis factor alpha and interferon gamma. J Cell Sci 2000;113:2085–2090

45. Sundstrom JM, Tash BR, Murakami T, Flanagan JM, Belew MC, Stanley BA, Gonsar KB, Antonetti DA. Identification and analysis of occludin phosphosites: a combined mass spectrometry and bioinformatics approach. J Proteome Res 2009;8:808–817

46. Criswell TL, Arteaga CL. Modulation of NFkappaB activity and E-cadherin by the type III transforming growth factor beta receptor regulates cell growth and motility. J Biol Chem 2007;282:32491–32500

47. Julien S, Puig I, Caretti E, Bonaventure J, Nelles L, van Roy F, Dargemont C, de Herreros AG, Bellacosa A, Larue L. Activation of NF-kappaB mediated by Akt upregulates Snail expression and induces epithelial mesenchyme transition. Oncogene 2007;26:7445–7456

48. Battle E, Sancho E, Franci C, Dominguez D, Monfar M, Baulida J, Garcia De Herreros A. The transcription factor snail is a repressor of E-cadherin gene expression in epithelial tumour cells. Nat Cell Biol 2000;2:84–89

49. Ikemouchi J, Matsuda M, Furuse M, Tsukita S. Regulation of tight junctions during the epithelium-mesenchyme transition: direct repression of the gene expression of claudins/occludin by Snail. J Cell Sci 2003;116:1959–1967

50. Martinez-Estrada OM, Cullerés A, Soriano FX, Peinado H, Bolós V, Martínez PO, Reina M, Cano A, Fabre M, Vilaró S. The transcription factors Slug and Snail act as repressors of Claudin-1 expression in epithelial cells. Biochem J 2006;394:449–457

51. Anrather J, Caizmádia V, Soares MP, Winkler H. Regulation of NF-kappaB Realpha phosphorylation and transcriptional activity by p21(ras) and protein kinase Czeta in primary endothelial cells. J Biol Chem 1999;274:13594–13603

52. Frey RS, Gao X, Javaid K, Siddiqui SS, Rahman A, Malik AB. Phosphatidylinositol 3-kinase gamma signaling through protein kinase Czeta of protein kinase Czeta induces NADPH oxidase-mediated oxidant generation and NF-kappaB activation in endothelial cells. J Biol Chem 2006;281:16128–16138

53. Garin G, Abe J, Mohan A, Lu W, Yan C, Newby AC, Rhaman A, Berk BC. Flow antagonizes TNF-alpha signaling in endothelial cells by inhibiting caspase-dependent PKC zeta processing. Circ Res 2007;101:97–105

54. Aiello LP, Bursell SE, Clermont A, Duh E, Ishii H, Takagi C, Mori F, Ciulla TA, Ways K, Ironside M, Smith LE, King GL. Vascular endothelial growth factor-induced retinal permeability is mediated by protein kinase C in vivo and suppressed by an orally effective beta-isoform-selective inhibitor. Diabetes 1997;46:1473–1480