



Vascular Expression of Permeability-Resistant Occludin Mutant Preserves Visual Function in Diabetes

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Diabetic retinopathy is one of the leading causes of vision loss and blindness. Extensive preclinical and clinical evidence exists for both vascular and neuronal pathology. However, the relationship of these changes in the neurovascular unit and impact on vision remains to be determined. Here, we investigate the role of tight junction protein occludin phosphorylation at S490 in modulating barrier properties and its impact on visual function. Conditional vascular expression of the phosphorylation-resistant Ser490 to Ala (S490A) form of occludin preserved tight junction organization and reduced vascular endothelial growth factor (VEGF)-induced permeability and edema formation after intraocular injection. In the retinas of streptozotocin-induced diabetic mice, endothelial-specific expression of the S490A form of occludin completely prevented diabetes-induced permeability to labeled dextran and inhibited leukostasis. Importantly, vascular-specific expression of the occludin mutant completely blocked the diabetes-induced decrease in visual acuity and contrast sensitivity. Together, these results reveal that occludin acts to regulate barrier properties downstream of VEGF in a phosphorylation-dependent manner and that loss of inner blood-retinal barrier integrity induced by diabetes contributes to vision loss.

Diabetic retinopathy (DR) is a progressive multifactorial disease with a complex pathogenesis with progressive disruption of the normal interaction between neuronal, glial, and vascular cells comprising the neurovascular unit (1). In addition to well-characterized vascular defects, human

studies and animal models reveal diabetes leads to increased neuronal cell death and thinning of the inner retina (1,2). Clinically, these alterations manifest by apparent vascular lesions in the retinal fundus of patients with diabetes and also by reduced contrast sensitivity and impaired color vision, demonstrating pathology in both vascular and neural sensory retina that may occur in DR (3,4). However, how these pathomechanisms interact in the retina remains unknown, and specifically, whether alterations in the blood retinal barrier permeability affect vision or neural cell death remains to be directly tested.

Vascular endothelial growth factor (VEGF) has been shown to have a central role in vascular alterations in DR, leading to increased vascular permeability and neovascularization (5–7). Targeting VEGF with antibodies or traps successfully reduces the occurrence of diabetic macular edema and improves vision or prevents further vision loss in approximately half of treated patients (8–11).

We previously demonstrated that VEGF, acting through the protein kinase C- β (PKC β) pathway, stimulates phosphorylation of the tight junction protein occludin at Ser490 (S490), increasing ubiquitination of occludin and trafficking of tight junctions to the cytoplasm and resulting in increased paracellular gaps and increased vascular permeability in retinal vascular endothelial cells (12,13). However, the relative contribution of occludin Ser490 phosphorylation to VEGF- or diabetes-induced vascular permeability in vivo has not been previously investigated. Further, the contribution of vascular permeability to visual loss has not been directly tested. Here, we describe a novel mouse model with conditional

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expression of a Ser490 to Ala (S490A) mutant form of occludin that prevents phosphorylation at this site and subsequent ubiquitination. Using vascular endothelial-restricted, Cre-controlled expression, we demonstrate that expression of this junctional stable form of occludin reduces VEGF-driven permeability and edema formation in vivo in a phosphorylation-specific manner. Importantly, the expressing S490A occludin mutant completely protects against diabetes-induced permeability and preserves visual function, without affecting neuronal cell loss or retinal thinning. To our knowledge, this is the first demonstration of a tight junction point mutant controlling VEGF- or diabetes-induced permeability in vivo, and the results reveal a critical role of barrier maintenance in preservation of vision.

RESEARCH METHODS AND DESIGN

Animal Experiments

All animal procedures were conducted in accordance with the Association for Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Vision Research and approved by the University of Michigan Institutional Animal Care and Use Committee.

Gene targeting to the ROSA26 locus with Zn-finger nucleases was used to create transgenic animals with CAG promoter, followed by floxed stop for conditional expression of wild-type (WT) occludin (WtOCC) or mutated Ser490 to Ala occludin (S490AOCC) using oocyte injection. Supplementary Fig. 1A provides a schematic of the construct. To allow conditional vascular expression of the transgenes, these mice were then crossed with Tek-Cre mice from The Jackson Laboratory or PDGF β -improved (i)Cre mice, a gift from Dr. Fruttiger (14). To control for the contribution of endogenous occludin, the PDGF-iCre mice expressing WtOCC or S490AOCC were crossed with *Occ*^{f/f} mice, a gift from Dr. Turner's laboratory (15) (Supplementary Fig. 3A). Inducible Cre was achieved by tamoxifen injection (30 μ g in 3 μ L volume) at postnatal day 3 into the milk sac.

For intravitreal injections, mice were anesthetized, and a 32-gauge needle was used to generate a hole for an intravitreal injection (1 μ L/eye) using a 5-mL Hamilton syringe of PBS or recombinant mouse VEGF (R&D Systems). The dose of VEGF and the time after the injection are specified in each figure legend.

For the diabetes studies, 2-month-old male Tek-Cre and Tek-S490AOCC mice were made diabetic by an intraperitoneal injection of 65 mg/kg streptozotocin (STZ) for 5 consecutive days. Blood glucose levels were measured after 2 days of the last injection, and animals with levels >250 mg/dL were considered diabetic.

Western Blotting

Retina lysates were prepared and Western blots performed as previously described (16). Detailed information

on the antibodies is provided in the Supplementary Material.

Retinal Vascular Permeability

For permeability measures after VEGF, mice were injected with a mixture of FITC-BSA (200 mg/kg body wt) and 70-kDa rhodamine B isothiocyanate (RITC)-dextran (50 mg/kg body wt). After 2 h of tracer circulation, retinas were harvested and processed as previously described (16). For representative purposes, retinal vascular leakage was also qualitatively assessed in retina whole mounts after 30 min of 70-kDa RITC-dextran-TR dye circulation. Stitched images of the whole retina were obtained using a DM6000 (Leica Microsystems) fluorescence microscope. Vascular permeability was also measured in retinal sections after 4 months of diabetes, as previously described (17). Results were normalized against background intensity and to the levels of tracer in the plasma and averaged, where *n* equals the number of eyes tested. These measurements were done in a masked fashion.

MRI

MRI was used to assess inner blood-retinal barrier (BRB) integrity in vivo using a dedicated small-rodent 7-T MRI system located at Trinity College Dublin, as previously described (18). A quantitative assessment of inner BRB integrity was made using dynamic contrast-enhanced MRI, in which the passage of gadolinium-diethylenetriamine pentaacetic acid from the vasculature into the retinal tissue was monitored for 10 min after injection.

Retinal Immunostaining and Microscopy

Retinal whole-mount staining was performed as previously described (19). Detailed information on antibodies is provided in the Supplementary Material. For tight junction border localization, images were obtained with a Leica TCS SP5 confocal microscope (Leica Microsystems) by projecting a z-stack of 10 serial images over a depth of 5 μ m, and border staining was quantified by a rank scoring system, as described previously (16).

The total number of positive inflammatory cells in diabetic retinas were manually counted in stitched images of each whole retina, obtained on a on a DM6000 fluorescence microscope (Leica Microsystems) and normalized per retinal area. Representative images from the superficial and deep capillary plexus were obtained on a Leica TCS SP5 confocal microscope (Leica Microsystems). Retinal vessel architecture was assessed by staining whole retinas with Alexa Fluor 488 conjugated isolectin B4 (IB4). Images of the superior, intermediate, and deep capillary plexus were obtained in a masked fashion, as described above, and vessel density, number of branch points, total vessel length, and number of terminal vessels were determined using Imaris software (Oxford Instruments).

Spectral Domain Optical Coherence Tomography

A spectral domain optical coherence tomography (BiopTigen) imaging system was used to measure retinal thickness. The total thicknesses of four points on the retina, 350 μm from the optic nerve head, were selected manually within the retinal image and averaged. Inner retina, defined as the inner nuclear layer through the nerve fiber layer, and outer retina, defined as the photoreceptor layer through outer plexiform layer, were also measured. These measurements were done in a masked fashion.

Visual Acuity and Contrast Sensitivity

An optokinetic tracking system (OptoMotry, Cerebral Mechanics) was used to test visual acuity and contrast sensitivity. Mice were provided an alternating, rotating sine wave grating stimulus on computer screens, in three-dimensional space. In a masked fashion, the tester recorded the presence or absence of head tracking, and a simple staircase method was used to determine the highest level of spatial frequency or lowest level of contrast visible to the mice. The tests were done at 100% contrast with a drift speed of 12 degrees/s, starting at a spatial frequency of 0.042 cycles/degree for acuity. Contrast sensitivity tests were done at a spatial frequency of 0.064 cycles/degree with a drift speed of 12 degrees/s, starting at 100% contrast. Contrast sensitivity is expressed as an inverse percentage to facilitate data interpretation.

Electroretinograms

A Celeris System (Diagnosys, Lowell, MA) was used to assess electroretinograms (ERGs). The mice received 0.5% tropicamide to stimulate eye dilation and 0.5% proparacaine to numb the eyes. After dark adaptation overnight, scotopic responses were recorded for 0.01, 0.1, 1, 10, and 32 $\text{candela}\cdot\text{s}/\text{m}^2$ intensity stimuli. The a- and b-wave amplitudes and implicit times were calculated by the software, and the oscillatory potentials were selected and summed. These measurements were done in a masked fashion.

DNA Fragmentation by TUNEL Assay

DNA fragmentation as a measure of cell death was detected by TUNEL assay using the Click-iT TUNEL Alexa Fluor 647 Imaging Assay (Thermo Fisher) in retinal cross sections, according to the manufacturer's instructions. Images from six sections spaced to cover the total retinal thickness were obtained using a DM6000 fluorescence microscope (Leica Microsystems) in a masked fashion, and TUNEL⁺ cells were manually counted.

Statistical Analysis

Results are expressed as mean $\pm$ SEM. The two-tailed Student *t* test was performed to assess the statistical difference between two groups. One-way ANOVA was used to calculate the statistical difference between three or more groups using Prism 8.0 (GraphPad Software), with *P* < 0.05 considered statistically significant.

Data and Resource Availability

The data sets generated and/or analyzed during the current study are available from the corresponding author upon reasonable request.

RESULTS

Occludin S490 Phosphorylation Contributes to VEGF-Induced Retinal Permeability in Mice

To determine whether occludin phosphorylation on Ser490 is required for VEGF-induced permeability *in vivo*, we first compared the VEGF-induced occludin phosphorylation time course in C57Bl6J mice with the permeability time course. Intravitreal injection of VEGF induced a rapid increase in occludin S490 phosphorylation, as early as 15 min, peaking at 1 h and lasting up to 36 h after VEGF injection (Fig. 1A). Similarly, using retinal cross-sectional imaging of extravasation of RITC 70-kDa dextran, we observed that the intravitreal injection of VEGF also induced a rapid and transient increase in BRB permeability, peaking at 36 h postinjection and resolving after 60 h (Fig. 1B).

To determine the role of occludin S490 phosphorylation in VEGF-induced permeability *in vivo*, we generated mice conditionally expressing WT (WtOCC^{+/+}) or the S490A mutant occludin (S490AOCC^{+/+}) in vascular endothelial cells using the Tek promoter (Supplementary Fig. 1A). These mice expressed EGFP from a downstream ribosome entry site that was restricted to the vasculature in all three capillary layers of the retina (Supplementary Fig. 1B), and Tek-Cre⁺; S490AOCC^{+/+} had a fourfold increase in occludin compared with Tek-Cre⁺ controls in whole-retina lysates (Supplementary Fig. 1C). Expression of the occludin mutant did not alter retinal vessel architecture of the mature animal (Supplementary Fig. 1D and E). Vessel permeability to FITC-BSA and 70-kDa RITC-dextran was assessed by measuring the amount of extravasated tracer into the retina 36 h after VEGF injection. No difference was found in the VEGF permeability response driven by Cre expression alone under the Tek promoter (Supplementary Fig. 2A and B). Expression of WT occludin induced an apparent decrease in VEGF-induced permeability (Supplementary Fig. 2A and B), although no statistical difference was observed compared with Tek-Cre⁺ controls. However, the expression of S490A mutant occludin decreased VEGF-induced permeability compared with Tek-Cre⁺ controls (Fig. 1C and D). As shown in the representative whole-mounted retinas, the 70-kDa RITC-dextran demonstrated extensive leak from the retinal vessels after VEGF injection in the Tek-Cre⁺ mice, but this leak was visibly diminished in the retinas of Tek-Cre⁺; S490AOCC^{+/+} animals (Fig. 1E).

The effect of the S490A occludin mutation was further corroborated in nonterminal experiments using contrast-enhanced MRI (gadolinium as a tracer: 742 Da), which provides a quantitative measure of permeability in living animals. VEGF-induced permeability was measured at

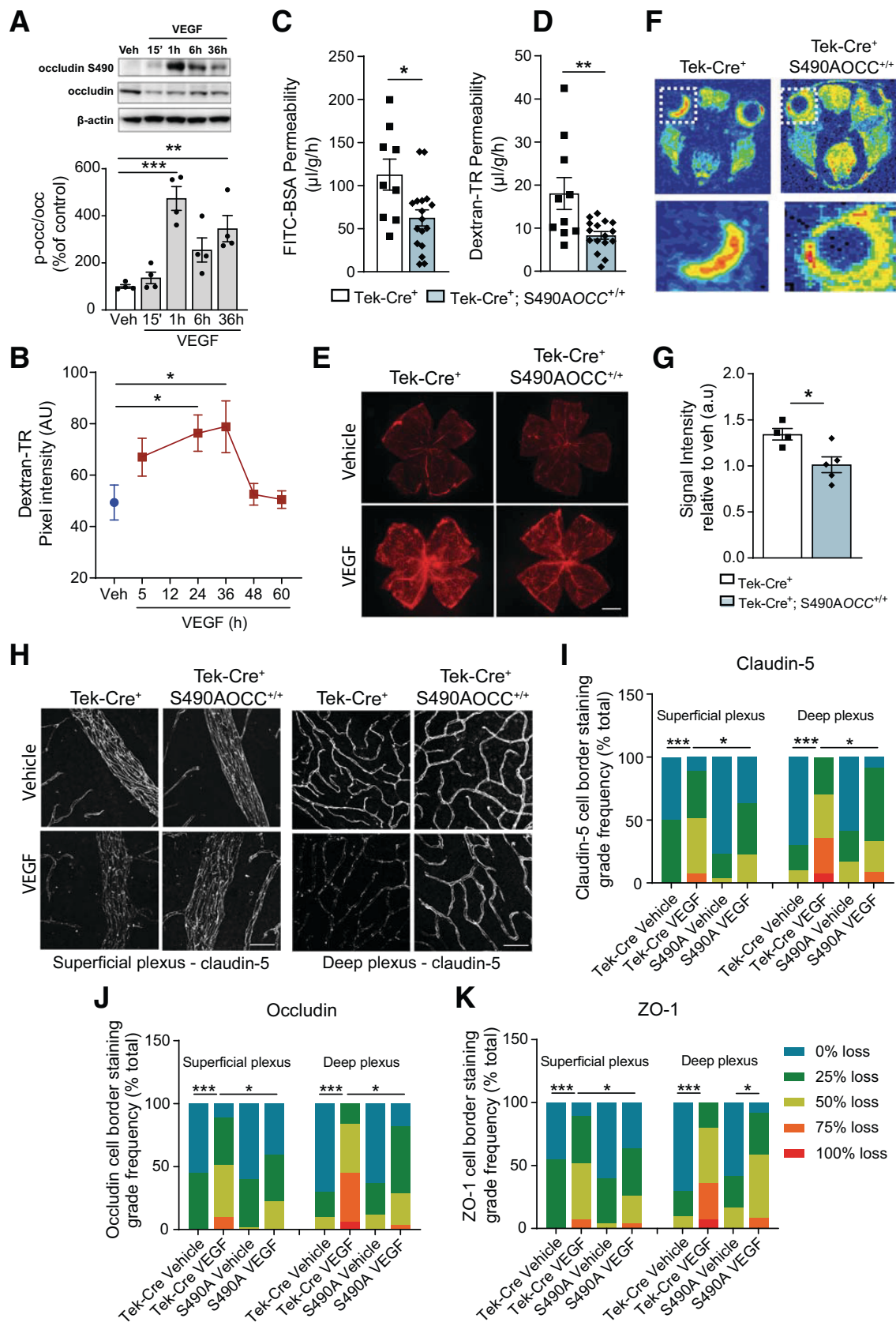


Figure 1—Occludin S490 phosphorylation contributes to VEGF-induced retinal permeability in mice. Mice were given an intravitreal injection of 200 ng VEGF in one eye and vehicle in the other. Retinas were collected at different times after VEGF injection to assess occludin

36 h in live animals. These studies confirmed that S490A occludin also protects against VEGF-induced BRB permeability to small molecules, as evidenced by the decreased accumulation of gadolinium within the eye compared with Tek-Cre⁺ control animals (Fig. 1F and G). Further, to confirm that the decrease in VEGF-induced permeability was not due to decreased VEGF signaling, we assessed extracellular signal-regulated kinase (ERK) 1/2 phosphorylation by Western blot as a measure of VEGFR2 downstream signaling. Intravitreal injection of VEGF promoted a robust increase in ERK1/2 phosphorylation in the retinas of both Tek-Cre and S490A-expressing mutant occludin animals (Supplementary Fig. 2C and D), indicating that VEGFR2 general activation was not hindered.

To investigate whether the S490A occludin mutant could prevent VEGF-induced tight junction disassembly in retinal vessels in vivo, we performed immunostaining for occludin, claudin-5, and zonula occludens 1 (ZO-1) tight junction proteins all known to have a critical role in maintaining barrier properties in retinal vessels. Border staining of these proteins was graded by masked observers by binning to categories based on the percentage of border staining (16). We chose 1 h and 36 h after the VEGF injection because these times corresponded with peak occludin phosphorylation and peak vascular hyperpermeability, respectively. After 1 h of VEGF, there were very minor alterations caused in tight junction organization (Supplementary Fig. 2E). However, at 36 h after the VEGF injection, all 3 proteins demonstrated reduced border staining (Fig. 1I–K and Supplementary Fig. 2F), consistent with occludin phosphorylation leading to subsequent junction endocytosis. As can be observed in Fig. 1H, VEGF induced a reduction of claudin-5 at the cell border in both the superficial and deep capillary plexus compared with the clear staining at the junctions of the cell borders in vehicle-injected eyes of Tek-Cre animals. Moreover, the effects of VEGF in tight junction organization at the cell border were more severe in the deep capillary layer than in the superficial layer, at least for this time point (Fig. 1H–K and Supplementary Fig. 2F). Strikingly, tight junction organization was significantly protected for all three proteins in both the superficial and deep capillary

plexus when the S490A occludin mutant was expressed (Fig. 1H–K and Supplementary Fig. 2F), demonstrating that the S490A occludin mutant maintains the tight junction strands intact after VEGF injection, consistent with preventing the increase in vascular permeability.

Given that overexpressing occludin alone was previously shown to reduce tumor necrosis factor-induced permeability in intestine (15) that and expression of the WtOCC yielded an apparent decrease in VEGF-induced permeability compared with controls, although not in a statistically significant manner (Supplementary Fig. 2A and B), we further explored the direct effect of inhibiting S490 phosphorylation. To directly compare the effect of the occludin point mutant to wild type occludin, we generated mice with floxed endogenous occludin, the tamoxifen-inducible PDGFb promoter driving Cre and either WT (PDGFbCre⁺; *Occ*^{fl/fl}; WtOCC^{+/+}) or S490A mutant occludin (PDGFbCre⁺; *Occ*^{fl/fl}; S490AOCC^{+/+}) to allow vascular endothelial-specific deletion of endogenous occludin and simultaneous expression of the transgene after induction by tamoxifen injection at postnatal day 3 (Supplementary Fig. 3A and B). Gene deletion of endogenous occludin made the effect of the S490A point mutant much more evident compared with WT occludin. Vascular permeability to FITC-BSA and 70-kDa dextran in response to the intravitreal injection of VEGF was reduced in PDGFbCre⁺; *Occ*^{fl/fl}; S490AOCC^{+/+} animals compared with PDGFbCre⁺; *Occ*^{fl/fl}; WtOCC^{+/+} (Fig. 2A and B). The expressing S490A occludin mutant also reduced VEGF-induced edema formation, as measured by retinal thickness using OCT, compared with WT mice expressing occludin (Fig. 2C and D). Knocking out endogenous occludin alone did not affect VEGF-induced permeability response to the solute tracers (Supplementary Fig. 3C and D). However, an increase in edema formation after VEGF injection was observed (Supplementary Fig. 3E).

Blocking Occludin S490 Phosphorylation in the Retinal Vasculature Prevents the Increase in BRB Permeability and Leukostasis Induced by Diabetes

Given that VEGF is a major driver of diabetes-induced retinal vascular permeability and that we previously ob-

S490 phosphorylation by Western blot (A), or BRB permeability to 70-kDa dextran in cross sections (B) (*n* = 6–8). AU, arbitrary units; TR, tetramethylrhodamine. Tek-Cre⁺ and Tek-Cre⁺; S490AOCC^{+/+} mice were given an intravitreal injection of 200 ng VEGF in one eye and vehicle in the other and retinal vascular permeability to FITC-BSA (C), and 70-kDa dextran (D) was determined after 36 h of VEGF intravitreal injection by extracting the extravasated dye into the tissue. Data were normalized to the vehicle injected eye of each mouse. E: Representative images of 70-kDa FITC-dextran fluorescence in whole-mounted retinas, showing vascular leakage sites induced by VEGF (*n* = 3). Scale bar: 1 mm. Vascular permeability to gadolinium (742 Da) was determined by dynamic contrast-enhanced MRI 36 h after VEGF (100 ng). F: Representative coronal images showing extravasated gadolinium in color scale and magnification of highlighted regions of VEGF-injected eyes. G: Signal was quantified and normalized to the vehicle injected eye of each mouse. a.u., arbitrary units. H: Representative images of claudin-5 staining in the superficial and deep capillary plexus in retina flat mounts, 36 h after VEGF injection (*n* = 3–4). Scale bar: 50 μ m. Masked scoring of claudin-5 (I), occludin (J), and ZO-1 (K) border staining, ranking in five categories of loss, of at least four pictures per retina. Data are represented as mean $\pm$ SEM. **P* < 0.05, ***P* < 0.01, ****P* < 0.001 by two-tailed Student *t* test (C, D, and G), one-way ANOVA, followed by the Dunnett (A and B) or the Sidak post hoc test (I–K). See also Supplementary Figs. 1 and 2.

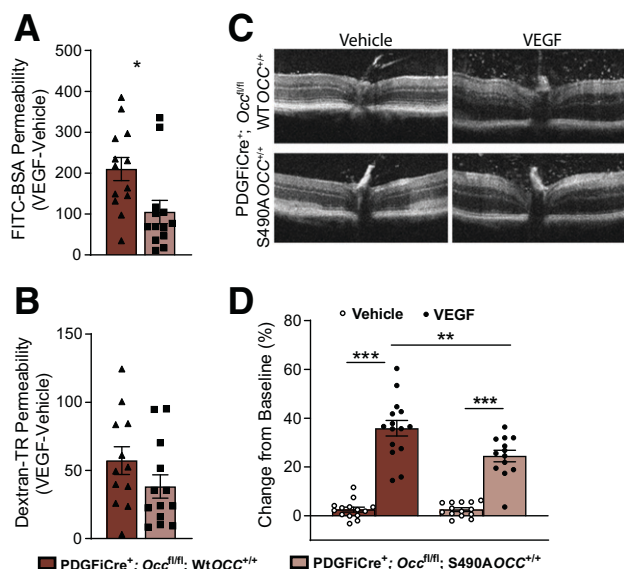


Figure 2—Blocking occludin S490 phosphorylation prevents VEGF-induced permeability and edema formation. PDGFiCre⁺; Occ^{fl/fl}; WtOCC^{+/+} and PDGFiCre⁺; Occ^{fl/fl}; S490AOCC^{+/+} mice were given an intravitreal injection of 200 ng VEGF in one eye and vehicle in the other, and after 36 h, retinal vascular permeability to FITC-BSA (A) and 70-kDa dextran (B) was determined by extracting the extravasated dye into the tissue. TR, tetramethylrhodamine. Data were normalized to the vehicle-injected eye of each mouse. VEGF-induced retinal edema formation was determined by measuring retinal thickness by OCT (C), and the percentage change from baseline was calculated (D). Data are represented as mean $\pm$ SEM. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ by two-tailed Student *t* test (A and B) or one-way ANOVA, followed by Sidak post hoc test (D). See also Supplementary Fig. 3.

served that VEGF and diabetes both promote loss of BRB integrity and reduced border junctional staining (20), we hypothesized that occludin S490 contributes an important role in diabetes-induced retinal vascular permeability. Therefore, we next tested whether occludin S490 phosphorylation is also required for diabetes-induced permeability in a mouse model of type 1 diabetes. Diabetes was induced by STZ injection in Tek-Cre⁺ and Tek-Cre⁺; S490AOCC^{+/+} mice. Conditional expression of the S490A occludin mutant had no impact on blood glucose levels or body weight of the mice (Supplementary Fig. 4A and B). Retinal vascular permeability to 70-kDa dextran was assessed at 4 months after diabetes induction by quantifying leaked FITC-dextran dye into the inner plexiform layer and outer nuclear layer of cross-sectioned retinas. Cross-section analysis and quantifying leaked dye in the retinal parenchyma without flushing dye from the vessels was found to better represent the changes in diabetes-induced permeability as opposed to the extraction methods used above, because flushing dye from vessels was often incomplete in diabetic animals, as observed by whole-mount microscopy. Diabetes induced a 3.3-fold increase the inner and a fourfold increase in the outer retina of FITC-dextran in control Tek-Cre⁺ animals. However,

vascular expression of S490A occludin mutant was able to completely prevent this diabetes-induced increase in permeability (Fig. 3A and B).

Inflammation with adhesion of leukocytes also has been reported to contribute to the pathophysiology of diabetic retinopathy (21,22). Upregulation of proinflammatory cytokines and chemokines attracts leukocytes to the vasculature and contributes to an activation of microglia resulting in vascular and neuronal damage (23). To investigate whether the expressing S490A occludin mutant reduced leukocyte number, whole retinas were stained for CD45, a leukocytes marker, and Iba1, a microglia/macrophage marker. The number of CD45⁺ and Iba1⁺ cells were counted throughout retinal whole mounts. At 4 months after diabetes induction, there was a 1.5-fold increase in the number of CD45⁺ cells on the vessel wall or perivascular space (Fig. 3D) and a 1.3-fold increase in Iba1⁺ cells in the retinal parenchyma (Fig. 3E) of Tek-Cre diabetic animals compared with nondiabetic controls. Cross-section staining revealed these changes in both the superficial (Fig. 3C) and deep capillary layers (Supplementary Fig. 5). However, in animals expressing S490A occludin, the diabetes-induced increase in both CD45⁺ and Iba1⁺ cells was completely prevented (Fig. 3B).

Vascular Expression of S490A Occludin Mutant Did Not Affect Retinal Thinning and Cell Death Induced by Diabetes

As diabetic retinopathy progresses, many cell types in the retina become affected, and neuronal changes as well as vascular alterations are now an established feature of the pathology (1,24). We next determined whether protection of the BRB prevented neuronal cell loss during diabetes. Measures of retinal thickness over time in the STZ model of C57Bl6J mice, Ins2 Akita, and *db/db* mouse model reveal retinal thinning in diabetes (25) as a marker of retinal degeneration. We performed retinal thickness measures by OCT after 2 and 4 months of diabetes induction. At 4 months after diabetes induction, retinal thinning of the inner, outer, and total retinal thickness was observed in Tek-Cre⁺ animals, and expression of the S490A point mutant of occludin failed to prevent this thinning (Fig. 4A–D). To further address cell loss, we measured cell death by performing a TUNEL assay in retinal cross sections after 4 months of diabetes induction. Images from six sections, spaced to cover the total retinal thickness, were obtained to quantify TUNEL⁺ cells. Diabetes increased cell apoptosis in Tek-Cre⁺ control animals as previously reported (2), and Tek-Cre⁺; S490AOCC^{+/+} animals also showed a similar increase in apoptosis, with most of the apoptotic cells observed in the outer nuclear layer (Fig. 4E and F). Diabetes did not decrease ganglion cell density, as measured by the number of positive RBPMs cells in retinal cross sections (Supplementary Fig. 6A and B).

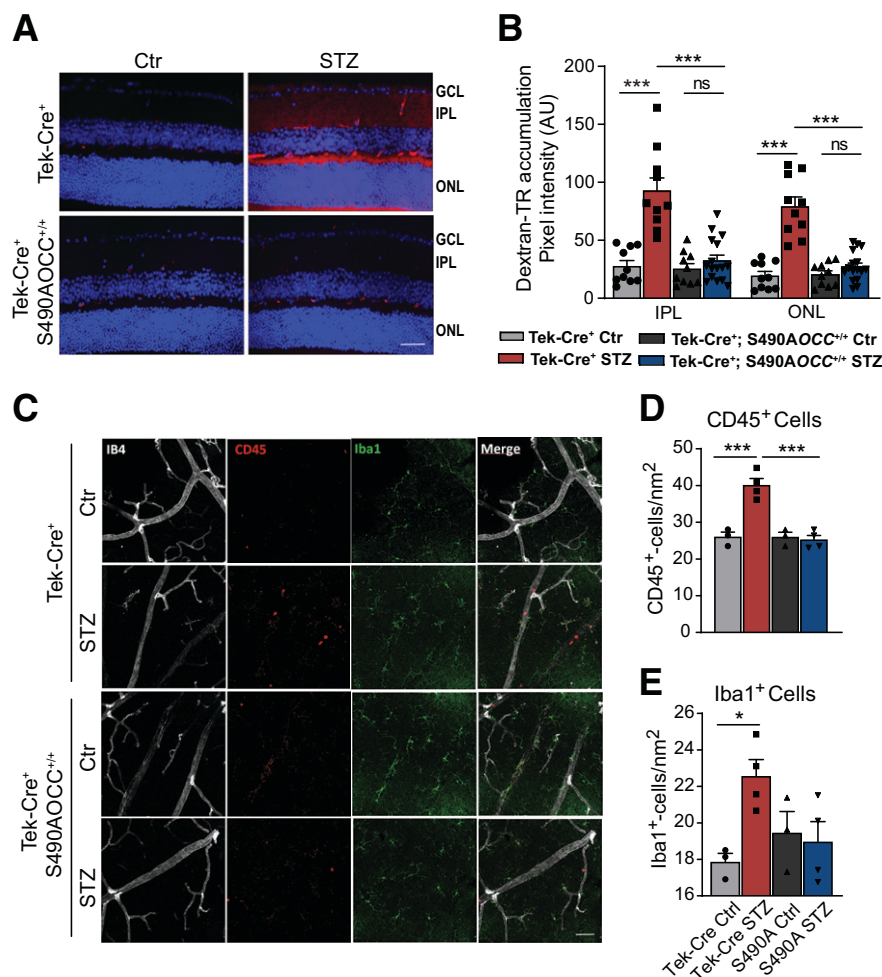


Figure 3—Blocking occludin phosphorylation at S490 prevents diabetes-induced BRB permeability and leukostasis. Diabetes was induced by STZ injection in Tek-Cre⁺ and Tek-Cre⁺; S490AOC^{+/+} mice. **A**: After 4 months, retinal vascular permeability to 70-kDa dextran (red) was determined in retinal cross sections counterstained with Hoechst (blue) for nuclei visualization. Scale bar: 50 μ m. Ctrl, control; GCL, ganglion cell layer; IPL, inner plexiform layer; ONL, outer nuclear layer. **B**: Quantification of the intensity of extravasated dye to the inner plexiform and outer nuclear layers. AU, arbitrary units; TR, tetramethylrhodamine. **C**: Representative confocal images from the superficial capillary plexus of retinas stained with vessel marker isolectin B4 (IB4) (gray), leukocyte marker CD45 (red), and microglia/macrophage marker Iba1 (green). Scale bar: 50 μ m. The number of CD45⁺ (**D**) and Iba1⁺ (**E**) cells were counted in the whole retina from stitched images. Ctrl, control. Data are represented as mean $\pm$ SEM. * P < 0.05, *** P < 0.001 by one-way ANOVA, followed by the Sidak post hoc test. See also Supplementary Figs. 4 and 5.

Blocking Occludin S490 Phosphorylation in the Retinal Vasculature Prevents the Decrease in Visual Acuity and Contrast Sensitivity Induced by Diabetes

To determine whether maintenance of the BRB protects visual function in diabetes, we assessed the visual acuity and contrast sensitivity of the mice by tracking the optokinetic response in a virtual cylinder. Diabetes led to a significant decrease of 17% in visual acuity and 45% in contrast sensitivity at 4 months (Fig. 5A and B), similar to previously reported diabetes changes (26,27). Strikingly, both defects observed at 4 months after diabetes induction were completely ameliorated by expressing S490A mutant of occludin specifically in the vascular endothelium (Fig. 5A and B).

To further assess neuronal function, scotopic ERGs (Supplementary Fig. 6C) were recorded at increasing light intensities and a- and b-wave amplitude, and implicit times were determined. Oscillatory potentials were isolated and the amplitude of the four peaks summed. Four months of diabetes did not induce a significant alteration in the a- or b-wave amplitude, a-wave implicit time, or oscillatory potentials summed amplitudes across all light intensities in both Cre control and S490A animals (Fig. 5C, D, F, and G). However, in the Tek-Cre animals, diabetes led to a significant decrease in the b-wave implicit time, a faster response that was normalized in S490AOC animals (Fig. 5E). These results suggest that the expressing S490A occludin mutant prevents alterations in inner retina function at 4 months after diabetes induction.

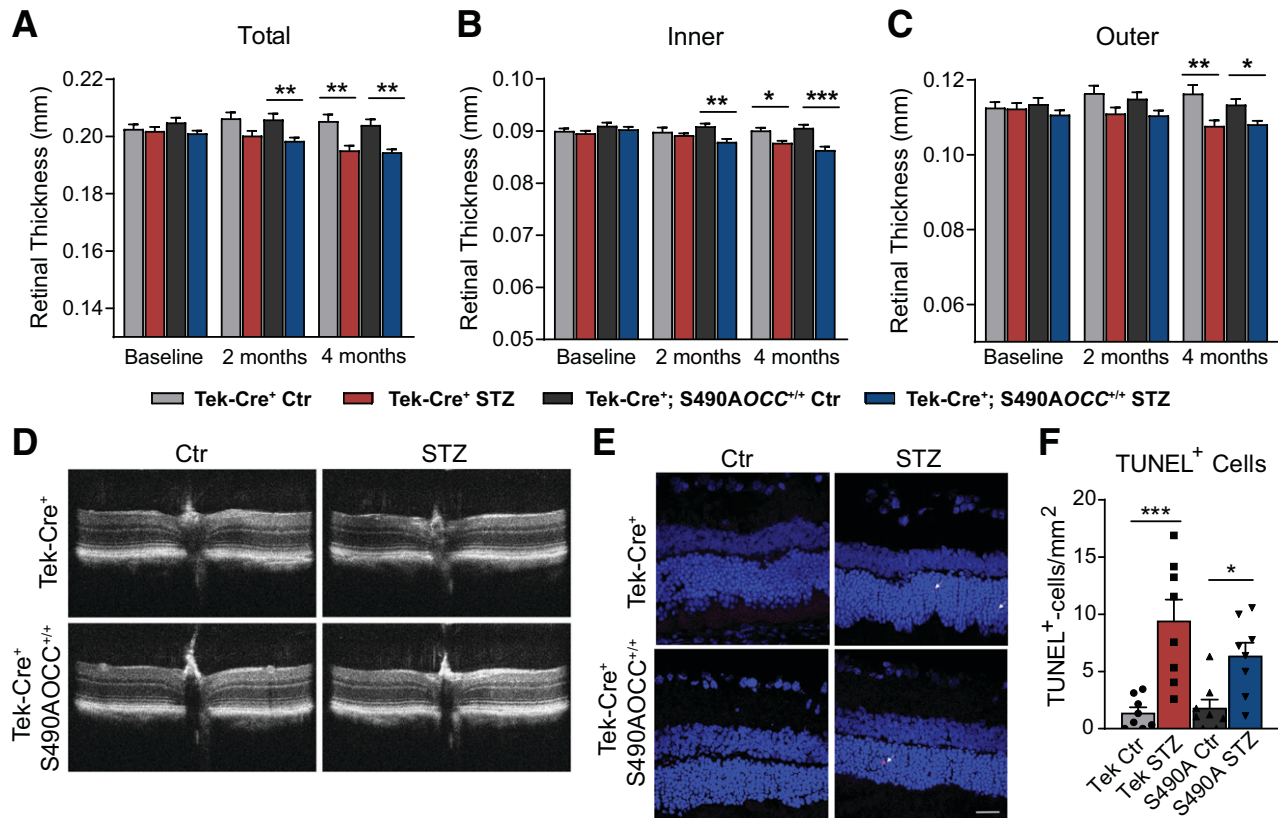


Figure 4—Blocking occludin S490A phosphorylation does not prevent neuronal cell death induced by diabetes. In vivo OCT was used to measure total (A), inner (inner nuclear layer through nerve fiber layer) (B), and outer (photoreceptor layer through outer plexiform layer) (C) retinal thickness at 2 and 4 months after diabetes induction ($n = 14$ –24). D: Representative OCT from Tek-Cre⁺ and Tek-Cre⁺; S490AOCCT^{+/+} mice after 4 months of diabetes induction. E: Representative images showing TUNEL⁺ cells (purple, arrows) and nuclear counterstaining with Hoechst (blue) in retinal sections. Scale bar: 50 μ m. F: Retinal cell death was determined by the number of TUNEL⁺ cells per retinal mm² after 4 months of diabetes induction. Ctr, control GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer. Data are represented as mean $\pm$ SEM. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ by one-way ANOVA, followed by the Sidak post hoc test. See also Supplementary Fig. 6.

DISCUSSION

The current study demonstrates that phosphorylation of occludin at S490 regulates both VEGF and diabetes-induced retinal vascular permeability. Further, by protecting the BRB by expressing the point mutant of occludin in vascular endothelium, visual acuity and contrast sensitivity were preserved in a diabetes model. Previous research found that phosphorylation of the tight junction protein occludin at S490 is a required step in VEGF-induced permeability in endothelial cell culture (12,13). Specifically, PKC β directly phosphorylates occludin S490 in a VEGF-dependent manner leading to subsequent ubiquitination by the ligase Itch. The ubiquitinated occludin is endocytosed through interaction with multiple chaperones and leads to tight junction disassembly with internalization of additional tight junction proteins such as claudin-5 and ZO-1.

In this work, expression of the occludin S490A mutant targeted to the vascular endothelium in mice reduced or prevented the increase in permeability of the BRB in response to VEGF or diabetes, demonstrating that

stabilizing occludin at the junctional complex prevents junction disassembly and consequent increase in VEGF-induced paracellular barrier permeability in vivo. However, the occludin mutant blocked half of the VEGF-induced permeability, and failure to block transcellular transport in the BRB might account for the remaining observable permeability. Previous studies have shown VEGF may also act through plasmalemma vesicle-associated protein (PLVAP) to increase transcellular transport (28). To our knowledge, this is the first example of a tight junction protein point mutant controlling permeability in vivo.

The current study corroborates and extends previous work revealing the role of occludin in regulating barrier properties. Specifically, we demonstrate that occludin phosphorylation at S490 regulates barrier properties downstream of VEGF in vivo. Similarly, other phosphorylation sites have been described to contribute a role in barrier regulation and cell signaling (29,30); for example, c-Src phosphorylation of occludin at Y398 and Y402 triggers its dissociation from ZO-1, leading to tight junction destabilization in epithelial cells (31), whereas Ser408

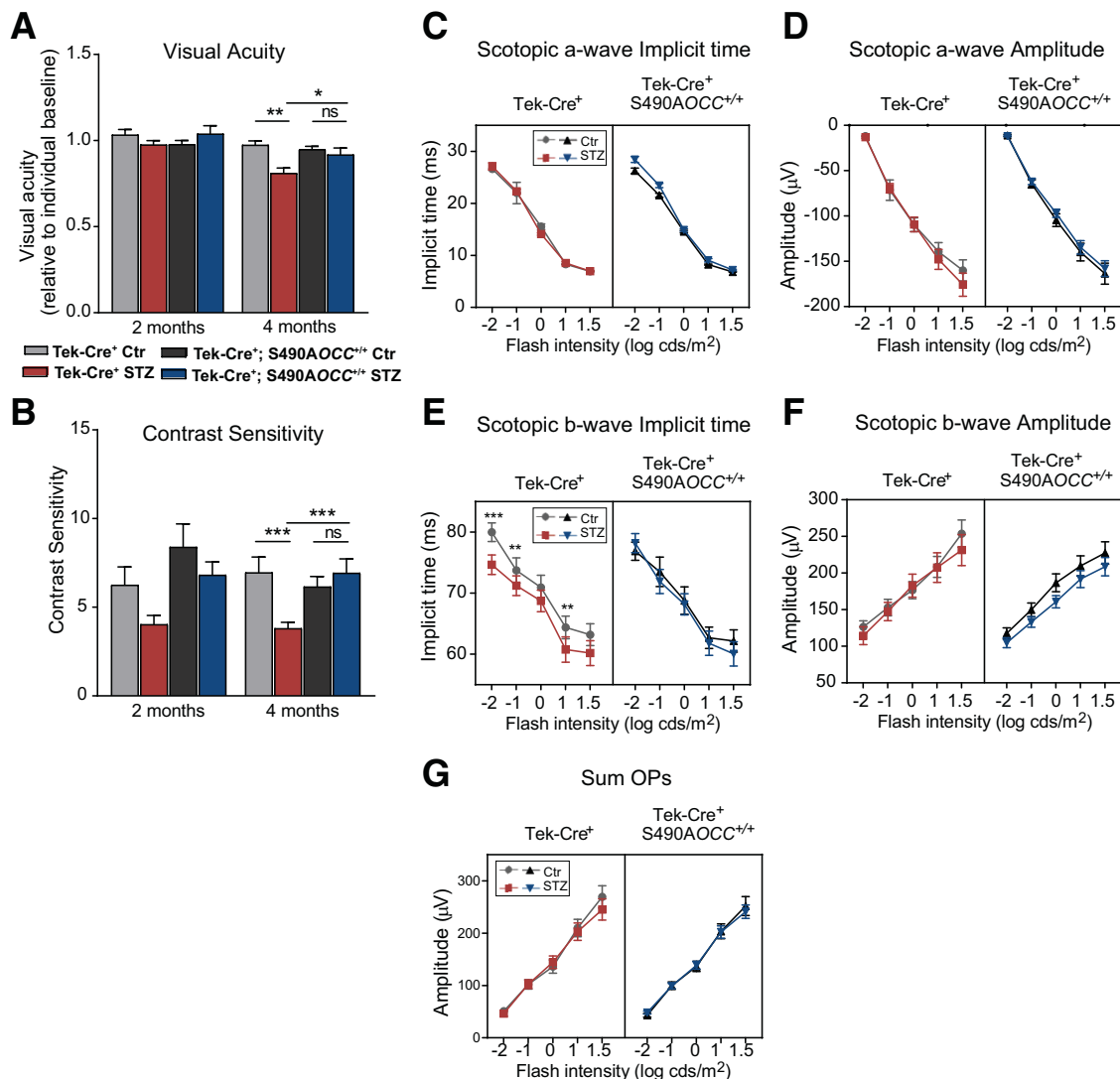


Figure 5—Expressing S490A occludin mutant prevents diabetes-induced decrease in visual function. Diabetes was induced by STZ injection in Tek-Cre⁺ and Tek-Cre⁺; S490AOCC^{+/+} mice. Visual function was assessed with an OptoMotry by measuring the optokinetic response after 2 and 4 months of diabetes induction. **A**: Visual acuity was measured as the spatial frequency thresholds of the grating at 100% contrast until animals no longer tracked ($n = 13$ –22). Ctr, control. **B**: Contrast sensitivity was determined as the minimum contrast that generates tracking ($n = 18$ –36). At 4 months after diabetes induction, scotopic ERG responses were recorded at increasing stimulus intensities. Implicit time and amplitude of a-wave (**C** and **D**) and b-wave (**E** and **F**) were calculated by the software. **G**: Oscillatory potentials were isolated and summed ($n = 22$ –50). cds, candelas. Data are represented as mean $\pm$ SEM. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ by one-way ANOVA, followed by the Sidak post hoc test. See also Supplementary Fig. 6.

phosphorylation controls paracellular pore formation in intestinal epithelia (32). Interestingly, occludin knockout mice revealed that occludin is not required for tight junction assembly (33). Likewise, we did not observe any basal retinal vascular permeability effect in PDGFiCre⁺; *Occl*^{fl/fl} mice. Lack of endogenous occludin did increase the effect of VEGF-induced retinal thickness, a measure of edema formation; however, no effect was observed in VEGF-induced solute flux in the animals with occludin gene deletion. This may be due to compensation of other MARVEL protein, such as MARVELD2 (tricellulin) in the occludin knockout, which has been shown to localize at bicellular junctions in the absence or

downregulation of occludin, both in vitro and in vivo (34,35). In contrast to deletion of occludin, expression of the S490A mutant reduced VEGF-induced permeability by half, demonstrating a dominant negative effect of the mutant. Further, expression of transgenic WtOCC or S490AOCC in the context of endogenous occludin deletion allowed direct quantification of the contribution of S490 phosphorylation on VEGF-induced permeability. These experiments demonstrated that S490 phosphorylation specifically contributes to the regulation of VEGF-induced permeability.

It was notable that the expression of S490A occludin had a partial reduction of permeability after a bolus

of VEGF but completely blocked diabetes-induced permeability. Importantly, the S490A mutant prevented an increase in inflammation as measured by CD45 leukocytes and Iba1 cell count. Leukostasis is known to block capillaries and lead to ischemia and infiltration of macrophages, and expansion of microglia are known to further aggravate BRB breakdown (23). Maintaining barrier integrity might prevent this expansion of inflammation and release of inflammatory factors. Specifically, the greater effectiveness of the S490A occludin point mutant in diabetes may be related to preventing regions of permeability and vascular dysfunction from expanding due to ischemia and relative hypoxia with associated inflammation. Blocking this process early may be protective of advancing barrier loss and associated inflammation and ultimately preserve visual function. Further investigation of the mechanism by which occludin S490A phosphorylation impacts leukostasis and potentially diapedesis would be of great interest.

The effect of anti-VEGF therapy on improving visual outcomes in patients with diabetes has been rigorously demonstrated by multicenter clinical trials (36–38). In addition, a phase III clinical trial showed blocking the downstream effector PKC β was effective in preventing vision loss and inhibiting macular edema in patients with DR (39,40). Our previous research demonstrated S490 of occludin as a target of PKC β (12), and the current study revealed that expressing a permeability-resistant S490A occludin mutant in the vascular endothelium was able to prevent the loss of visual acuity and contrast sensitivity in a model of type 1 diabetes. These results suggest that loss of the BRB contributes to visual dysfunction in DR beyond cystoid edema formation and that early protection of the vasculature can preserve vision. The data strongly implicate BRB dysfunction as a cause of visual deficit in the STZ mouse model of DR.

Recent studies demonstrate the required role of the blood-brain barrier in maintenance of brain function, with conditional knockout in adult mice of claudin-5 leading to impairments in learning and memory and to anxiety-like behavior, with eventual seizures and death by 3–4 weeks (41). In addition, suppression of claudin-5 levels at the inner retina can induce retinopathy in both mouse and nonhuman primate models (18). Here the S490A occludin mutant preserved claudin-5 at the cell border maintaining retinal barrier properties.

Importantly, in the current study, expression of the occludin point mutant did not alter neural apoptosis or retinal thinning at the 4-month time point. The observed retinal neural cell loss may affect other components of vision not studied or may impact vision after more extensive cell loss. Nevertheless, at the 4-month time point, loss of vision was clearly associated with altered barrier properties because preservation of the vascular barrier maintained vision.

ERG studies revealed diabetes-induced changes in inner retinal signaling that were preserved by maintaining the BRB. At this time point, diabetes had no detectable effect on a- or b-wave amplitude. That no change was observed in scotopic ERG a- and b-wave amplitudes despite retinal thinning in diabetes was not surprising, because targeted genetic studies reveal similar levels of apoptosis and inner retinal thinning without change in the scotopic ERG (42). Multifocal ERG studies in humans have shown that diabetes induces focal changes, which often coincide with vascular lesions (43,44) that full-field ERG may fail to detect. However, diabetes decreased the b-wave implicit time that was reversed by expressing the occludin mutant, suggesting altered inner retinal signaling in diabetes that is preserved by maintaining BRB function.

Determining how loss of the barrier is causative for loss of vision warrants future studies on specific retinal neural cell-regulated electrochemical signaling. In the brain, barrier maintenance has been linked with proper neuronal activity in the context of epilepsy. Blood-brain barrier dysfunction has been shown to be temporally and anatomically associated with epileptic seizures in patients (45), and disruption of endothelial tight junctions directly results in a hypersynchronous epileptiform activity in rodent models (46,47). Notably, diabetes decreased the b-wave implicit time that was corrected in the S490A-expressing animals, suggesting that the electrical microenvironment in the inner nuclear layer of the retina is altered by loss of the barrier, potentially decreasing the threshold signaling of ON-bipolar and Müller cells. This altered signaling may impact the delicate balance required for the visual response and lead to the observed reduced visual acuity and contrast sensitivity, similar to kainate-induced blood-brain barrier permeability that has been proposed to alter neural signaling through altered electrical environment (48). However, changes in neural signaling, including altered glutamate or other neurotransmitters, may also contribute to the altered acuity and contrast sensitivity in diabetes that is corrected by barrier maintenance.

In summary, the findings of this study highlight the role of BRB maintenance in the preservation of visual function in diabetes and mechanistically reveal occludin S490 phosphorylation as an important regulator of BRB permeability in response to VEGF and diabetes. Preservation of barrier properties by expressing a point mutant of the tight junction protein occludin in the vascular endothelium was shown to maintain vision in a model of diabetic retinopathy.

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References

- Simó R, Stitt AW, Gardner TW. Neurodegeneration in diabetic retinopathy: does it really matter? *Diabetologia* 2018;61:1902–1912
- Barber AJ, Lieth E, Khin SA, Antonetti DA, Buchanan AG, Gardner TW. Neural apoptosis in the retina during experimental and human diabetes. Early onset and effect of insulin. *J Clin Invest* 1998;102:783–791
- Abcouwer SF, Gardner TW. Diabetic retinopathy: loss of neuroretinal adaptation to the diabetic metabolic environment. *Ann N Y Acad Sci* 2014;1311:174–190
- Duh EJ, Sun JK, Stitt AW. Diabetic retinopathy: current understanding, mechanisms, and treatment strategies. *JCI Insight* 2017;2:e93751
- Simó R, Sundstrom JM, Antonetti DA. Ocular Anti-VEGF therapy for diabetic retinopathy: the role of VEGF in the pathogenesis of diabetic retinopathy. *Diabetes Care* 2014;37:893–899
- Aiello LP, Avery RL, Arrigg PG, et al. Vascular endothelial growth factor in ocular fluid of patients with diabetic retinopathy and other retinal disorders. *N Engl J Med* 1994;331:1480–1487
- Titchenell PM, Antonetti DA. Using the past to inform the future: anti-VEGF therapy as a road map to develop novel therapies for diabetic retinopathy. *Diabetes* 2013;62:1808–1815
- Sun JK, Glassman AR, Beaulieu WT, et al.; Diabetic Retinopathy Clinical Research Network. Rationale and application of the protocol S anti-vascular endothelial growth factor algorithm for proliferative diabetic retinopathy. *Ophthalmology* 2019;126:87–95
- Writing Committee for the Diabetic Retinopathy Clinical Research Network; Gross JG, Glassman AR, Jampol LM, et al. Panretinal photocoagulation vs intravitreal ranibizumab for proliferative diabetic retinopathy: a randomized clinical trial. *JAMA* 2015;314:2137–2146
- Wells JA 3rd, Glassman AR, Jampol LM. Targeting the effect of VEGF in diabetic macular edema. *N Engl J Med* 2015;373:481–482
- Diabetic Retinopathy Clinical Research Network; Wells JA, Glassman AR, Ayala AR, et al. Aflibercept, bevacizumab, or ranibizumab for diabetic macular edema. *N Engl J Med* 2015;372:1193–1203
- Murakami T, Frey T, Lin C, Antonetti DA. Protein kinase c β phosphorylates occludin regulating tight junction trafficking in vascular endothelial growth factor-induced permeability in vivo. *Diabetes* 2012;61:1573–1583
- 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
- Claxton S, Kostourou V, Jadeja S, Chambon P, Hodivala-Dilke K, Fruttiger M. Efficient, inducible Cre-recombinase activation in vascular endothelium. *Genesis* 2008;46:74–80
- Marchiando AM, Shen L, Graham WV, et al. Caveolin-1-dependent occludin endocytosis is required for TNF-induced tight junction regulation in vivo. *J Cell Biol* 2010;189:111–126
- Muthusamy A, Lin CM, Shanmugam S, Lindner HM, Abcouwer SF, Antonetti DA. Ischemia-reperfusion injury induces occludin phosphorylation/ubiquitination and retinal vascular permeability in a VEGFR-2-dependent manner. *J Cereb Blood Flow Metab* 2014;34:522–531
- Barber AJ, Antonetti DA, Kern TS, et al. The Ins2Akita mouse as a model of early retinal complications in diabetes. *Invest Ophthalmol Vis Sci* 2005;46:2210–2218
- Hudson N, Celkova L, Hopkins A, et al. Dysregulated claudin-5 cycling in the inner retina causes retinal pigment epithelial cell atrophy. *JCI Insight* 2019;4:e130273
- Liu X, Dreffs A, Díaz-Coránguez M, et al. Occludin S490 phosphorylation regulates vascular endothelial growth factor-induced retinal neovascularization. *Am J Pathol* 2016;186:2486–2499
- Antonetti DA, Barber AJ, Hollinger LA, Wolpert EB, Gardner TW. Vascular endothelial growth factor induces 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
- Joussen AM, Murata T, Tsujikawa A, Kirchhof B, Bursell SE, Adamis AP. Leukocyte-mediated endothelial cell injury and death in the diabetic retina. *Am J Pathol* 2001;158:147–152
- Abcouwer SF. Angiogenic factors and cytokines in diabetic retinopathy. *J Clin Cell Immunol* 2013;Suppl. 1:1–12
- Altmann C, Schmidt MHH. The role of microglia in diabetic retinopathy: inflammation, microvasculature defects and neurodegeneration. *Int J Mol Sci* 2018;19:110
- Antonetti DA, Klein R, Gardner TW. Diabetic retinopathy. *N Engl J Med* 2012;366:1227–1239
- Lin CM, Sheskey S, Antonetti DA. Assessments of Retinal Structure and Visual Function in Mouse Diabetes Models. Acute and Chronic Complications. *Diabetes Jun* 2016;65(Suppl. 1):A157
- Akimov NP, Rentería RC. Spatial frequency threshold and contrast sensitivity of an optomotor behavior are impaired in the Ins2Akita mouse model of diabetes. *Behav Brain Res* 2012;226:601–605
- Miller WP, Yang C, Mihailescu ML, et al. Deletion of the Akt/mTORC1 repressor REDD1 prevents visual dysfunction in a rodent model of type 1 diabetes. *Diabetes* 2018;67:110–119
- Wisniewska-Kruk J, van der Wijk AE, van Veen HA, et al. Plasmalemma vesicle-associated protein has a key role in blood-retinal barrier loss. *Am J Pathol* 2016;186:1044–1054
- Cummins PM. Occludin: one protein, many forms. *Mol Cell Biol* 2012;32:242–250
- Sundstrom JM, Tash BR, Murakami T, et al. Identification and analysis of occludin phosphosites: a combined mass spectrometry and bioinformatics approach. *J Proteome Res* 2009;8:808–817
- Elias BC, Suzuki T, Seth A, et al. Phosphorylation of Tyr-398 and Tyr-402 in occludin prevents its interaction with ZO-1 and destabilizes its assembly at the tight junctions. *J Biol Chem* 2009;284:1559–1569
- Raleigh DR, Boe DM, Yu D, et al. Occludin S408 phosphorylation regulates tight junction protein interactions and barrier function. *J Cell Biol* 2011;193:565–582
- Saitou M, Furuse M, Sasaki H, et al. Complex phenotype of mice lacking occludin, a component of tight junction strands. *Mol Biol Cell* 2000;11:4131–4142
- Ikenouchi J, Sasaki H, Tsukita S, Furuse M, Tsukita S. Loss of occludin affects tricellular localization of tricellulin. *Mol Biol Cell* 2008;19:4687–4693

35. Kitajiri S, Katsuno T, Sasaki H, Ito J, Furuse M, Tsukita S. Deafness in occludin-deficient mice with dislocation of tricellulin and progressive apoptosis of the hair cells. *Biol Open* 2014;3:759–766
36. Nguyen QD, Brown DM, Marcus DM, et al.; RISE and RIDE Research Group. Ranibizumab for diabetic macular edema: results from 2 phase III randomized trials: RISE and RIDE. *Ophthalmology* 2012;119:789–801
37. Brown DM, Nguyen QD, Marcus DM, et al.; RIDE and RISE Research Group. Long-term outcomes of ranibizumab therapy for diabetic macular edema: the 36-month results from two phase III trials: RISE and RIDE. *Ophthalmology* 2013;120:2013–2022
38. Comyn O, Sivaprasad S, Peto T, et al. A randomized trial to assess functional and structural effects of ranibizumab versus laser in diabetic macular edema (the LUCIDATE study). *Am J Ophthalmol* 2014;157:960–970
39. PKC-DRS2 Group; Aiello LP, Davis MD, Girach A, et al. Effect of ruboxistaurin on visual loss in patients with diabetic retinopathy. *Ophthalmology* 2006;113:2221–2230
40. Aiello LP, Vignati L, Sheetz MJ, et al.; PKC-DRS and PKC-DRS2 Study Groups. Oral protein kinase c β inhibition using ruboxistaurin: efficacy, safety, and causes of vision loss among 813 patients (1,392 eyes) with diabetic retinopathy in the Protein Kinase C β Inhibitor-Diabetic Retinopathy Study and the Protein Kinase C β Inhibitor-Diabetic Retinopathy Study 2. *Retina* 2011;31:2084–2094
41. Greene C, Kealy J, Humphries MM, et al. Dose-dependent expression of claudin-5 is a modifying factor in schizophrenia. *Mol Psychiatry* 2018;23:2156–2166
42. Braunger BM, Pielmeier S, Demmer C, et al. TGF- β signaling protects retinal neurons from programmed cell death during the development of the mammalian eye. *J Neurosci* 2013;33:14246–14258
43. Han Y, Bearse MA Jr, Schneck ME, Barez S, Jacobsen CH, Adams AJ. Multifocal electroretinogram delays predict sites of subsequent diabetic retinopathy. *Invest Ophthalmol Vis Sci* 2004;45:948–954
44. Palmowski AM, Sutter EE, Bearse MA Jr, Fung W. Mapping of retinal function in diabetic retinopathy using the multifocal electroretinogram. *Invest Ophthalmol Vis Sci* 1997;38:2586–2596
45. Rüber T, David B, Lückters G, et al. Evidence for peri-ictal blood-brain barrier dysfunction in patients with epilepsy. *Brain* 2018;141:2952–2965
46. Seiffert E, Dreier JP, Ivens S, et al. Lasting blood-brain barrier disruption induces epileptic focus in the rat somatosensory cortex. *J Neurosci* 2004;24:7829–7836
47. Marchi N, Angelov L, Masaryk T, et al. Seizure-promoting effect of blood-brain barrier disruption. *Epilepsia* 2007;48:732–742
48. Fredriksson L, Stevenson TK, Su EJ, et al. Identification of a neurovascular signaling pathway regulating seizures in mice. *Ann Clin Transl Neurol* 2015;2:722–738