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Plasma Kallikrein Mediates Vascular Endothelial Growth Factor–Induced Retinal Dysfunction and Thickening

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PURPOSE. Plasma kallikrein is a serine protease and circulating component of inflammation, which exerts clinically significant effects on vasogenic edema. This study examines the role of plasma kallikrein in VEGF-induced retinal edema.

METHODS. Intravitreal injections of VEGF and saline vehicle were performed in plasma prekallikrein-deficient (KLKB1−/−) and wild-type (WT) mice, and in both rats and mice receiving a selective plasma kallikrein inhibitor, VA999272. Retinal vascular permeability (RVP) and retinal thickness were measured by Evans blue permeation and optical coherence tomography, respectively. The retinal kallikrein kinin system was examined by Western blotting and immunohistochemistry. Retinal neovascularization was investigated in KLKB1−/− and WT mice subjected to oxygen-induced retinopathy.

RESULTS. Vascular endothelial growth factor–induced RVP and retinal thickening were reduced in KLKB1−/− mice by 68% and 47%, respectively, compared to VEGF responses in WT mice. Plasma kallikrein also contributes to TNFα-induced retinal thickening, which was reduced by 52% in KLKB1−/− mice. Systemic administration of VA999272 reduced VEGF-induced retinal thickening by 57% (P < 0.001) in mice and 53% (P < 0.001) in rats, compared to vehicle-treated controls. Intravitreal injection of VEGF in WT mice increased plasma prekallikrein in the retina, which was diffusely distributed throughout the inner and outer retinal layers. Avascular and neovascular areas induced by oxygen-induced retinopathy were similar in WT and KLKB1−/− mice.

CONCLUSIONS. Vascular endothelial growth factor increases extravasation of plasma kallikrein into the retina, and plasma kallikrein is required for the full effects of VEGF on RVP and retinal thickening in rodents. Systemic plasma kallikrein inhibition may provide a therapeutic opportunity to treat VEGF-induced retina edema.

Keywords: VEGF, plasma kallikrein, retinal edema, SD-OCT

Vascular endothelial growth factor (VEGF) contributes to macular thickening and vision loss associated with diabetic macular edema (DME) and retinal vein occlusion (RVO). Intravitreal injections of VEGF inhibitors are often highly effective in reducing macular thickness and improving visual acuity for these conditions. However, while VEGF blockade has emerged as a primary therapy for these diseases, this pharmacologic approach has a number of limitations. Multiple intravitreal injections of anti-VEGF agents are often required to reach and maintain the full pharmacodynamic response, while a substantial number of patients are refractory or have incomplete response. Vascular endothelial growth factor blockade is likely to inhibit both the pathological as well as the potential beneficial effects of VEGF in the eye. In addition, the use of anti-VEGF therapies for the treatment of macular edema is currently limited to an intraocular route of administration due, in part, to complications associated with systemic VEGF blockade.

Inhibition of the downstream factors that mediate the edematous effects of VEGF rather than blockade of VEGF itself, may provide additional therapeutic opportunities for the treatment of macular edema. Vascular endothelial growth factor–induced macular edema has been primarily attributed to increases in retinal vascular permeability (RVP), which facilitates the influx of plasma components across the blood-retinal barrier. Since vascular hyperpermeability is a hallmark of inflammation and the kallikrein–kinin system (KKS) is an abundant circulating component of innate inflammation, we examined the role of plasma kallikrein (PKal) in VEGF-induced retinal edema.

The KKS is activated in response to vascular injury and is a clinically significant mediator of vasogenic edema caused by hereditary angioedema. This system is composed of the zymogen plasma prekallikrein (PPK) that is converted to the catalytically active serine protease PKal by factor XIIa (FXIIa). Plasma kallikrein provides positive feedback by zymogen activation of FXII to FXIIa and also cleaves high molecular...
weight kininogen (HK) to liberate the nonapeptide hormone bradykinin. This process leads to local increases of bradykinin that elicit proinflammatory effects including vasodilation, vascular permeability, and immune cell activation, as well as direct effects on neurons and glial cells.12,13 Diabetes and hypertension increase levels of KKS components in retina, and inhibition of PPK reduces retinal vascular hyperpermeability in experimental rodent models.14,15 Recently, we have shown that vitreous from DME patients with high concentrations of VEGF also have elevated levels of KKS components.16 Since VEGF-induced macular edema has been attributed, in large part, to plasma protein and fluid extravasation, we examined the role of PPK in VEGF-induced RVP and retinal thickening. In addition, we investigated the potential role of PPK in the angiogenic response to oxygen-induced retinopathy.

**METHODS**

**Animals**

We have previously generated and characterized the line of plasma prekallikrein gene-deficient mice (KLKB1−/−) that was used in this study.16,17 These mice were backcrossed with C57Bl/6 mice (Jackson Laboratories, Bar Harbor, ME, USA) for nine generations, and C57Bl/6 from Jackson Laboratories were used as wild-type (WT) controls. Blood pressure and heart rate were measured on conscious mice using tail cuff plethysmography (Visitec Systems, Inc., Apex, NC, USA). Male Sprague-Dawley (SD) rats at 10 weeks of age were obtained from Charles River Laboratories (Wilmington, MA, USA). Anesthesia involved intramuscular injection of ketamine (80 mg/kg; Charles River Laboratories (Wilmington, MA, USA)). Experiments were performed in male Sprague-Dawley (SD) rats at 10 weeks of age were obtained from Charles River Laboratories (Wilmington, MA, USA). Anesthesia involved intramuscular injection of ketamine (80 mg/kg; VEDCO, St. Joseph, MO, USA) and xylazine (10 mg/kg; Sigma-Aldrich, Milwaukee, WI, USA). Experiments were performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and with approval from the Institutional Animal Care and Use Committee of the Joslin Diabetes Center.

**Intravitreal Injections and PKal Inhibitor Administration**

Intravitreal injections were performed as previously described.14,16 Eyes were dilated with 1% tropicamide and single infusions were made using a 10-μl Hamilton syringe with a 31-gauge needle. Mice and rats received 1 and 5 μL of the described reagents, respectively: phosphate-buffered saline (PBS); VEGF (10 or 100 ng/eye; no. PHC9394; Life Technologies, Carlsbad, CA, USA); tumor necrosis factor-α (TNFα) (100 ng/eye; no. 210- TA; R&D Systems, Inc., Minneapolis, MN, USA); and human plasma kallikrein (100 ng/eye; no. HPK1303; Enzyme Research Labs, South Bend, IN, USA). For the interventional studies, animals received systemic administration of the PKal inhibitor (R)-2-amino-N-(S)-1-(carbamimidoyl-piperidin-4-ylcarbamoyl)-2-phenyl-ethyl-3-(4-ethoxy-phenoxy)propionamide ditrifluoroacetate (VA999272)18 at rates of 0.58 mg/kg/d in rats and 1.60 mg/kg/d in mice or corresponding volumes of vehicle (10% polyethylene glycol (PEG)400 in PBS). VA999272 was continuously administered using subcutaneous mini-osmotic pumps (1000SD and 100TD; Alzet, Cupertino, CA, USA) initiated 1 and 2 days prior to intravitreal injection in rats and mice, respectively.

**Retinal Vascular Permeability Measurements**

Evans blue dye permeation was used to quantify RVP as described previously.14,16 Briefly, under anesthesia, Evans blue dye (Sigma-Aldrich) was infused through a jugular vein catheter for 10 seconds at a dose of 180 mg/kg. Following circulation of the dye for 1 hour, mice were perfused with saline followed by neutral-buffered 10% formalin and perfused eyes were enucleated. The retinas were extracted with dimethyl formamide, and resultant supernatant was used to determine retinal Evans blue dye content.

**Optical Coherence Tomography**

Spectral-domain optical coherence tomography (SD-OCT) (840SDOCT System; Bioptigen, Durham, NC, USA) was performed as described previously.14 Briefly, rectangular volumes of retina were obtained that consisted of 1000 A-scans by 100 B-scans over a 1.5-× 1.5-mm area for mice (2.5 × 2.5 mm for rats) centered upon the optic nerve head (ONH). Retinal thickness was measured at 600 μm (800 μm for rats) relative to the ONH center. Retinal thickness was measured using calipers from the retinal pigment epithelium (RPE) to the retinal nerve fiber layer (RNFL). Caliper measurements were taken at each site, corresponding to the temporal, nasal, superior, and inferior quadrants of the retina. The measurements were averaged to produce a single thickness value for each retina. Retinal layers were assigned on OCT B-scans using Bioptigen Diver software version 2.4.

**Analyses of KKS Components in Retina**

Extracts were prepared from PBS-perfused retina in tissue protein extraction reagent (TPER, Thermo Scientific, Waltham, MA, USA) with protease inhibitor cocktail (no. P8340; Sigma-Aldrich) and analyzed by Western blotting using anti-KLK1, plasma kallikrein (no. AF2498, R&D) and anti-high molecular weight kininogen antibody (no. ab1004; Abcam, Cambridge, MA, USA). Results were visualized by enhanced chemiluminescence (20X LumiGLO; Cell Signaling, Danvers, MA, USA) and quantified using ImageJ software (National Institutes of Health, Bethesda, MD, USA). Antibody specificity was confirmed using plasma from WT, KLKB1−/−, and HK−/− (Supplementary Fig. S1). For immunohistochemistry, whole eyes were harvested and mounted in dry ice-cooled cryotmatrix compound (OCT, Thermo Scientific), and 12-μm transverse frozen tissue sections were prepared using a cryostat. Immunofluorescent staining of retinal sections was performed using anti-KLK1 (AF2498, R&D) and anti-CD31 (557355; BD Pharmingen, San Jose, CA, USA). Secondary antibodies anti-goat Alexa 594 and anti-rat Alexa 488 (Abcam) were used to immunofluorescently label PPK and CD31. DAPI (4',6-diamidino-2-phenylindole) was used for nuclear staining within the retinal layers. Images were captured using an Olympus IX53 fluorescence microscope system (Center Valley, PA, USA). Higher-magnification images (<×40) were taken on a confocal microscope (LSM70; Zeiss, Oberkochen, Germany).

**PKal and Trypsin-Like Protease Enzyme Activity Assays**

Plasma kallikrein was assayed for protease activity using the fluorogenic substrate H-D-Pro-Phe-Arg-AFC (AFC-059; Enzyme System Products, Santa Ana, CA, USA). Enzyme activity was measured by monitoring the accumulation of fluorescence liberated from the substrate over 5 minutes, and the linear rate of fluorescence increase per minute was calculated. Human trypsin-like protease enzymes plasmin, thrombin, trypsin, KLK1, factor XIa, and factor XIIa were assayed for enzymatic activity using an appropriate fluorogenic substrate. Protease activity was measured by monitoring the accumulation of AFC liberated (excitation 410 nm, emission 510 nm) from the

**Role of Plasma Kallikrein in VEGF-Induced Retinal Edema**


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substrate over 5 minutes. The linear rate of fluorescence increase per minute was expressed as percentage (%) activity. The Km for the cleavage of each substrate was determined by standard transformation of the Michaelis-Menten equation. The compound inhibitor assays were performed at substrate Km concentration, and activities were calculated as the concentration of inhibitor giving 50% inhibition (IC50) of the uninhibited enzyme activity (100%).

Oxygen-Induced Retinopathy (OIR)

Mouse pups were exposed to 75% O2 from postnatal day (p)7 to p12 in an OxyCycler system (BioSpherix, Inc., Lacona, NY, USA) and returned to room air. At p17, pups were killed and whole eyes were isolated. Pups with body weight less than 5 g were not included in the analysis. Eyes were incubated in 10% formalin for 1 hour and whole retinas were recovered. Retinal vascular tissues were stained by incubating whole retina overnight with isoelectric B4-Alexa Fluor 594 (Molecular Probes, Eugene, OR, USA). Whole retinas were flat mounted on microscope slides and imaged at ×4 using an Olympus fluorescence microscope. Areas of vascular obliteration and neovascularization were analyzed using Adobe Photoshop (San Jose, CA, USA).

Statistics

Statistical analysis was performed with SigmaPlot statistical software (Version 12.5; Jandel, San Raphael, CA, USA). Multiple group comparisons for statistical significance were performed by using Fisher least significant difference method of pairwise comparison for 1-way ANOVA. For unequal variance between groups, Kruskal-Wallis 1-way ANOVA on ranks using the Tukey comparison for 1-way ANOVA. For unequal variance between groups, Kruskal-Wallis 1-way ANOVA on ranks using the Tukey comparison for 1-way ANOVA. For unequal variance between groups, Kruskal-Wallis 1-way ANOVA on ranks using the Tukey comparison for 1-way ANOVA. For unequal variance between groups, Kruskal-Wallis 1-way ANOVA on ranks using the Tukey comparison for 1-way ANOVA. For unequal variance between groups, Kruskal-Wallis 1-way ANOVA on ranks using the Tukey comparison for 1-way ANOVA.

RESULTS

Effects of KLKB1 Deficiency on VEGF-Induced RVP and Retinal Thickening

Age-matched KLKB1+/− and WT mice displayed similar body weights and systemic blood pressure (Table 1). The effects of intravitreal injection of VEGF (100 ng/eye) on RVP in KLKB1+/− and WT mice were measured at 2 hours and 24 hours post injection (Fig. 1). At 2 hours, RVP was increased by 267% in eyes of WT mice receiving VEGF compared with eyes injected with PBS vehicle (35.4 ± 5.3 vs. 9.6 ± 1.2 μl/g/h, P < 0.001). Vascular endothelial growth factor increased RVP in KLKB1+/− mice by 229% (37.8 ± 3.8 vs. 11.5 ± 2.4 μl/g/h, P < 0.001), which was similar to the responses observed in WT mice. In a second set of mice, VEGF-induced RVP measured 24 hours after intravitreal injection was increased by 435% in WT mice compared to PBS-injected controls (70.6 ± 14 vs. 13 ± 2 μl/g/h, P < 0.001). Vascular endothelial growth factor increased RVP by 220% in KLKB1+/− mice (35.3 ± 6 vs. 15.2 ± 3 μl/g/h, P = 0.032) at 24 hours post injection, which was reduced by 68% (P = 0.011) compared to the VEGF response in WT mice.

Retinal thicknesses in WT and KLKB1+/− mice were measured by SD-OCT at baseline (preinjection) and at 24 hours post intravitreal injection with VEGF or PBS vehicle. Representative retinal SD-OCT B-scans with segmentation of the retinal layers illustrating retinal thickness after intravitreal PBS or VEGF injections in WT and KLKB1+/− mice are shown in Figure 2A. Measurements of total retinal thickness (RPE to RNFL) and retinal segments are shown in Supplementary Table S1. The total retinal thicknesses of WT and KLKB1+/− mice at baseline were 204.8 ± 0.6 and 205.5 ± 0.9 μm, respectively (NS). At 24 hours post intravitreal injection, VEGF increased retinal thickness by 35.6 ± 3.5 μm (17.4%) and 20.0 ± 2.3 μm (9.7%) (P < 0.001) in WT and KLKB1+/− mice, respectively, compared with baseline thicknesses (Fig. 2B). Intravitreal injection of PBS vehicle increased retinal thicknesses in WT mice by 6.6 ± 2.8 μm (3.2%) and KLKB1+/− mice by 4.7 ± 1.4 μm (2.3%) (Fig. 2B). Subtraction of the small injection/PBS effect on retinal thickness from the VEGF responses revealed 14.1% and 7.4% increases in VEGF-specific retinal thickening in WT and KLKB1+/− mice, respectively. Vascular endothelial growth factor injection increased retinal layer thickness compared with baseline in WT mice by 15.9 ± 3.5, 7.7 ± 2.2, and 13.9 ± 2.4 μm (P < 0.001) for inner plexiform layer (IPL)-RNFL, inner nuclear layer (INL), and outer nuclear layer (ONL) and in KLKB1+/− mice by 8.4 ± 2.0 and 6.2 ± 1.8 μm (P < 0.005) for IPL-RNFL and ONL, respectively (Fig. 2C). The VEGF induction of retinal layer thickness in KLKB1+/− mice was reduced by 47.2% and 44.9% (P < 0.001) for total (RPE-RNFL) and ONL and 49.5% and 58.3% (P < 0.05) for IPL-RNFL and INL. No significant differences were observed in the RPE-inner segment (IS) layer among groups.

### Table 1. Physiological Characteristics of WT and KLKB1+/− Mice

<table>
<thead>
<tr>
<th>Group</th>
<th>Age, d</th>
<th>BW, g</th>
<th>SBP, mm Hg</th>
<th>MBP, mm Hg</th>
<th>DBP, mm Hg</th>
<th>HR, bpm</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>10</td>
<td>114 ± 9</td>
<td>24.8 ± 0.8</td>
<td>108.7 ± 1.7</td>
<td>94.0 ± 1.5</td>
<td>86.5 ± 1.5</td>
</tr>
<tr>
<td>KLKB1−/−</td>
<td>17</td>
<td>104 ± 10</td>
<td>25.2 ± 0.8</td>
<td>106.6 ± 2.2</td>
<td>93.7 ± 3.0</td>
<td>86.8 ± 3.5</td>
</tr>
</tbody>
</table>

BW, body weight; SBP, systolic blood pressure; MBP, mean blood pressure; DBP, diastolic blood pressure; HR, heart rate; bpm, beats per minute.
Effects of Systemic PKal Inhibition on VEGF-Induced Retinal Thickening in Mice

The role of PKal in VEGF-induced thickening was examined in rodents receiving systemic administration of VA999272 (Fig. 3A), which is a soluble, low molecular weight (480.6 Da), and competitive reversible inhibitor of PKal. The VA999272 is a potent PKal inhibitor with IC$_{50}$ of 3.98$^{6}$ 3.61, 1.18$^{6}$ 0.25, and 6.54$^{6}$ 0.76 nM (mean $^{6}$ SD) for purified enzyme from human, mouse, and rat, respectively (Fig. 3B). The IC$_{50}$s of VA999272 for closely related trypsin-like serine proteases are 33.5$^{6}$ lM, KLK1 tissue kallikrein; $^{>}$10$^{6}$ lM, factor XIIa; $^{>}$10$^{6}$ lM, factor XIa; 9.1$^{6}$ lM, thrombin; 23.1$^{6}$ lM, plasmin; and 38.9$^{6}$ lM, trypsin; these demonstrate $^{>}$1000-fold selectivity to PKal (Table 2). The in vivo pharmacokinetics of VA999272 following subcutaneous dosing at 10 mg/kg to male SD rats are shown in Figure 3C. Analysis of VA999272 concentrations in the plasma showed mean Cmax at 974 ng/mL, mean Tmax at 90 minutes, and T1/2 at 213 minutes. These results show that VA999272 is a potent and selective PKal inhibitor, which displays sustained plasma exposure following subcutaneous administration.

Next we examined the effects of systemically administered VA999272 or vehicle alone on VEGF-induced retinal thickening. Retinal thickness was measured by SD-OCT at baseline (preinjection) and at both 24 and 48 hours post injection with VEGF (100 ng/eye) or PBS (Supplementary Table S2). Baseline retinal thicknesses were not significantly different among groups, and intravitreal injection of PBS did not affect retinal thickness (Fig. 3D). Vascular endothelial growth factor increased total retinal thickness in vehicle-treated mice by 25.6$^{6}$ 4.9 and 37.0$^{6}$ 5.5 $\mu$m at 24 and 48 hours, respectively, compared to baseline retinal thickness (206$^{6}$ 1 $\mu$m, $P$ < 0.001). In mice that received VA999272, VEGF increased total retinal thickness by 16.5$^{6}$ 3.8 $\mu$m at 24 hours and 16.1$^{6}$ 3.8 $\mu$m at 48 hours, compared to baseline. The effects of VEGF on retinal thickness were reduced by 36% ($P$ = 0.039) and 57% ($P$ < 0.001) at 24 and 48 hours, respectively, in mice receiving VA999272 compared to mice receiving vehicle.

Effects of VEGF on Levels of KKS Components in the Retina

Next we examined the effects of intravitreal injection of VEGF on PKal system components in the retina. Vascular endothelial growth factor increased total retinal thickness in vehicle-treated mice by 25.6 ± 4.9 and 37.0 ± 5.5 $\mu$m at 24 and 48 hours, respectively, compared to baseline retinal thickness (206 ± 1 $\mu$m, $P$ < 0.001). In mice that received VA999272, VEGF increased total retinal thickness by 16.5 ± 3.8 $\mu$m at 24 hours and 16.1 ± 3.8 $\mu$m at 48 hours, compared to baseline. The effects of VEGF on retinal thickness were reduced by 36% ($P$ = 0.039) and 57% ($P$ < 0.001) at 24 and 48 hours, respectively, in mice receiving VA999272 compared to mice receiving vehicle.
plexiform and nuclear layers. Immunohistochemical staining for PPK was also observed at relatively higher levels colocalized with CD31-staining vessels (Fig. 5D).

Effect of KLKB1 Deficiency on TNFα-Induced Retinal Thickening

To investigate the potential role of PPK in the effects of another mediator of retinal edema, we examined the effect of KLKB1 deficiency on TNFα-induced retinal thickening. WT mice were administered PKal inhibitor VA999272 (1.60 mg/kg/d) or vehicle alone (10% PEG400 in PBS) at 0.5 μL/h via a subcutaneously implanted osmotic pump for 2 days prior to intravitreal injections of VEGF (100 ng/eye) or PBS alone. Total retinal thickness (RPE to RNFL) was measured by SD-OCT at baseline (preinjection) and 24 and 48 hours post injections. *P < 0.05, **P < 0.01 indicate difference compared with corresponding PBS injection. *P < 0.05 and **P < 0.001 indicate comparison of VEGF responses in mice administered VA999272 (n = 11) and vehicle alone (n = 9).

| Table 2. Selectivity of VA999272 to Plasma Kallikrein |
|---------------------------|-------------|-------------|-------------|-------------|-------------|-------------|
|                          | PKal        | Thrombin    | Plasmin     | KLK1        | Trypsin     | Factor Xla  |
| Mean IC₅₀ (μM)            | 3.98 ± 3.61 | 9.1         | 23.1        | 33.5        | 38.9        | >10         |
| Fold selectivity to human | 1           | 2291        | 5821        | 8414        | 9772        | >2513       |

Half-maximal inhibitory concentration (IC₅₀) of VA999272 to plasma kallikrein and closely related serine proteases. Fold compound selectivity is shown in comparison to the IC₅₀ for human plasma kallikrein.

Effects of KLKB1 Deficiency on the Angiogenic Response to Oxygen-Induced Retinopathy

Vascular endothelial growth factor receptor signaling is critical for retinal neovascularization in mice with OIR. To determine whether PKal plays a role in angiogenesis in this model, we compared responses in WT and KLKB1−/− mice. Mouse pups were exposed to 75% O₂ from p7 to p12, returned to room air, and killed on p17. Isolectin B4 staining was used to visualize neovascular tufts in retinal whole mounts, and

FIGURE 3. PKal inhibitor VA999272 structure, in vitro pharmacology, and in vivo effect on VEGF-induced retinal thickening. (A) Compound structure of VA999272. (B) Inhibition curves of VA999272 against mouse, rat, and human PKal enzymatic activities. PKal were assayed for protease activity using a fluorogenic substrate in the presence of inhibitor. The mean PKal IC₅₀ for mouse, rat, and human is 1.18 ± 0.25, 6.54 ± 0.76, and 3.98 ± 3.61 nM, respectively. (C) Pharmacokinetic profile of VA999272 in rat. Plasma exposure following a single bolus subcutaneous injection of VA999272 (10 mg/kg). (D) Effect of VA999272 on VEGF-induced retinal thickening. WT mice were administered PKal inhibitor VA999272 (1.60 mg/kg/d) or vehicle alone (10% PEG400 in PBS) at 0.5 μL/h via a subcutaneously implanted osmotic pump for 2 days prior to intravitreal injections of VEGF (100 ng/eye) or PBS alone. Total retinal thickness (RPE to RNFL) was measured by SD-OCT at baseline (preinjection) and 24 and 48 hours post injections. *P < 0.05, **P < 0.01 indicate difference compared with corresponding PBS injection. *P < 0.05 and **P < 0.001 indicate comparison of VEGF responses in mice administered VA999272 (n = 11) and vehicle alone (n = 9).
FIGURE 4. Effect of VEGF on plasma kallikrein system components in the retina. Western blot analyses of (A) plasma prekallikrein (PPK) and plasma kallikrein (PKal) and (B) high molecular weight kininogen (HK) in retinal extracts obtained from WT mice at 24 hours post intravitreal injections of VEGF (100 ng/eye) or PBS vehicle alone. Comparisons are *P < 0.05 and **P < 0.01.

FIGURE 5. Immunohistochemical staining of PPK in the retina. WT and KLKB1–/− mice received intravitreal injections of VEGF (100 ng/eye) or PBS vehicle alone, as indicated. After 24 hours, eyes were harvested, and frozen retinal sections were prepared. Immunofluorescent staining of retinal sections was performed for CD31 (green) and PPK (red). Nuclear stain with DAPI delineates retinal layers. The position of the vitreous compartment (vit) is shown. Arrowhead indicates colocalization of endothelial CD31 with PPK within a microvessel. Magnification: ×20 (A–C); ×40 (D).
Avascular areas were indicated by white overlay (Figs. 7A, 7B). No significant differences were observed between WT and KLKB1/C0/C0 mice in the avascular area (33.3 ± 3.3 vs. 31.8 ± 3.3%) and neovascularization area (5.7 ± 1.6 vs. 6.6 ± 2.3%) after OIR (Figs. 7C, 7D).

Effect of PKal Inhibition on VEGF-Induced Retinal Thickening in Rats

To further investigate the significance of PKal in VEGF-induced retinal edema using another species, we examined VEGF-induced retinal thickening in rats receiving systemic subcutaneous administration of VA999272 (0.58 mg/kg/d) or vehicle (10% PEG400 in PBS) via osmotic pump. After 1 day of treatment, rats received intravitreal injections of VEGF (10 ng/eye) or PBS vehicle. Retinal thicknesses (RPE to RNFL) were measured by SD-OCT at baseline and at 24 and 48 hours post injection (Fig. 8A; Supplementary Table S3). The changes in retinal thickness from baseline for PBS or VEGF injection at 24 and 48 hours in vehicle-treated rats and 48 hours in PKal inhibitor (VA999272)-treated rats are shown in Figure 8B. Intravitreal injection of VEGF increased retinal thickness in vehicle-treated animals by 37.6 ± 2.0 μm at 24 hours and 42.4 ± 4.3 μm at 48 hours compared to increases of 7.2 ± 2.0 and 11.8 ± 2.8 μm in eyes receiving an injection with PBS ($P < 0.001$). In VA999272-treated rats, VEGF increased thickness at 48 hours to 21.4 ± 4.6 μm, which was 53% ($P < 0.001$) lower than VEGF’s effect in rats receiving vehicle.

**DISCUSSION**

This study demonstrates that PKal mediates, in part, VEGF-induced retinal edema. Plasma kallikrein does not appear to play a role in the direct effects of VEGF, since VEGF-induced RVP measured at 40 minutes and 2 hours post intravitreal injection is not affected by PKal inhibition, PPK deficiency, and bradykinin receptor antagonists.6,21 In contrast, we found that PKal was required for the full effects of VEGF on RVP and retinal thickening at 24 hours post intravitreal injection, suggesting that PKal may contribute to the sustained effects of VEGF on blood–retinal barrier function. Vascular endothelial growth factor increased retinal thickness by approximately 30 to 40 μm in both rats and mice, which corresponded to up to a 21% increase in total retinal thickness. We show that KLKB1 deficiency and PKal inhibition were similarly effective in reducing VEGF-induced retinal thickening in mice. Moreover, PKal inhibition was similarly effective in reducing VEGF-stimulated edema in rats and mice; resulting in up to a 57% reduction in thickening. The similar efficacies of VA999272 and
Deficiency on VEGF-induced retinal thickening suggest that a systemically administered PKal inhibitor accessed the necessary site(s) of PKal action that contributes to retinal edema in this experimental model.

Intravitreal injection of VEGF in mice increased the rate of Evans blue permeation by 4-fold, whereas retinal thickness measured by SD-OCT was increased by 14% at 24 hours. In addition, we show that KLKB1 deficiency reduced VEGF-stimulated RVP and retinal thickness by 68% and 47%, respectively. Although retinal vascular hyperpermeability contributes to retinal edema, little is known about the quantitative relationship between the rate of RVP and extent of retinal edema in mice. Evans blue is used to measure the rate of albumin permeability whereas SD-OCT measures retinal thickening, which is affected by the rates of both fluid influx and efflux. In a previous proteomic study using mass spectrometry-derived spectral/peptide counts, we have demonstrated that albumin represents approximately 1.5% of the total retinal protein content in untreated WT C57Bl/6 mice. While spectral peptide counts provide only a semiquantitative estimate of protein abundance, these data suggest that a 4-fold increase in extravasation of albumin, and other plasma proteins, would be expected to exert a relatively small effect on the total retinal protein content. It has been proposed, based on Starling’s law, that increased interstitial protein concentration in the retina decreases the osmotic pressure gradient and thereby contributes to macular edema. However, our group and others have shown that total vitreous protein and VEGF concentrations do not correlate, and vitreous from subjects with macular hole often has higher vitreous protein concentrations than vitreous from DME and PDR subjects. These findings suggest that total extracellular protein concentration in the vitreous does not fully explain the effects of RVP on retinal edema. We have previously shown that PKal is elevated in DME vitreous compared with macular hole vitreous and that intravitreal injection of PKal and bradykinin induces retinal edema. Taken together, these data suggest that VEGF-induced extravasation of PKal into the retina contributes to its effects on retinal edema, in part, by increasing intraocular bradykinin.

Transport of albumin and a variety of other proteins across the vascular endothelium is mediated by both transcellular and paracellular mechanisms of endothelial permeability. Barber and Antonetti have reported that VEGF increases paracellular vascular permeability in the retina, suggesting that this route may...
contribute to VEGF’s effects on albumin transport across the blood-retinal barrier. However, additional studies are needed to determine if VEGF may also facilitate the transcellular route, as well as the relative contributions of PKal to both routes. Vascular endothelial growth factor binding to its receptor, VEGF-R2, rapidly activates a cascade of signaling elements, including Src kinases, PKC, and eNOS, which facilitates vascular permeability via multiple mechanisms including phosphorylation and ubiquitination of occludin, resulting in the disruption of tight junctions and S-nitrosylation of beta-catenin leading to disassembly of adherens junctions. While these effects of VEGF likely contribute to RVP, they may not fully recapitulate VEGF’s effects in DME, which is a chronic condition of vascular hyperpermeability with enhancement of leakage often located at or adjacent to areas of retinal thickening. Although intravitreal concentrations of VEGF are increased in DME, the levels of VEGF correlate only weakly with macular thickness. Moreover, there is only a modest correlation between macular thickness and visual acuity. Therefore we hypothesized that VEGF may not act alone in mediating edema and vision loss but rather may recruit additional factors that amplify retinal permeability. We show that intravitreal injection of VEGF increases retinal PPK and HK, indicating that VEGF stimulates recruitment of the KKS into the retina. A similar response was observed following intravitreal injection of TNFα suggesting that retinal KKS extravasation is not selective to VEGF but could be a consequence of increasing blood-retina barrier permeability induced by multiple factors.

Although VEGF appeared to exert larger effects on retinal thickness and retinal PPK levels compared with TNFα, KLKB1 deficiency reduced both VEGF- and TNFα-induced retinal thickening by approximately 50%. These findings suggest that PPK mediates a fraction of retinal thickening response induced by these permeability factors; however, additional mechanisms contribute to retinal edema in these models.

While anti-VEGF therapies are often highly effective for DME and RVO, the administration of these agents generally requires monthly repeated intravitreal injections. Long-acting or controlled-release anti-VEGF agents are potential strategies to reduce the frequency of intravitreal injections; however, these approaches may also have greater effects on inhibiting the beneficial physiological actions of VEGF on the retina. While VEGF inhibition is a safe and effective therapy for macular edema, there is also considerable experimental evidence in animal models for essential functions of VEGF in normal retinal homeostasis. Conditional knockout of VEGF expression in adult mouse RPE resulted in vision loss. Indeed, VEGF has been shown to exert beneficial effects on the choriocapillaris, Müller cell, endothelium, and retinal neuron survival. Although adverse effects of VEGF blockade are not clinically evident with the current monthly cycle of VEGF inhibitor injections, more intensive VEGF inhibition may further reduce levels of VEGF that might be important for retinal physiology. In contrast to the role of KLKB1 in VEGF-induced retinal edema described above, we show that KLKB1 deficiency does not alter the angiogenic
response to OIR in mice, which has been previously attributed to VEGF-mediated neovascularization. These findings suggest that KKLBI is not required for VEGF’s angiogenic effects that are critical for vascular growth and survival.

In conclusion, systemic administration of PKal inhibitors may provide an opportunity to ameliorate the edematous effects of VEGF in the retina without blocking the direct effects of VEGF that are critical for retinal, vascular, and systemic/renal homeostasis. Further characterization of the mediators for VEGF-induced macular edema and vision loss, rather than complete VEGF blockade, may reveal new therapeutic targets for DME and RVO.

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