

COMP-Ang1 Stabilizes Hyperglycemic Disruption of Blood-Retinal Barrier Phenotype in Human Retinal Microvascular Endothelial Cells

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PURPOSE. Current treatments for diabetic retinopathy (DR) have considerable limitations, underpinning the need for new therapeutic options. In this article, the ability of an engineered angiopoietin-1 variant (COMP-Ang1) to ameliorate the injurious effects of hyperglycemia on barrier integrity in a human retinal microvascular endothelial cell (HRMvEC) model is comprehensively investigated.

METHODS. Confluent HRMvECs were treated (0–72 hours) with D-glucose (5 or 30 mM) in the absence and presence of COMP-Ang1 (10–200 ng/mL). L-glucose (30 mM) was used as osmotic control. Posttreatment, intact cell monolayers were monitored for permeability to FITC-dextran 40 kDa. Cells were also harvested for analysis of interendothelial junction targets by RT-qPCR and Western blotting. The impact of receptor tyrosine kinase Tie2 gene silencing on COMP-Ang1 efficacy was also evaluated.

RESULTS. Treatment with 30 mM D-glucose (but not L-glucose) demonstrated a time-dependent elevation in the mean rate of FITC-dextran diffusion across intact HRMvEC monolayers, in parallel with significant reductions in mRNA/protein levels of occludin, claudin-5, ZO-1, and VE-Cadherin. These effects were all attenuated by COMP-Ang1 in a concentration-dependent fashion, with 200 ng/mL recovering barrier function by ~88%, and recovering reduced interendothelial junction protein levels by more than 50%. Finally, Tie2 knockdown by small interfering RNA silencing blocked the ability of COMP-Ang1 to mitigate against hyperglycemia-induced permeabilization of HRMvECs and depletion of junctional expression levels.

CONCLUSIONS. In summary, this article presents a reproducible in vitro cell study that quantifies the concentration-dependent efficacy of COMP-Ang1 to mitigate the injurious effects of hyperglycemic challenge on HRMvEC barrier properties via Tie2-mediated signaling.

Keywords: endothelial, diabetic retinopathy, COMP-Ang1, Tie2, blood-retinal barrier

Type-1/2 diabetes mellitus (DM), with its high mortality rate directly linked to vascular and renal complications, is fast becoming one of the leading global causes of death, placing enormous strain on health care systems.¹ Diabetic retinopathy (DR) is one of the main global causes of blindness in working-age individuals, affecting both type-1/2 diabetic individuals. DR is a degenerative disease of retinal capillaries and associated neuroglial cells, collectively manifesting impaired retinal vasoregulation, elevated microvascular permeability, microaneurysm formation, cell death, and ultimately, widespread nonperfusion and ischemia of the inner retina.^{2–4}

Several novel therapeutic approaches to DR have focused on limiting damage to the microvascular endothelial cells that form the retinal capillary lumen at the frontline of hyperglycemic attack. Both in vitro and in vivo strategies targeting connexin 43 (Cx43) gap junctions,^{5,6} VEGF receptors-1/2,⁷ and the Tie2 receptor pathway⁸ have previously been used, with varying degrees of success. Using human primary retinal endothelial

cells, Stewart et al.⁹ also recently reported beneficial effects of dexamethasone to reverse the permeabilizing effects of hyperglycemia by restoring the cellular balance between the Tie2-activating ligand, angiopoietin-1 (Ang1), and its context-dependent antagonist, angiopoietin-2 (Ang2).

With respect to Ang1, substitution of the N-terminal portion of this ligand with the short coiled-coil domain of cartilage oligomeric matrix protein has yielded a soluble and stable Ang1 variant (COMP-Ang1) with therapeutic potential for treating endothelial inflammation and vascular leakage.¹⁰ In this study, we hypothesized that COMP-Ang1 could ameliorate the injurious effects of hyperglycemia on barrier integrity in a human retinal microvascular endothelial cell (HRMvEC) model of DR. In support of this hypothesis, a limited number of mainly in vivo studies have previously reported the ability of COMP-Ang1 to improve microvascular function. Cho et al.¹¹ showed that COMP-Ang1 can promote wound healing through enhancement of angiogenesis and blood flow in diabetic db/db



mice, whereas Chen et al.¹² demonstrated that COMP-Ang1 can protect endothelial barriers in rats with acute necrotizing pancreatitis. Subretinal and intravitreal delivery of COMP-Ang1 cloned into an adeno-associated virus vector (AAV2.COMP-Ang1) exhibited microvasculature-related improvements in mouse models of AMD¹³ and diabetic retinopathy,¹⁴ respectively. To our knowledge however, there are no *in vitro* retinal microvascular endothelial cell studies delineating the precise mechanistic impact of COMP-Ang1 on hyperglycemia-induced injury to the interendothelial junction complex.

To address this issue, we pursued three key aims: (1) to establish a reproducible model of hyperglycemia-induced HRMvEC barrier disruption; (2) to quantitatively assess the impact of COMP-Ang1 treatment on HRMvEC barrier properties under hyperglycemic challenge; and (3) to evaluate the role of the Tie2 receptor signaling pathway in the observed effects of COMP-Ang1.

MATERIALS AND METHODS

Materials

Unless otherwise stated, all reagents were purchased from Sigma-Aldrich (Dublin, Ireland). Western blotting primary antisera were purchased from the following sources: anti-occludin IgG, anti-claudin-5 IgG, and anti-ZO-1 IgG (Thermo Fischer Scientific, Dublin, Ireland); anti-VE-Cadherin IgG (Abcam, Cambridge, UK); anti- β -actin (Cell Signaling Technologies Inc., Danvers, MA, USA). Horseradish peroxidase (HRP)-conjugated secondary antisera for all targets were purchased from Cell Signaling Technologies Inc. COMP-Ang1 was purchased from Enzo Life Sciences (Exeter, UK). For Tie2 experiments, dual Ambion Silencer Select small interfering RNA (siRNA) constructs (assay IDs 13983 and s13984 against TEK/Tie2) were purchased from Thermo Fisher Scientific. An anti-Tie2 IgG and associated donkey anti-goat secondary IgG were purchased from R&D Systems (Abingdon, UK).

Cell Culture

Primary HRMvECs were acquired from Cell Systems (Kirkland, WA, USA) and routinely cultured in EndoGRO MV Endothelial Medium (MerckMillipore, Cork, Ireland) containing 100 units of penicillin and 100 μ g of streptomycin per milliliter of medium. All cells (passages 5–12) were grown on attachment factor-coated tissue culture grade plastic-ware and maintained in a humidified atmosphere of 5% CO₂/95% air at 37°C. Confluent cells were treated with either D-glucose (5 or 30 mM) or L-glucose (osmotic control, 30 mM) for up to 72 hours. For specific experiments, COMP-Ang1 (10, 100, or 200 ng/mL) was incorporated into the treatment regimen. Following treatments, intact cell monolayers were either monitored for changes in barrier function via trans-endothelial permeability assay or cells were harvested for analysis of mRNA and protein expression. Details on the preparation of mRNA and whole cell protein lysates have been described in detail previously.¹⁵ All samples were stored at –80°C until required. Effects of all treatments on cell viability (including siRNA transfection described below) were routinely monitored using either MTT formazan dye or crystal violet assay.

Trans-endothelial Permeability Assay

HRMvECs were seeded into transwell inserts at a density of 500,000 cells/well and allowed to grow to confluency. The analysis of HRMvEC monolayer permeability following various treatments used transwell diffusion of FITC-labeled 40 kDa dextran as previously described in detail by Walsh et al.¹⁶

Permeability results were expressed as the mean rate of FITC-dextran trans-endothelial exchange (%FD₄₀TEE.min⁻¹).

Reverse-Transcriptase Quantitative PCR (RT-qPCR)

Following experiments, HRMvECs were harvested for extraction of total RNA, preparation of cDNA, and amplification of target cDNA sequences by RT-qPCR (Thermocycler; Roche Diagnostics, West Sussex, UK) as previously described in Guinan et al.¹⁷ with minor modifications. PCR reaction mixtures (12 μ L) were as follows: 6 μ L of FastStart Universal SYBR Green/Rox Mastermix (Roche Diagnostics, West Sussex, UK), 2 μ L RNase-free water, 2 μ L cDNA, and 1 μ L each 10 μ M forward and reverse primers. PCR reaction conditions were as follows: denaturation at 95°C for 10 minutes followed by 40 cycles of (1) denaturation at 95°C for 15 seconds, (2) annealing at 59°C for 60 seconds, and (3) elongation at 72°C for 15 seconds. Each cDNA sample was assayed in triplicate and results analyzed by the comparative CQ method. For normalization purposes, β -actin was routinely used. All primer pairs were screened for correct product size (1% agarose gel electrophoresis) and underwent melt-curve analysis for primer-dimers. β -actin (218 bp): Forward 5'-catccgcaaagacctgtacg-3'; Reverse 5'-cctgcttgctgatccacatc-3'; Occludin (224 bp): Forward 5'-ccttcacccccatctgacta-3'; Reverse 5'-gcaggtgctcttttgaagg-3'; Claudin-5 (240 bp): Forward 5'-gaggcgtgctctacctgtt-3'; Reverse 5'-agtacttcacggggaagctg-3'; ZO-1 (218 bp): Forward 5'-gaacgaggcatcatccctaa-3'; Reverse 5'-ccagcttctcgaagaaccac-3'; VE-Cadherin (185 bp): Forward 5'-cagccaaagtgtgtgagaa-3'; Reverse 5'-cggtaaacctgccaactc-3'; Tie2 (222 bp): Forward 5'-tcataactggtgacctcc-3'; Reverse 5'-agtcctgagttgaactgca-3'.

Western Immunoblotting

Following experiments, HRMvEC cell lysates were harvested, resolved by 8% SDS-PAGE under reducing conditions, and electroblotted as previously described.¹⁸ Primary antisera: 0.5 μ g/mL anti-occludin, anti-claudin-5, and anti-ZO-1 mouse monoclonal IgGs; 0.2 μ g/mL anti-VE-Cadherin mouse monoclonal IgG; 1/1000 anti-Tie2 goat monoclonal IgG; and 0.2 μ g/mL anti- β -actin rabbit monoclonal IgG. Secondary antisera: 1:2000 HRP-conjugated goat anti-mouse IgG (occludin, claudin-5, ZO-1, and VE-Cadherin), 1:2000 HRP-conjugated donkey anti-goat IgG (Tie2), and 1:3000 HRP-conjugated goat anti-rabbit IgG (β -actin). Membranes were developed using a Luminata Western HRP kit (MerckMillipore), followed by chemiluminescent imaging using a G-Box gel-documentation system (Syngene, Cambridge, UK). Scanning densitometry of Western blots was performed using NIH ImageJ software (<http://imagej.nih.gov/ij/>; provided in the public domain by the National Institutes of Health, Bethesda, MD, USA), with β -actin routinely used as a loading control to facilitate densitometric normalization of bands.

Tie2 siRNA Studies

To assess the extent to which COMP-Ang1 potentially exerts its effects through the Tie2 receptor, molecular inhibition of Tie2 using siRNA knockdown was performed. Scrambled (Ambion Silencer Select Negative Control No.1 and No.2) and Tie2 (Ambion Silencer Select Tie2 siRNA, ID: s13983 and s13984) siRNAs were used. For siRNA transfection into HRMvECs, a Microporator system was used (Life Technologies, Paisley, UK) as previously described.¹⁷ Optimization studies were initially performed on HRMvECs to confirm Tie2 knockdown efficiency and to determine optimal siRNA working conditions. For these optimization studies, we tested siRNA constructs either individually (0–50 nM) or in combination/duplex (0–50 nM

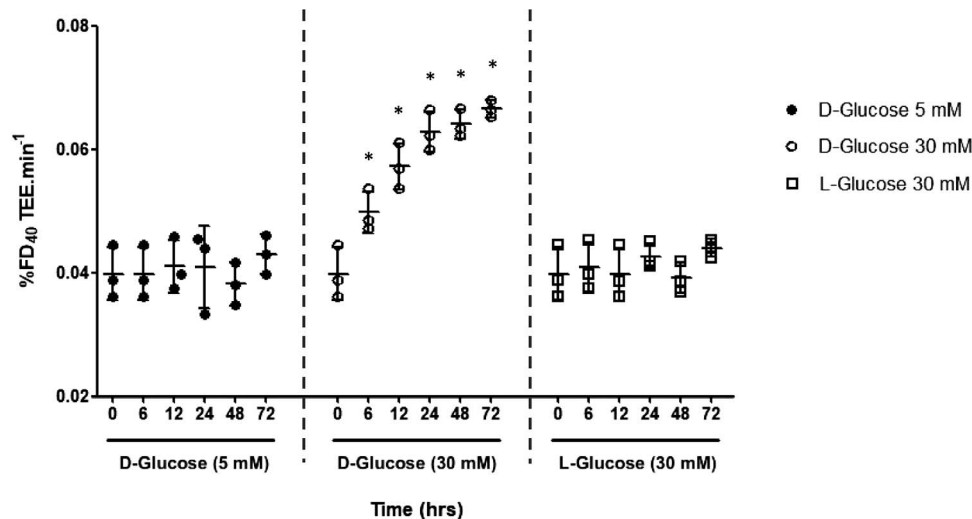


FIGURE 1. D-glucose causes permeabilization of HRMvECs. Cells were treated for up to 72 hours with either 5 mM D-glucose (normoglycemic), 30 mM D-glucose (hyperglycemic), or 30 mM L-glucose (osmotic control). HRMvEC monolayer barrier integrity was then monitored by transendothelial permeability assay (i.e., mean rate of FITC-dextran diffusion: %FD₄₀ TEE.min⁻¹). **P* ≤ 0.05 versus 0-hour control.

per construct) for up to 72 hours. Levels of Tie2 knockdown were assessed by RT-qPCR and Western Blotting. Mock transfections (excluding siRNA) were routinely included for control purposes.

Statistical Analysis

Results are expressed as mean ± SD. Experimental points were performed in triplicate with a minimum of three independent experiments (*n* = 3). Statistical comparisons between control and experimental groups were by ANOVA in conjunction with a Dunnett's post hoc test for multiple comparisons. A Student's *t*-test was also routinely used for pairwise comparisons. A value of *P* ≤ 0.05 was considered significant.

RESULTS

Hyperglycemia Reduces HRMvEC Barrier Function

To better understand the precise impact of hyperglycemia on retinal barrier function *in vitro*, we investigated the effects of D-glucose on HRMvEC permeability. Confluent HRMvECs were treated for up to 72 hours with either 5 mM D-glucose (normoglycemia), 30 mM D-glucose (hyperglycemia), or 30 mM L-glucose (osmotic control). Trans-endothelial permeability assays clearly demonstrated a time-dependent elevation in the mean rate of FITC-dextran diffusion across intact HRMvEC monolayers in response to hyperglycemia, showing a 70% increase after 72 hours with 30 mM D-glucose. Diffusion rates over the same period remained unchanged under normoglycemic conditions and in the 30-mM L-glucose control (Fig. 1). In parallel studies, we also examined how these conditions affected the expression of interendothelial junctions at both the mRNA and protein levels. In response to 30 mM D-glucose, HRMvECs showed a time-dependent decrease (68%–74%) in mRNA levels for occludin, claudin-5, zonula occludens-1 (ZO-1), and VE-Cadherin (Fig. 2A). This was also reflected at the protein level with decreases ranging from 74% to 89% (Figs. 2B, 2C). Importantly, 30 mM L-glucose did not elicit any changes in the mRNA or protein levels for any of these proteins over the 72-hour treatment period (Supplementary Figs. S1A–C).

COMP-Ang1 Significantly Attenuates the Hyperglycemia-Induced Reduction in HRMvEC Barrier Function

We next investigated the ability of COMP-Ang1 to attenuate the effects of hyperglycemia on HRMvEC barrier properties. Confluent HRMvECs were treated for up to 72 hours with either 5 mM D-glucose (normoglycemia) or 30 mM D-glucose (hyperglycemia) in the absence and presence of recombinant COMP-Ang1 (10–200 ng/mL). Under hyperglycemic conditions, a significant elevation in the mean rate of FITC-dextran diffusion across intact HRMvEC monolayers was observed. We also observed that hyperglycemia-induced permeabilization could be significantly reduced in a concentration-dependent manner by COMP-Ang1, with 200 ng/mL recovering barrier function by approximately 88% (Fig. 3). In parallel studies, we examined the effects of COMP-Ang1 on the hyperglycemia-induced reduction of interendothelial junctions. Our data clearly demonstrated that COMP-Ang1 could significantly reverse, in a concentration-dependent manner, the injurious effects of hyperglycemia on occludin, claudin-5, ZO-1, and VE-Cadherin with recovery of depleted mRNA levels ranging from 13% (at 10 ng/mL COMP-Ang1) to 53% (at 200 ng/mL COMP-Ang1) (Figs. 4A–D). Similar concentration-dependent recovery effects with COMP-Ang1 treatment also were observed for all interendothelial junction protein levels (Figs. 5A–D). Finally, treatment of HRMvECs with up to 200 ng/mL COMP-Ang1 under either normoglycemic or hyperglycemic conditions had no observable effects on cell viability (data not shown).

COMP-Ang1 Stabilizing Effects in HRMvECs Under Hyperglycemia Operate Through the Tie2 Receptor Pathway

In a final round of experiments, we investigated the mechanistic contribution of the Tie2 receptor pathway toward mediating the beneficial effects of COMP-Ang1 on HRMvECs during hyperglycemic challenge. In pre-optimization studies with HRMvECs, a duplex combination of two Ambion Silencer Select Tie2 siRNAs elicited up to 85% knockdown of Tie2 mRNA levels and 75% knockdown of Tie2 protein levels (50 nM per siRNA, 12 hours post-transfection). Scrambled siRNA

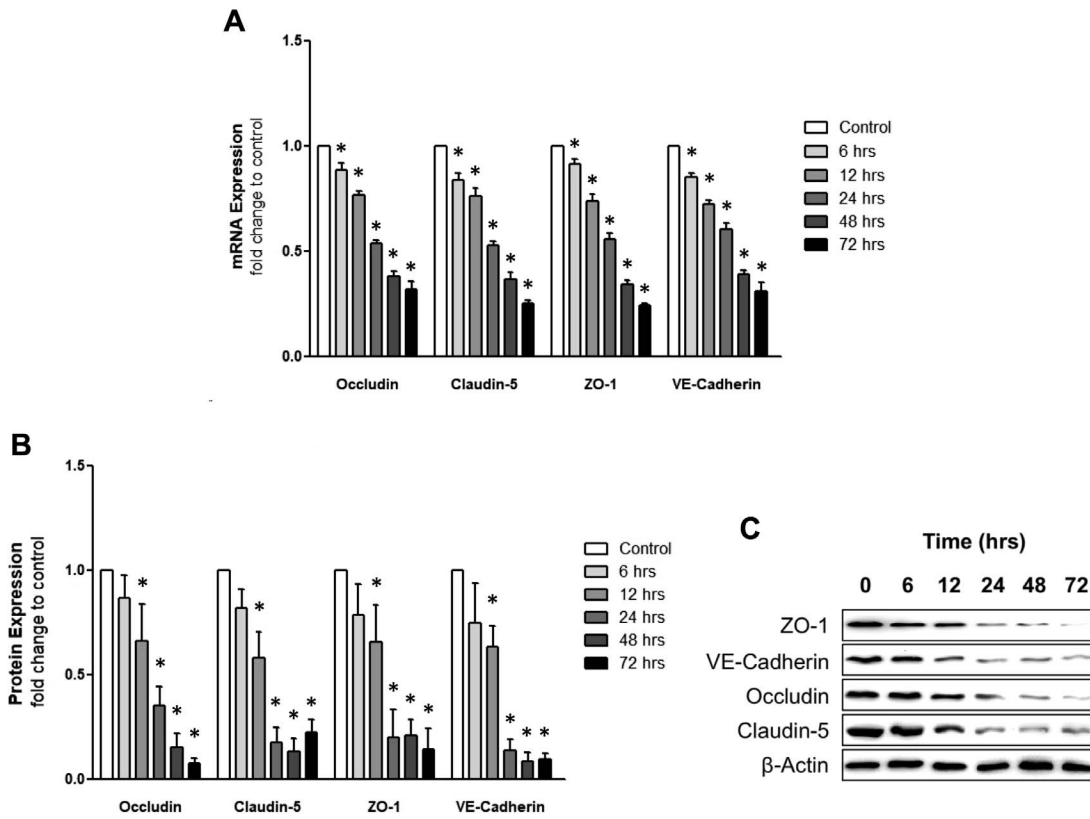


FIGURE 2. D-glucose reduces interendothelial junction expression in HRMVECs. Cells were treated for up to 72 hours with 30 mM D-glucose (hyperglycemic). HRMVECs were then harvested and monitored by (A) quantitative PCR and (B) Western blotting for levels of occludin, claudin-5, ZO-1, and VE-Cadherin. Histograms in (B) represent densitometric fold changes in Western blot band intensity relative to 0-hour controls. A representative set of blots (0–72 hours) is shown in (C). * $P \leq 0.05$ versus 0-hour control.

had no effect (Supplementary Figure S2). Moreover, siRNA treatment of HRMVECs had no observable effects on cell viability under these conditions (data not shown). In subsequent experiments, Tie2 knockdown was found to completely block the ability of COMP-Ang1 to mitigate against hypergly-

cemia-induced permeabilization of HRMVECs (Fig. 6). Likewise, the ability of COMP-Ang1 to reverse the considerable hyperglycemia-induced depletion in mRNA (Fig. 7) and protein (Fig. 8) expression levels of interendothelial junction targets was completely blocked as a consequence of Tie2 silencing.

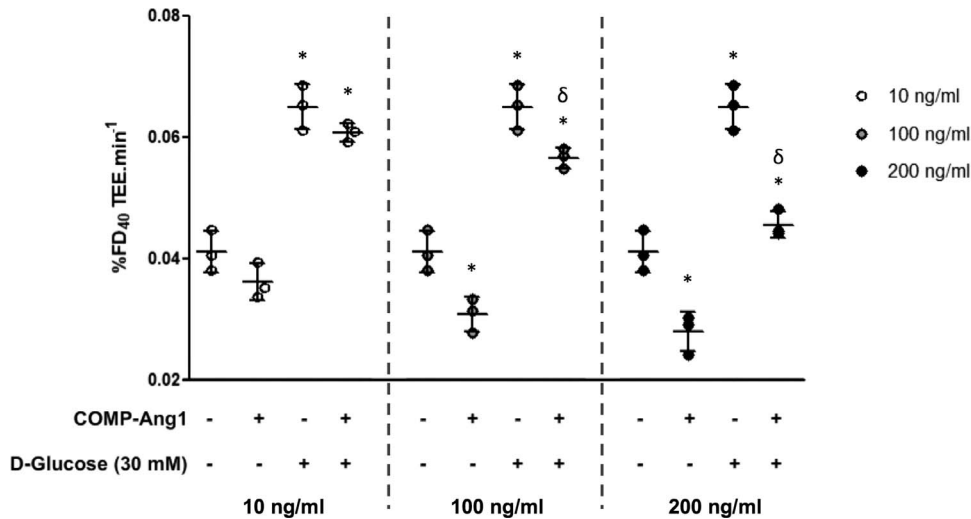


FIGURE 3. COMP-Ang1 attenuates hyperglycemia-induced permeabilization of HRMVECs. Cells were treated for 48 hours with either 5 mM D-glucose (normoglycemic) or 30 mM D-glucose (hyperglycemic) in the absence and presence of COMP-Ang1 (10, 100, or 200 ng/mL). HRMVEC monolayer barrier integrity was then monitored by transendothelial permeability assay (i.e., mean rate of FITC-dextran diffusion: %FD₄₀ TEE.min⁻¹). * $P \leq 0.05$ versus untreated normoglycemic control. $\delta P \leq 0.05$ versus 30 mM D-glucose.

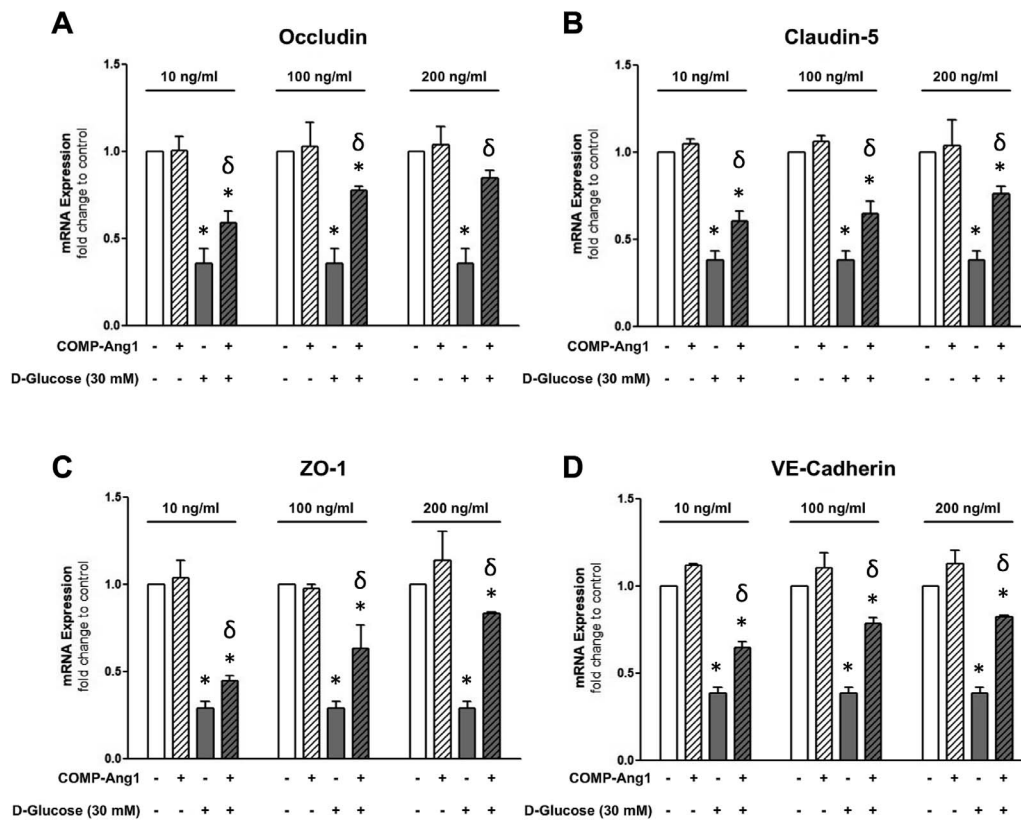


FIGURE 4. COMP-Ang1 attenuates hyperglycemia-induced reduction in interendothelial junction mRNA expression in HRMVECs. Cells were treated for 48 hours with either 5 mM D-glucose (normoglycemic) or 30 mM D-glucose (hyperglycemic) in the absence and presence of COMP-Ang1 (10, 100, or 200 ng/mL). HRMVECs were then harvested and monitored by qPCR for mRNA expression of (A) Occludin, (B) Claudin-5, (C) ZO-1, and (D) VE-Cadherin. * $P \leq 0.05$ versus untreated normoglycemic control; $\delta P \leq 0.05$ versus 30 mM D-glucose.

DISCUSSION

Current therapeutic options for DR, a blinding disease that seriously affects quality of life in type-1/2 diabetic patients, have significant limitations. Rates of improvement in visual function following 5-year ranibizumab treatment were 33% and 23%, respectively, for nonproliferative and proliferative DR.¹⁹ Likewise, other anti-VEGF agents like VEGF-Trap Eye achieved similar gains in only approximately 34% of DR patients.²⁰ Moreover, frequent intravitreal injections of anti-VEGF agents carry risks of pain, vitreous hemorrhage, retinal detachment, and endophthalmitis.²¹ Laser photocoagulation therapy, a traditional mainstay of DR therapy, carries the disadvantage of creating burns that can reduce peripheral vision, central vision, and/or night vision.²² Improved therapeutic options for DR are therefore clearly warranted.

In recent years, attention has focused on using COMP-Ang1, a soluble, stable angiotensin-1 variant,¹⁰ in models of DR. Recent studies using diabetic murine models have reported the ability of COMP-Ang1 to improve microvascular function by promoting wound healing and enhanced visual acuity.^{11,13,14} By delineating the precise mechanistic impact of COMP-Ang1 on the retinal interendothelial junction in vitro, our study lends important insight into physiologically elevated microvascular leakage and vessel destabilization during DR pathology.

To this end, we first established a comprehensive and reproducible model of hyperglycemia-induced HRMVEC barrier disruption with the intention of broadening the experimental depth of existing HRMVEC model equivalents (e.g., testing multiple drug concentrations and glucose exposure times to establish dose/time-dependency criteria, quantitatively investi-

gating a broad range of adherens and tight junction proteins at both the mRNA and protein levels, and using L-glucose as an osmotic control in all studies given the now-established confounding signaling effects of mannitol²³). This undertaking is also necessitated by the fact that certain widely used retinal cell models such as bovine retinal endothelial cells (BRECs) exhibit poor/variable glucose responsiveness.²⁴ Following chronic hyperglycemic challenge with 30 mM D-glucose, intact HRMVEC monolayers exhibited a robust time-dependent increase in the transendothelial diffusion rate of FITC-dextran, in parallel with decreased expression (both mRNA and protein) of the interendothelial junction proteins essential to maintenance of barrier integrity (i.e., occludin, claudin-5, ZO-1, and VE-Cadherin). These results mirror the hyperpermeabilizing effects of high glucose recently reported for rat retinal endothelial cells,⁶ as well as in human primary retinal endothelial cells,⁹ which additionally showed reduced mRNA levels of claudin-5, occludin, and JAM-A. With respect to this latter study of Stewart et al.,⁹ it can be noted, however, that glucose had no effect on VE-Cadherin levels in HRMVECs, a clear contrast to the current study. This may point to heterogeneity in different commercial preparations of HRMVECs and/or variations in experimental approach.

In subsequent experiments, we demonstrated that all of the aforementioned harmful glucose-dependent effects on barrier integrity and interendothelial junctions can be substantively ameliorated by cotreatment of HRMVECs with COMP-Ang1. An additional statistical comparison (not shown) between the COMP-Ang1/normoglycemia versus COMP-Ang1/hyperglycemia conditions for our experiments also regularly demonstrates statistically significant differences, leading us to deduce that

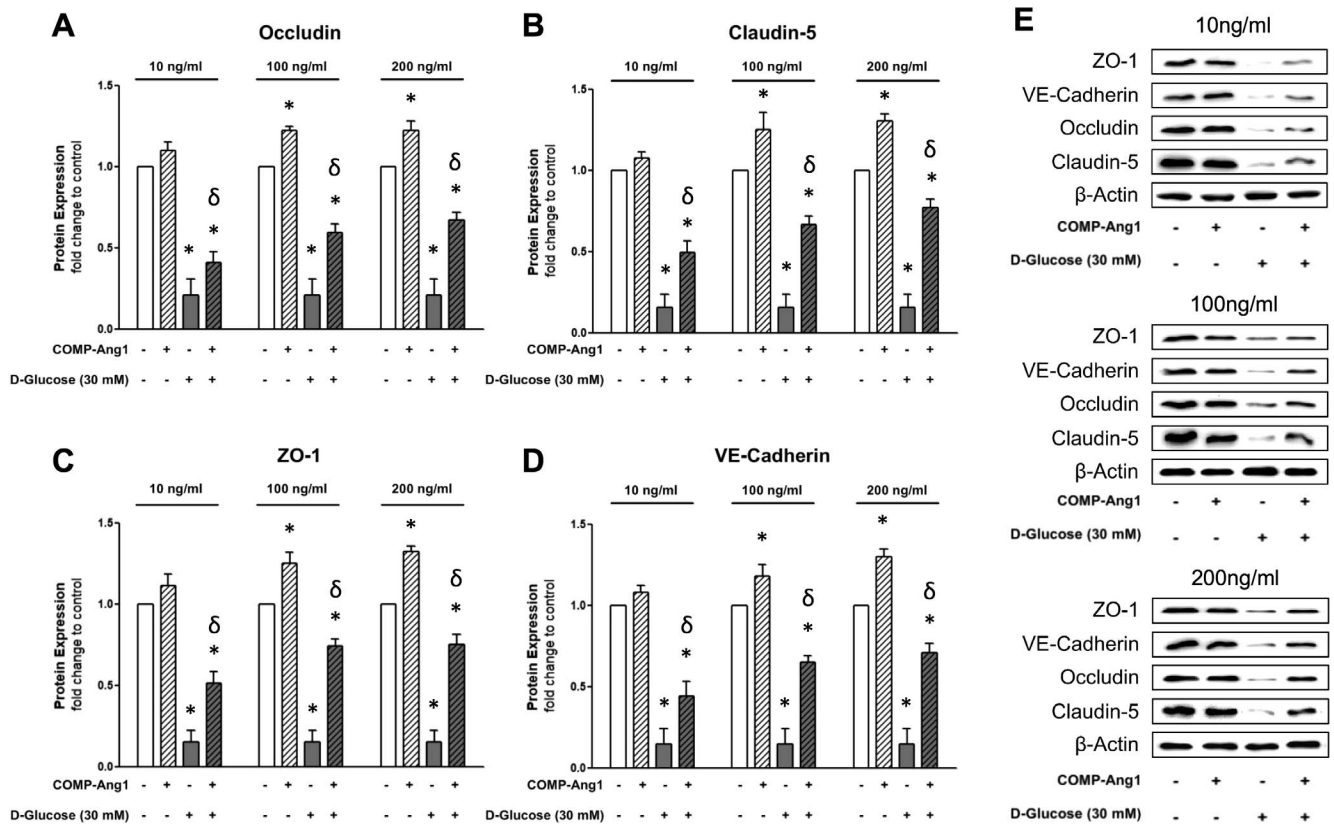


FIGURE 5. COMP-Ang1 attenuates hyperglycemia-induced reduction in interendothelial junction protein expression in HRMVECs. Cells were treated for 48 hours with either 5 mM D-glucose (normoglycemic) or 30 mM D-glucose (hyperglycemic) in the absence and presence of COMP-Ang1 (10, 100, or 200 ng/mL). HRMVECs were then harvested and monitored by Western blotting for protein expression of (A) Occludin, (B) Claudin-5, (C) ZO-1, and (D) VE-Cadherin. Histograms represent densitometric fold changes in Western blot band intensity relative to untreated normoglycemic control. A representative set of blots for 10, 100, and 200 ng/mL COMP-Ang1 is shown in (E). * $P \leq 0.05$ versus untreated normoglycemic control; $\delta P \leq 0.05$ versus 30 mM D-glucose.

COMP-Ang1 is not fully restoring the damaging effects of hyperglycemia, even at the 200 ng/mL concentration.

Consistent with our in vitro findings, COMP-Ang1 was previously found to prevent endotoxin-induced vascular

leakage in mice, in part by restoring expression of platelet endothelial cell adhesion molecule-1 (PECAM-1) within interendothelial junctions.²⁵ Our findings also bring some valuable mechanistic insight into the recent in vivo study of Cahoon et

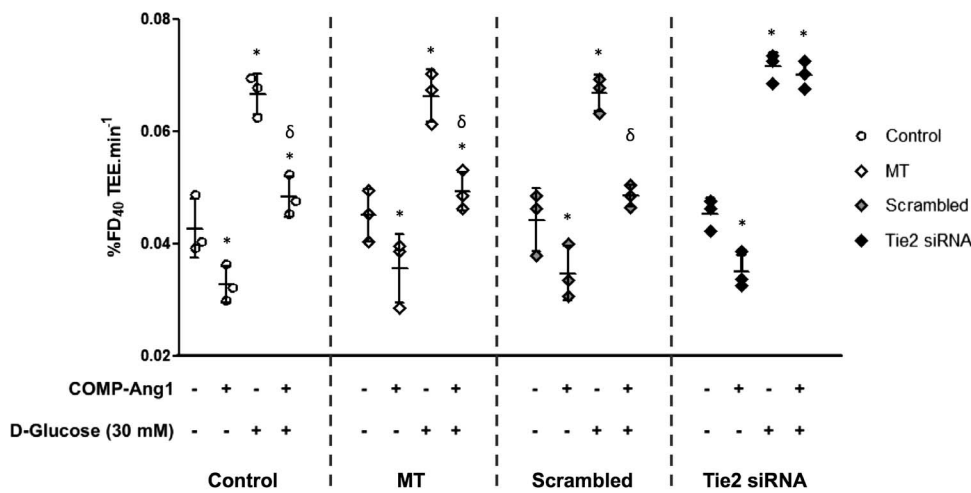


FIGURE 6. COMP-Ang1 attenuates hyperglycemia-induced permeabilization of HRMVECs: impact of Tie2 siRNA. Based on gene silencing treatment, cells were divided into four groups: Control, Mock Transfected (i.e., no siRNA), Scrambled siRNA, and Tie2 siRNA. Cells were then treated for 48 hours with either 5 mM D-glucose (normoglycemic) or 30 mM D-glucose (hyperglycemic) in the absence and presence of 200 ng/mL COMP-Ang1. HRMVEC monolayer barrier integrity was then monitored by transendothelial permeability assay (i.e., mean rate of FITC-dextran diffusion: %FD₄₀ TEE.min⁻¹). * $P \leq 0.05$ versus untreated normoglycemic control. $\delta P \leq 0.05$ versus 30 mM D-glucose.

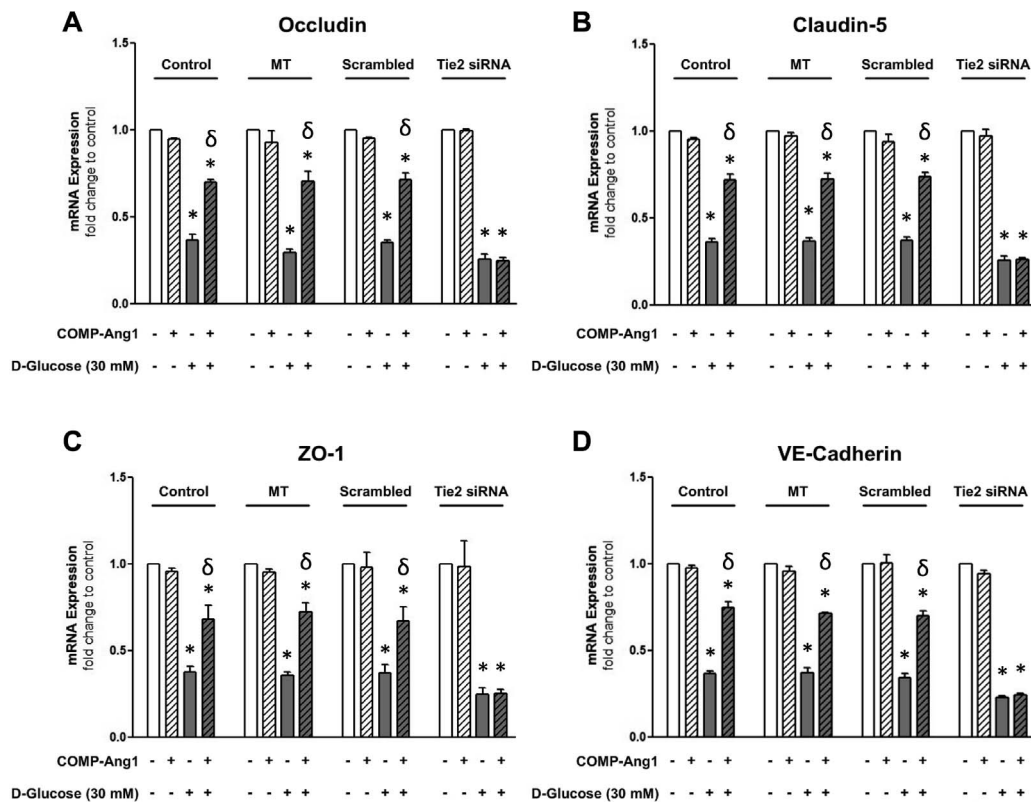


FIGURE 7. COMP-Ang1 attenuates hyperglycemia-induced reduction in interendothelial junction mRNA expression in HRMVECs: impact of Tie2 siRNA. Based on gene silencing treatment, cells were divided into four groups: Control, Mock Transfected (i.e., no siRNA), Scrambled siRNA, and Tie2 siRNA. Cells were treated for 48 hours with either 5 mM D-glucose (normoglycemic) or 30 mM D-glucose (hyperglycemic) in the absence and presence of 200 ng/mL COMP-Ang1. HRMVECs were then harvested and monitored by qPCR for mRNA expression of (A) Occludin, (B) Claudin-5, (C) ZO-1, and (D) VE-Cadherin. * $P \leq 0.05$ versus untreated normoglycemic control; $\delta P \leq 0.05$ versus 30 mM D-glucose.

al.,¹⁴ who demonstrated how Evans Blue extravasation and GFP-microsphere leakage from the retinal capillary microvasculature in diabetic Ins2Akita mice can be robustly prevented by COMP-Ang1 treatment, likely via restoration of the depleted interendothelial barrier. It should be noted that this latter study also reported the ability of COMP-Ang1 to increase transendothelial electrical resistance (TER) and VE-Cadherin expression in HRMVECs under normoglycemia, but unlike the present article, does not examine drug effects under hyperglycemia.

Within the adult vasculature, Ang1 agonist-induced Tie2 activation is known to promote endothelial barrier stabilization and vascular quiescence, and as such, has become an important focus of therapeutic strategies targeting pathologies of vascular activation, such as DR.^{26,27} We therefore decided to confirm that the beneficial COMP-Ang1 effects on HRMVECs under hyperglycemia were putatively mediated through the canonical Ang1:Tie2 receptor system, the latter Tie2 receptor tyrosine kinase being highly enriched within the endothelium. In support of this, an early study by Cho et al.¹⁰ clearly demonstrates the ability of COMP-Ang1 to temporally activate Tie2 in human umbilical vein endothelial cells (HUVECs). The functional relevance of this observation is further demonstrated by Oh et al.,⁸ who showed that COMP-Ang1 can suppress vascular leakage in mice and improve cell survival and angiogenesis in HUVECs through the Tie2 receptor system. Other HUVEC studies by Sturk et al.,²⁸ Moon et al.,²⁹ and Qiu et al.³⁰ also provide evidence of COMP-Ang1-mediated Tie2 activation with downstream consequences for tyrosine phosphorylation of growth factor receptor-bound (Grb) adaptor

proteins, neovascularization and wound-healing processes, and paracrine signaling to nonvascular cell types (e.g., dorsal root ganglion neurons).

To confirm a role for Tie2 in our HRMVEC experiments, we optimized a commercially sourced siRNA duplex to achieve robust silencing of Tie2 expression in HRMVECs. The inability of COMP-Ang1 to ameliorate hyperglycemia-induced effects in HRMVECs after siRNA-mediated Tie2 silencing strongly supports our hypothesis that the beneficial effects of COMP-Ang1 on barrier integrity during hyperglycemia are mediated through a COMP-Ang1:Tie2 interaction. Interestingly, COMP-Ang1 also reduces HRMVEC permeability under normoglycemic conditions, an effect that does not appear to be influenced by Tie2 silencing (see Fig. 6). This may reflect a duality in COMP-Ang1 mode-of-action toward paracellular permeability possibly stemming from COMP-Ang1 operating through distinct signaling pathways under normoglycemic versus hyperglycemic states. Alternately, it may be attributable to alterations in endothelial Tie2 receptor levels in response to elevated glucose. Future investigations will address this interesting issue more closely.

In summary, this article presents a reproducible in vitro cell study confirming the concentration-dependent efficacy of COMP-Ang1 to mitigate the injurious effects of hyperglycemic challenge on HRMVEC barrier properties. A clear role for COMP-Ang1:Tie2 interaction also is confirmed. These observations provide timely mechanistic support to recent murine studies and serve as a basis for further investigations into the therapeutic value of modulating the Tie2 signaling axis during inflammatory microvascular diseases of the eye.

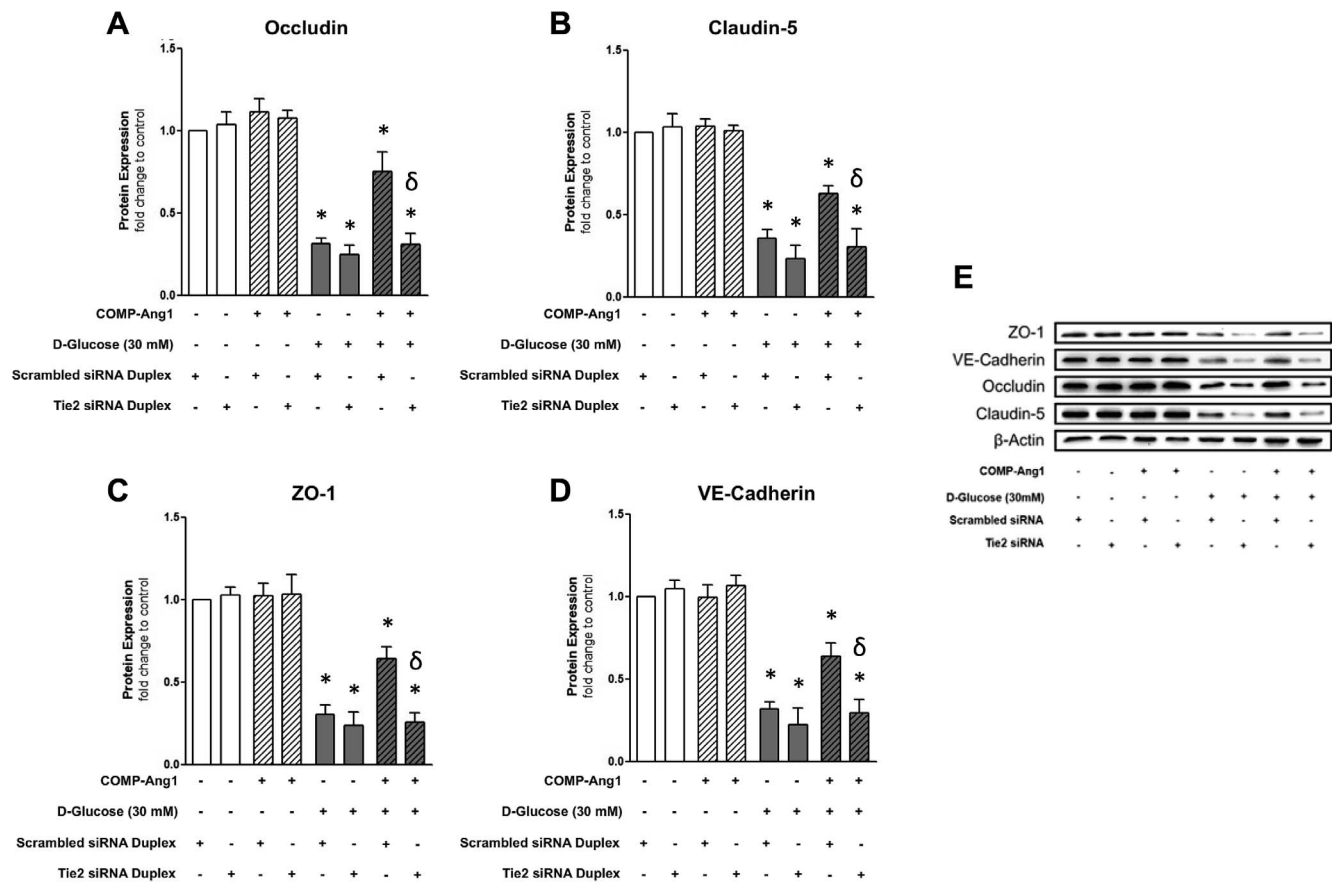


FIGURE 8. COMP-Ang1 attenuates hyperglycemia-induced reduction in interendothelial junction protein expression in HRMVECs. Impact of Tie2 siRNA. Cells were treated for 48 hours with either 5 mM D-glucose (normoglycemic) or 30 mM D-glucose (hyperglycemic). Within each glucose group, the following four treatments were used: Scrambled siRNA, Tie2 siRNA, COMP-Ang1 + Scrambled siRNA, COMP-Ang1 + Tie2 siRNA. Cells were treated with 200 ng/mL COMP-Ang1. HRMVECs were then harvested and monitored by Western blotting for protein expression of (A) Occludin, (B) Claudin-5, (C) ZO-1, and (D) VE-Cadherin. Histograms represent densitometric fold changes in Western blot band intensity relative to normoglycemic scrambled controls (without COMP-Ang1). A representative set of blots is shown in (E). * $P \leq 0.05$ versus normoglycemic scrambled controls (without COMP-Ang1); $\delta P \leq 0.05$ versus hyperglycemic scrambled controls (with COMP-Ang1).

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References

- Huang ES, Basu A, O'Grady M, Capretta JC. Projecting the future diabetes population size and related costs for the U.S. *Diabetes Care*. 2009;32:2225-2229.
- Archer DB. Diabetic retinopathy: some cellular, molecular and therapeutic considerations. *Eye*. 1999;13:497-523.
- Stitt AW, Lois N, Medina RJ, Adamson P, Curtis TM. Advances in our understanding of diabetic retinopathy. *Clin Sci (Lond)*. 2013;125:1-17.
- Eshaq RS, Aldalati AMZ, Alexander JS, Harris NR. Diabetic retinopathy: breaking the barrier. *Pathophysiology*. 2017;24:229-241.
- Tien T, Barrette KF, Chronopoulos A, Roy S. Effects of high glucose-induced Cx43 downregulation on occludin and ZO-1 expression and tight junction barrier function in retinal endothelial cells. *Invest Ophthalmol Vis Sci*. 2013;54:6518-6525.
- Kim D, Mouritzen U, Larsen BD, Roy S. Inhibition of Cx43 gap junction uncoupling prevents high glucose-induced apoptosis and reduces excess cell monolayer permeability in retinal vascular endothelial cells. *Exp Eye Res*. 2018;173:85-90.
- Spoerri PE, Afzal A, Li Calzi S, et al. Effects of VEGFR-1, VEGFR-2, and IGF-IR hammerhead ribozymes on glucose-mediated tight junction expression in cultured human retinal endothelial cells. *Mol Vis*. 2006;12:32-42.
- Oh N, Kim K, Kim SJ, et al. A designed angiopoietin-1 variant, dimeric COMP-Ang1 activates Tie2 and stimulates angiogenesis and vascular stabilization in N-glycan dependent manner. *Sci Rep*. 2015;5:15291.
- Stewart EA, Saker S, Amoaku WM. Dexamethasone reverses the effects of high glucose on human retinal endothelial cell permeability and proliferation in vitro. *Exp Eye Res*. 2016;151:75-81.
- Cho CH, Kammerer RA, Lee HJ, et al. COMP-Ang1: a designed angiopoietin-1 variant with nonleaky angiogenic activity. *Proc Natl Acad Sci U S A*. 2004;101:5547-5552.
- Cho CH, Sung HK, Kim KT, et al. COMP-angiopoietin-1 promotes wound healing through enhanced angiogenesis, lymphangiogenesis, and blood flow in a diabetic mouse model. *Proc Natl Acad Sci U S A*. 2006;103:4946-4951.

12. Chen YF, Kong PT, Li HC, et al. Cartilage oligomeric matrix protein-angiopoietin-1 has a protective effect of vascular endothelial barrier in rat with acute necrotizing pancreatitis. *Pancreas*. 2016;45:142-147.
13. Lambert NG, Zhang X, Rai RR, et al. Subretinal AAV2.COMP-Ang1 suppresses choroidal neovascularization and vascular endothelial growth factor in a murine model of age-related macular degeneration. *Exp Eye Res*. 2016;145:248-257.
14. Cahoon JM, Rai RR, Carroll LS, et al. Intravitreal AAV2.COMP-Ang1 prevents neurovascular degeneration in a murine model of diabetic retinopathy. *Diabetes*. 2015;64:4247-4259.
15. Rochfort KD, Collins LE, Murphy RP, Cummins PM. Down-regulation of blood-brain barrier phenotype by proinflammatory cytokines involves NADPH oxidase-dependent ROS generation: consequences for interendothelial adherens and tight junctions. *PLoS One*. 2014;9:e101815.
16. Walsh TG, Murphy RP, Fitzpatrick P, et al. Stabilization of brain microvascular endothelial barrier function by shear stress involves VE-cadherin signaling leading to modulation of pTyr-occludin levels. *J Cell Physiol*. 2011;226:3053-3063.
17. Guinan AF, Rochfort KD, Fitzpatrick PA, et al. Shear stress is a positive regulator of thimet oligopeptidase (EC3.4.24.15) in vascular endothelial cells: consequences for MHC1 levels. *Cardiovasc Res*. 2013;99:545-554.
18. Rochfort KD, Cummins PM. Thrombomodulin regulation in human brain microvascular endothelial cells in vitro: role of cytokines and shear stress. *Microvasc Res*. 2015;97:1-5.
19. Bressler SB, Oda I, Glassman AR, et al. Changes in diabetic retinopathy severity when treating diabetic macular edema with ranibizumab: drcr.net protocol i 5-year report. *Retina*. 2018;38:1896-1904.
20. Heier JS, Boyer D, Nguyen QD, et al. The 1-year results of CLEAR-IT 2, a phase 2 study of vascular endothelial growth factor trap-eye dosed as-needed after 12-week fixed dosing. *Ophthalmology*. 2011;118:1098-1106.
21. Rosenfeld PJ, Brown DM, Heier JS, et al. Ranibizumab for neovascular age-related macular degeneration. *N Engl J Med*. 2006;355:1419-1431.
22. Comer GM, Ciulla TA. Pharmacotherapy for diabetic retinopathy. *Curr Opin Ophthalmol*. 2004;15:508-518.
23. Li M, Xu L, Feng G, Zhang Y, Wang X, Wang Y. High glucose downregulates myocardin expression in rat glomerular mesangial cells via the ERK signaling pathway. *Oncotarget*. 2017;8:87390-87400.
24. Lopez-Quintero SV, Ji XY, Antonetti DA, Tarbell JM. A three-pore model describes transport properties of bovine retinal endothelial cells in normal and elevated glucose. *Invest Ophthalmol Vis Sci*. 2011;52:1171-1180.
25. Hwang JA, Lee EH, Lee SD, Park JB, Jeon BH, Cho CH. COMP-Ang1 ameliorates leukocyte adhesion and reinforces endothelial tight junctions during endotoxemia. *Biochem Biophys Res Commun*. 2009;381:592-596.
26. Fukuhara S, Sako K, Noda K, Zhang J, Minami M, Mochizuki N. Angiopoietin-1/Tie2 receptor signaling in vascular quiescence and angiogenesis. *Histol Histopathol*. 2010;25:387-396.
27. Moss A. The angiopoietin: Tie2 interaction: a potential target for future therapies in human vascular disease. *Cytokine Growth Factor Rev*. 2013;24:579-592.
28. Sturk C, Dumont DJ. Tyrosine phosphorylation of Grb14 by Tie2. *Cell Commun Signal*. 2010;8:30.
29. Moon HE, Byun K, Park HW, et al. COMP-Ang1 potentiates EPC treatment of ischemic brain injury by enhancing angiogenesis through activating AKT-mTOR pathway and promoting vascular migration through activating Tie2-FAK pathway. *Exp Neurobiol*. 2015;24:55-70.
30. Qiu L, He B, Hu J, Zhu Z, Liu X, Zhu J. Cartilage oligomeric matrix protein angiopoietin-1 provides benefits during nerve regeneration in vivo and in vitro. *Ann Biomed Eng*. 2015;43:2924-2940.