

A Potential Role for Angiopoietin 2 in the Regulation of the Blood–Retinal Barrier in Diabetic Retinopathy

Sampathkumar Rangasamy,¹ Ramprasad Srinivasan,¹ Joann Maestas,¹ Paul G. McGuire,¹ and Arup Das^{2,3}

PURPOSE. Although VEGF has been identified as an important mediator of the blood–retinal barrier alteration in diabetic retinopathy, the hypothesis for this study was that other molecules, including the angiopoietins (Ang-1 and -2), may play a role. The expression of angiopoietins was analyzed in an animal model of diabetic retinopathy, and the role of Ang-2 in the regulation of diabetes-induced alterations of vascular permeability was characterized.

METHODS. Diabetes was induced in rats, and human retinal endothelial cells (HRECs) were grown in media with 5.5 or 30.5 mM glucose. Levels of Ang-1 and -2 mRNA and protein were analyzed. Fluorescence-based assays were used to assess the effect of Ang-2 on vascular permeability *in vivo* and *in vitro*. The effect of Ang-2 on VE-cadherin function was assessed by measuring the extent of tyrosine phosphorylation.

RESULTS. Ang-2 mRNA and protein increased in the retinal tissues after 8 weeks of diabetes and in high-glucose-treated cells. Intravitreal injection of Ang-2 in rats produced a significant increase in retinal vascular permeability. Ang-2 increased HREC monolayer permeability that was associated with a decrease in VE-cadherin and a change in monolayer morphology. High glucose and Ang-2 produced a significant increase in VE-cadherin phosphorylation.

CONCLUSIONS. Ang-2 is upregulated in the retina in an animal model of diabetes, and hyperglycemia induces the expression of Ang-2 in isolated retinal endothelial cells. Increased Ang-2 alters VE-cadherin function, leading to increased vascular permeability. Thus, Ang-2 may play an important role in increased vasopermeability in diabetic retinopathy. (*Invest Ophthalmol Vis Sci.* 2011;52:3784–3791) DOI:10.1167/iovs.10-6386

Diabetic retinopathy is the leading cause of visual impairment and blindness in diabetic patients in both developed and developing nations.¹ One of the early events in diabetic retinopathy is the alteration of the blood–retinal barrier (BRB) leading to the increased permeability of blood vessels, resulting in diabetic macular edema. The development of macular edema is a progressive pathologic process characterized by

hyperglycemia-induced damage to the vessel wall. The integrity of the BRB is maintained by the presence of specialized intracellular junctional molecules between adjacent endothelial cells as well as by adhesive interactions between endothelial cells and associated pericytes. Dysregulation of these junctions and the associated loss of cell–cell contact in response to hyperglycemia can lead to altered retinal vascular permeability.²

Vascular endothelial growth factor (VEGF) has been the primary factor implicated in the alteration of retinal vascular function leading to diabetic macular edema. This finding has led to several ongoing clinical trials of anti-VEGF treatments.^{3,4} Treatment with anti-VEGF appears to have limitations, as the improvement in retinal thickness is transient, and the edema tends to recur in most patients, suggesting that other factors play a role.⁵ Indeed, one such factor that has been suggested to play a role, along with VEGF, in the regulation of endothelial cell permeability is Ang-2.⁶

The angiopoietins are a family of growth factors that bind to the endothelial receptor tyrosine kinase Tie-2 and regulate vascular development and function.⁷ Angiopoietin (Ang)-1 and -2 share 60% amino acid identity and bind with similar affinity to Tie-2. The activity of Tie-2 is differentially regulated by the two ligands. Ang-1 is a strong agonist of the Tie-2 receptor, and Ang-2 acts as an agonist or antagonist in a context-dependent manner.⁸ The primary source of Ang-1 has been shown to be from nonendothelial cells, including pericytes (periendothelial cells), but little is known about its regulation of expression.⁹ Ang-2 is predominantly expressed in endothelial cells, stored in vesicles known as Weibel–Palade bodies, and is rapidly released in response to specific stimuli.¹⁰ Emerging evidence indicates that Ang-2 is upregulated in response to hyperglycemia and plays an important role in the pathogenesis of retinal diseases.^{11–16} A potential role for Ang-2 in altering vascular permeability, however, is not well understood.

The cadherins are a family of proteins that mediate calcium-dependent homophilic adhesion between cells. Of particular importance to the endothelial cells of the vasculature is the vascular endothelial cadherin or (VE)-cadherin.^{17–19} The integrity of the VE-cadherin junctions between adjacent endothelial cells is considered to be critical for normal barrier function and is likely to involve interactions between VE-cadherin and the tight junction proteins occludin and claudin-5.^{20,21} Loss of VE-cadherin function by proteolysis or by phosphorylation has been implicated in the pathologic changes related to altered vascular permeability seen in diabetic retinopathy.^{22,23} Several factors have been shown to regulate the function of VE-cadherin; however, the role of Ang-2 as a mediator of altered VE-cadherin function has not been reported.

In the present study, we hypothesized that Ang-2 may be an important mediator in the alteration of the blood–retinal barrier in diabetes. We examined the expression of Ang-2 in the diabetic retina and in human microvascular endothelial cells exposed to high glucose. The effect of Ang-2 on vascular

From the ¹Department of Cell Biology and Physiology, ²Division of Ophthalmology, Department of Surgery, University of New Mexico School of Medicine, Albuquerque, New Mexico; and the ³New Mexico VA Health Care System, Albuquerque, New Mexico.

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Corresponding author: Paul G. McGuire, Department of Cell Biology and Physiology, MSC08 4750, 1 University of New Mexico, Albuquerque, NM 87131; pmcguire@salud.unm.edu.

permeability was assessed in vivo and in vitro using fluorescence-based assays. We also assessed the effect of Ang-2 on VE-cadherin by measuring the extent of tyrosine phosphorylation of this adherens junction protein.

METHODS AND METHODS

Reagents and Antibodies

Recombinant Ang-1 and human Tie-2/Fc chimera were obtained from R&D Systems (Minneapolis, MN), and recombinant Ang-2 was purchased from Abcam (Cambridge, MA). The polyclonal antibody to VE-cadherin (ALX-210-232) was obtained from Enzo Life Sciences (Plymouth Meeting, PA). Rabbit polyclonal antibodies to phosphorylated VE-cadherin (phospho-Y658 and phospho-Y731) were purchased from Abcam. All other reagents were purchased from Sigma-Aldrich (St. Louis MO), unless otherwise stated.

Cell Culture

Human retinal microvascular endothelial cells (HRECs) (ACBRI-181) were obtained from Cell Systems (Kirkland, WA). The cells were grown on fibronectin-coated cell culture dishes in MCDB-131 supplemented with 10% FBS, 10 ng/mL EGF, 1 μ g/mL hydrocortisone, 0.2 mg/mL endothelial culture medium [EndoGro; Millipore, Billerica, MA], and 0.09 mg/mL heparin (pH 7.3; VEC Technologies, Rensselaer, NY). The cells were grown to confluence and incubated in MCDB-131 in the presence of either high (30.5 mM) or low (5.5 mM) glucose for 3 to 5 days. Some cells were grown in 5.5 mM glucose and 25 mM mannitol as an osmotic control. Passages 3 to 8 were used for all experiments.

Animal Model

Male Sprague-Dawley rats (Harlan Animal Research Laboratory, Indianapolis, IN) were used for these studies. Diabetes was induced by a single intraperitoneal injection of streptozotocin (60 mg/kg body weight) in 10 mM citrate buffer (pH 4.5). Animals with plasma glucose concentrations greater than 250 mg/dL 24 to 48 hours after streptozotocin injection were considered diabetic and were used in the study at 8 weeks after the induction of diabetes. Blood glucose levels and body weight were monitored regularly. Animals received insulin (0.5 IU) as needed to maintain body weight. The tolerated maximum difference in weight between diabetic and nondiabetic rats was 40%. Age-matched nondiabetic animals were used as comparison controls. All animal studies were consistent with and adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Real-Time PCR

Total mRNA was extracted from the retinas of control and diabetic rats at 8 weeks after the induction of diabetes, and from HRECs treated with low glucose, high glucose, or low glucose and mannitol. RNA was extracted (RNAqueous Kit; Ambion, Austin, TX) and first-strand cDNA was synthesized from 1 μ g total RNA (High-Capacity cDNA Reverse Transcription Kit; Applied Biosystems, Inc. [ABI], Foster City, CA). The following genetic analysis assays (TaqMan; ABI) for real-time RT-PCR were obtained from ABI: rat Ang-2 (Rn01756774), rat Ang-1 (Rn00585552), human Ang-2 (Hs01048043), human VEGF (Hs00900055), and 18s RNA (Hs99999901). Amplification and detection were then performed on a fast real-time PCR system (7500 Fast; ABI). Data were derived by using the comparative Ct method for duplicate reactions with normalization to the housekeeping gene 18s RNA.²⁴

Assessment of Ang-2-Induced BRB Breakdown in Sprague-Dawley Rats

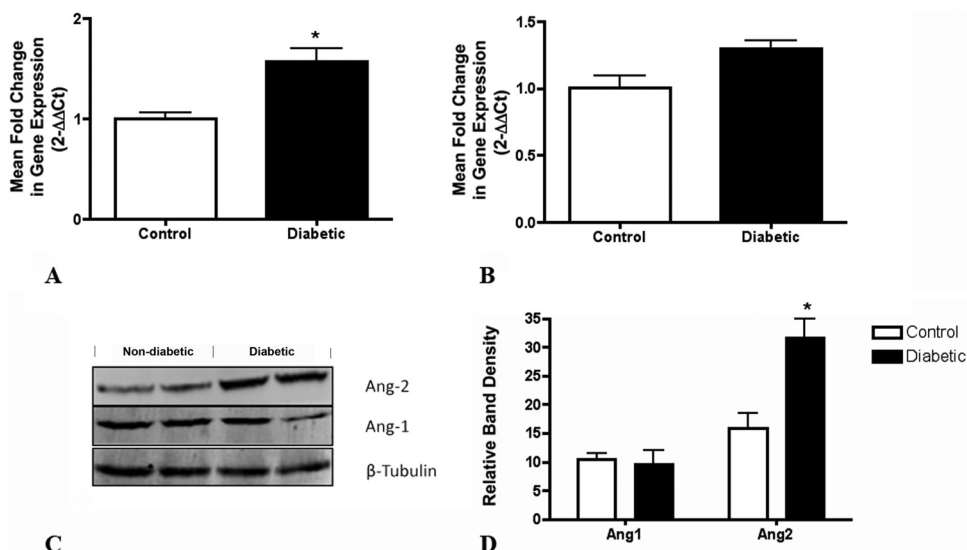
Vascular permeability in the retina of Sprague-Dawley rats was measured by using a modification of the Evans blue dye procedure.²⁵ The rats were injected via the femoral vein with 45 mg/kg of FITC-labeled albumin 16 hours after an intraocular injection of 100 ng of recombinant Ang-2 (A9771; Sigma-Aldrich). The fluorescence of retinal extracts and a 1:1000 dilution of plasma were measured at 485 nm (Ex) and 515 nm (Em). The concentrations of FITC-albumin was calculated from a standard curve of FITC-labeled albumin in PBS, and BRB permeability was calculated as follows and was expressed as microliters of plasma per gram retinal wet weight⁻¹ · hour⁻¹:

$$\frac{\text{FITC-albumin retina (mg)/retina dry weight (g)}}{\text{FITC-albumin concentration (mg)/plasma (\mu\text{L})} \times \text{circulation time (h)}}$$

Human Retinal Endothelial Cell Permeability Assay

In vitro permeability assays were performed using cell culture inserts (Falcon; BD Biosciences, Bedford, MA) containing a membrane of 0.4- μ m pore size. Human retinal microvascular endothelial cells (20,000 cells) were plated onto membranes coated with fibronectin and grown in MCDB-131 complete medium. The cells were allowed to grow to confluence and form a monolayer. Ang-2 was added in 0.2 mL of medium to the insert, and the lower chamber was filled with 0.8 mL of medium and incubated at 37°C for 24 hours. After incubation, FITC-labeled dextran (40 kDa) was added to the insert at a final concentration of 100 μ M and a 50- μ L aliquot was collected from the

FIGURE 1. Ang-2 was significantly increased in the rat retina after 8 weeks of diabetes. (A) Retinas from diabetic animals ($n = 4$) demonstrated significantly more Ang-2 mRNA than retinas from nondiabetic controls ($n = 6$). (B) No significant change in Ang-1 mRNA was detected. *Significantly greater than in the nondiabetic controls ($P = 0.031$). (C) A representative Western blot demonstrates a significant elevation in Ang-2 expression in the diabetic compared with control retina. (D) Quantitation of Ang-1 and -2 protein levels in control nondiabetic retinas ($n = 5$) and diabetic retinas ($n = 3$). *Significantly greater than nondiabetic controls ($P = 0.0184$). Data are expressed as the mean \pm SEM.



lower chamber after 30 minutes. The aliquots were diluted 1:1 in PBS and the FITC-dextran concentration was measured fluorometrically at an excitation wavelength of 485 nm.

Measurement of Transendothelial Cell Resistance

Measurements of transendothelial electrical resistance were performed with the electrical cell-substrate impedance sensing (ECIS) system (*Zθ*; Applied Biophysics, Troy, NY) as described previously.²⁶ Briefly, HRECs were seeded onto fibronectin-coated gold microelectrodes in ECIS culture wells (8W10E⁺) and incubated overnight at 37°C. The cells were transferred to serum-free medium, and the resistance of the confluent monolayers was monitored for 1 hour to establish the baseline resistance, which for HRECs was approximately 1200 Ω. Test substances were added to each well, and monitoring was continued for up to 15 hours. Resistance values for 2000 to 4000 cells per well were averaged for each acquisition time point. The data from duplicate or triplicate wells were averaged and presented as normalized resistance versus time.

Western Blot

Cells, concentrated conditioned medium, or retinal tissues were solubilized in 2× sample buffer, separated by 10% SDS PAGE gels under reducing conditions, and transferred to polyvinylidene difluoride membranes (Hybond-P; GE Health Care, Piscataway, NJ). The membranes were blocked with blocking buffer (Odyssey; LI-COR, Lincoln, NE) and probed with primary antibodies to Ang-1, Ang-2, Tie-2, Caspase 3, cleaved Caspase 3, VE-cadherin, and phospho-VE-cadherin (phospho-Y685 and phospho-Y731). IRDye800 conjugated anti-rabbit and anti-mouse antibodies were used to visualize the bands. The membranes were analyzed with an infrared imaging system (Odyssey; LI-COR), and where relevant, the signal intensity was determined with imaging software (LI-COR) and exported to computer (Prism; GraphPad, San Diego, CA) for graphic representation as the mean ± SD. Analyses of conditioned media samples were normalized to total number of cells, as estimated by crystal violet staining. Media are collected, and the cells were fixed with 1% glutaraldehyde for 15 minutes. After they were washed, the cells were incubated with 0.1% crystal violet in deionized water for 30 minutes. They were then washed for 30 minutes and extracted with 0.2% Triton X-100 and the absorbance read at 590 nm.²⁷ For cell and tissue extracts, the blots were normalized to β-tubulin levels.

Immunofluorescence Microscopy

HRECs were grown to confluence in chamber slides and treated with Ang-2 for 24 hours. The cells were washed in PBS and fixed in 3:1 methanol/acetic acid for 2 minutes and washed with PBS. They were then stained with an anti-VE-cadherin antibody and examined using a fluorescence microscope. Images were captured and pseudocolored with an image-management program (Meta Morph; Universal Imaging Corp., Downingtown, PA).

Statistical Methods

For all quantitative experiments, statistical analyses of data were performed with either an unpaired *t*-test or a one-way ANOVA (Prism4 software; GraphPad). Differences among means were tested by ANOVA and corrected using the Bonferroni multiple comparison post test. Values for *P* < 0.05 were considered significant.

RESULTS

Angiopoietin Expression in the Retinas of Diabetic Animals

The expression of Ang-1 and Ang-2 was determined by real-time PCR and Western blot analyses in the retinas of animals

after 8 weeks of diabetes. Compared to the control nondiabetic animals, there was no significant change in the expression of Ang-1 mRNA in the retinas of the diabetic animals (Fig. 1A). In contrast, the expression of Ang-2 mRNA was significantly elevated in the retinas of diabetic animals compared with that in the controls (Fig. 1B). The elevated expression of Ang-2 mRNA correlated with differences in protein levels as determined by Western blot analysis of whole retina extracts (Fig. 1C). Normalization to tubulin levels, and relative quantitation of band density, revealed no significant difference in Ang-1 protein amounts between the diabetic and control nondiabetic animals. In contrast, the level of Ang-2 protein was significantly elevated in the retinas of diabetic animals compared with the level in the controls (Fig. 1D). Quantitation of Tie-2 receptor levels in retinal extracts from diabetic and control animals, demonstrated a modestly significant downregulation of Tie-2 in the diabetic animals compared with that in the controls (2.82 ± 0.294 vs. 1.83 ± 0.201 ; *P* = 0.0491). Since Ang-1 and -2 compete for binding to the Tie-2 receptor with similar affinities, differences in their levels may determine which protein binds and the resulting cellular response. The relative ratio of Ang-2 to -1 was determined and found to be nearly three times higher in the diabetic retinas compared with that in the controls (3.62 ± 0.65 vs. 1.25 ± 0.63).

High-Glucose Regulation of Ang-2 Expression in Retinal Microvascular Endothelial Cells

To determine whether hyperglycemia itself can modify Ang-2 expression, HRECs were treated with low glucose (5.5 mM), high glucose (30.5 mM) or low glucose and mannitol (25 mM) for 72 hours, and the Ang-2 mRNA expression levels were determined by PCR analysis. The Ang-2 mRNA levels were found to be elevated in high glucose-treated cells compared with cells grown in normoglycemic conditions (Fig. 2A). The level of secreted Ang-2 protein in the endothelial cell-conditioned medium was evaluated by Western blot. The high-glucose-treated cultures showed a nearly threefold increase in the level of Ang-2 protein in the medium compared with that in cells grown in normal levels of glucose. We also found that incubation of cells in 25 mM mannitol, an osmotic control, did not significantly alter the secretion of Ang-2 in the retinal endothelial cells (Fig. 2B). Ang-1 was not detected in HRECs in culture, consistent with its reported pattern of expression (data not shown). Taken together, these results demonstrate that the hyperglycemic condition induces a high level of Ang-2 secretion by the retinal microvascular endothelial cells.

Ang-2 Regulation of Retinal Endothelial Cell Permeability and Monolayer Resistance In Vitro

The ability of Ang-2 to alter the retinal endothelial cell barrier was examined in vitro. Endothelial cells stimulated with recombinant Ang-2 at concentrations of 20, 100, and 200 ng/mL demonstrated a dose-dependent increase in monolayer permeability (Fig. 3A). This increase in permeability was not associated with a change in VEGF expression in these cells after Ang-2 treatment (Fig. 3B). Endothelial cells were also examined by using ECIS to specifically determine the functional integrity of the endothelial intercellular junctions. Untreated cells grown in serum-free medium demonstrated a uniform and consistent level of resistance across the monolayer. The addition of Ang-2 at either 100 or 200 ng/mL resulted in a 4% or 9% reduction in resistance, respectively (Fig. 3C). As a positive control, some cultures were treated with 50 ng/mL VEGF, which resulted in a 19% reduction in resistance after 15 hours of incubation.

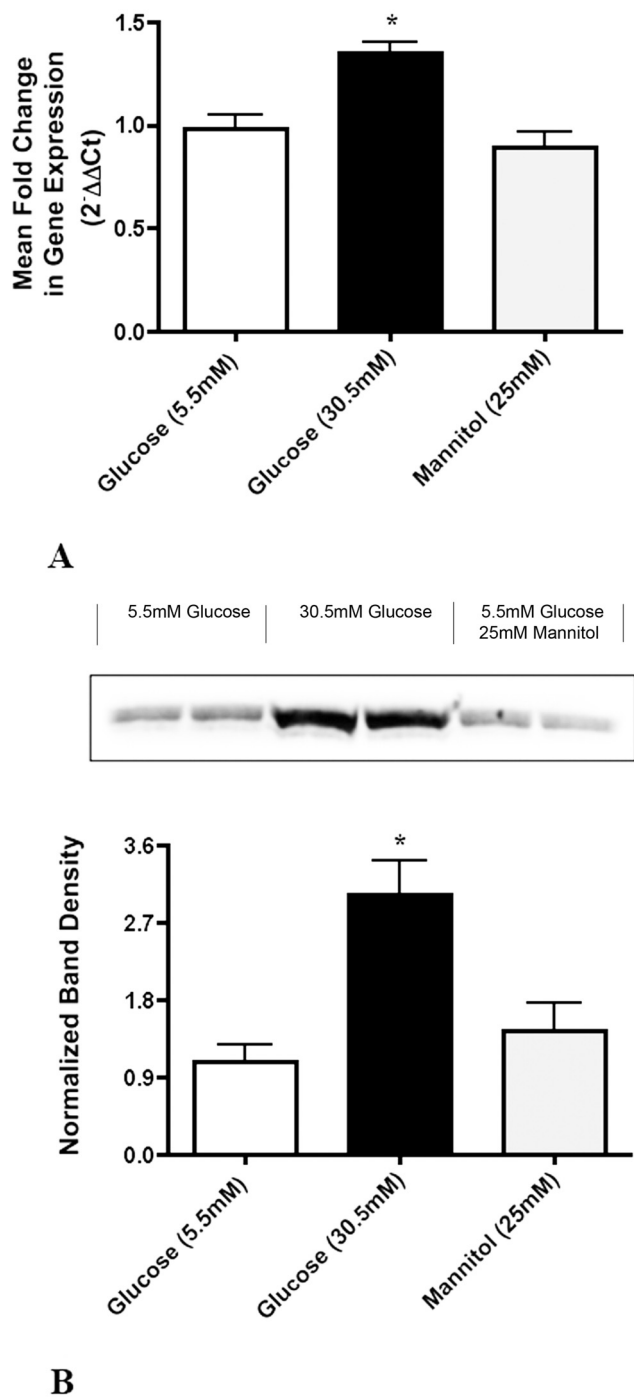


FIGURE 2. High glucose increased the expression and secretion of Ang-2 in isolated human retinal microvascular endothelial cells. **(A)** Ang-2 mRNA levels from cells grown in 30.5 mM glucose was significantly greater than that from cells grown in 5.5 mM glucose or 5.5 mM glucose plus 25 mM mannitol (5.5 mM glucose vs. 30.5 mM glucose, $P < 0.01$; 25 mM mannitol vs. 30.5 mM glucose, $P < 0.05$). **(B)** The relative level of Ang-2 protein in the conditioned medium of cells grown in 5.5 glucose, 30.5 glucose, or 5.5 mM glucose and 25 mM mannitol. Quantitation of band density and normalization to total cell number revealed a significant elevation of Ang-2 protein in the high glucose-treated cells (5.5 mM glucose vs. 30.5 mM glucose, $P < 0.01$; 25 mM mannitol vs. 30.5 mM glucose, $P < 0.05$). Data are expressed as the mean \pm SEM.

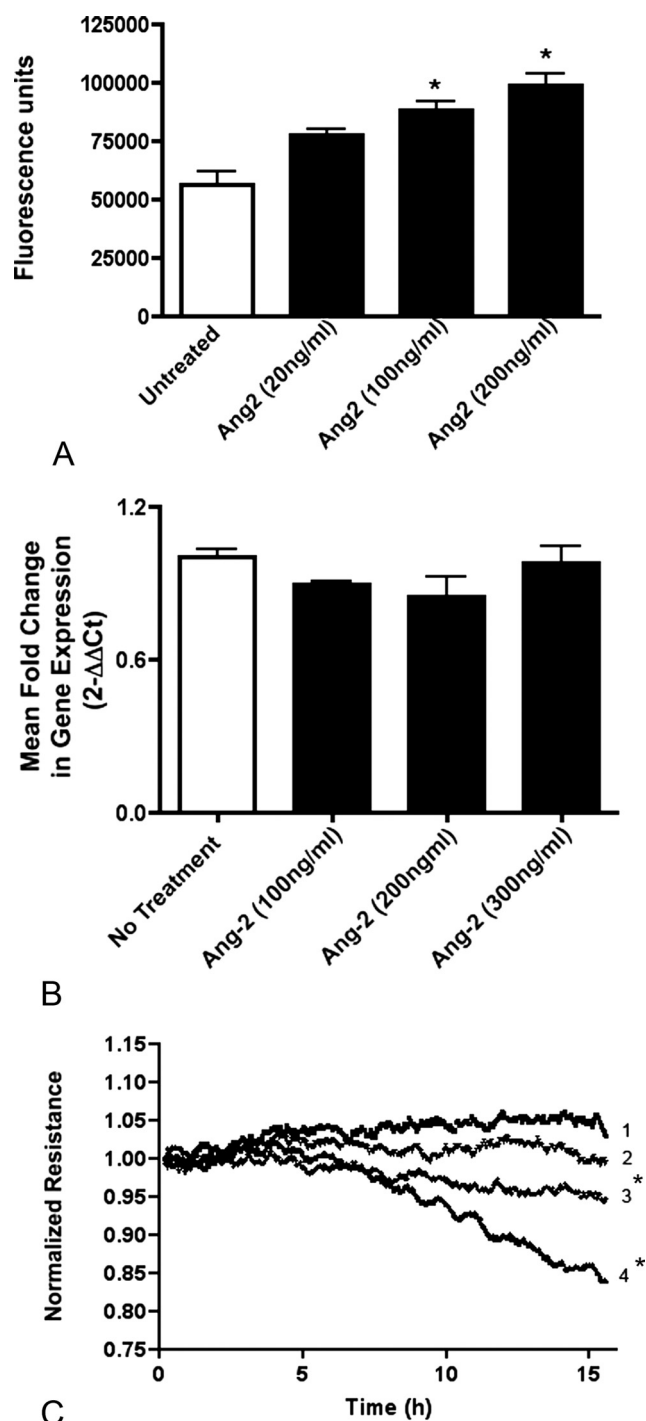


FIGURE 3. Ang-2 alters the endothelial cell barrier. **(A)** Monolayer permeability to FITC-dextran after Ang-2 treatment. *Significantly greater than untreated cells (untreated versus Ang-2 [20 ng/mL], $P > 0.05$; untreated versus Ang-2 [100 ng/mL], $P < 0.01$; untreated versus Ang-2 [200 ng/mL]; $P < 0.001$). Values represent the mean \pm SEM from triplicate wells. **(B)** Expression of VEGF by human retinal endothelial cells treated with increasing concentrations of Ang-2 for 24 hours. No significant difference was noted at any concentration. **(C)** Representative ECIS tracing plotted as normalized resistance reflecting the decrease in resistance of endothelial cell monolayers to 100 ng/mL Ang-2 (tracing 2), 200 ng/mL Ang-2 (tracing 3), 50 ng/mL VEGF (tracing 4) compared with untreated cells (tracing 1). *Significantly less than untreated cells (VEGF versus untreated, $P < 0.001$; Ang-2 vs. untreated, $P < 0.01$).

Ang-2 Alteration of Endothelial Cell Morphology

Previous studies have shown that the expression of VE-cadherin by microvascular retinal endothelial cells plays an important role in the maintenance of the endothelial barrier both *in vivo* and *in vitro*.¹⁹ The change in permeability observed in the retinal endothelial cells treated with Ang-2 was accompanied by structural changes in the morphology of the endothelial monolayer. The control untreated cells demonstrated a continuous pattern of VE-cadherin staining associated with the lateral cell borders and only occasional gaps between cells (Fig. 4A). In cultures treated for 24 hours with recombinant Ang-2, the cells displayed an increased number of openings or gaps between cells at multiple points throughout the monolayer and an associated loss of VE-cadherin staining at these locations (Fig. 4B). There was no evidence of increased apoptosis in the Ang-2-treated cells based on levels of cleaved caspase-3. There was no decrease in the level of full-length caspase-3 and no detection of the cleaved form after Ang-2 treatment (Fig. 4C). These data suggest a mechanism by which Ang-2 modifies the BRB through the alteration of VE-cadherin between adjacent endothelial cells.

Ang-2 Regulation of Retinal Vascular Permeability *In Vivo*

Ang-2 also modulates vascular permeability *in vivo*. Normal nondiabetic rats receiving a single intraocular injection of recombinant purified Ang-2 showed a nearly threefold increase in retinal vascular permeability 16 hours after injection compared with animals receiving a control injection (Fig. 5). The injection of Ang-2 did not induce the expression of Tie-2 in these animals. Western blot analysis of retinal extracts from PBS and Ang-2 injected animals showed no significant difference in the levels of Tie-2 protein (2.93 ± 0.29 vs. 2.35 ± 0.40 ; $P = 0.313$).

Ang-2 Mediates Phosphorylation of VE-Cadherin in Retinal Endothelial Cells and in the Retina

Several studies have reported that tyrosine phosphorylation of various adhesion and tight junctional proteins may lead to the loss of junctional integrity and altered vascular permeability. HREC incubated in either high glucose or Ang-2 demonstrated significantly more phosphorylated VE-cadherin compared with cells grown in low glucose or mannitol (Fig. 6). The high-glucose response was mediated by Ang-2, as cells in high glucose treated with 200 ng/mL recombinant Ang-1 or the Ang-1 and Ang-2 inhibitor Tie-2/Fc chimera showed significantly reduced levels of phosphorylated VE-cadherin. Similar phosphorylation of other junctional proteins, including the tight junction proteins ZO-1 and claudin-5, was examined. No change in the phosphorylation state of these proteins was observed (data not shown).

Extracts of the retina from mice receiving an intraocular injection of Ang-2 demonstrated increased phosphorylation of VE-cadherin compared with rats injected with PBS alone. In addition, the retinas from diabetic rats displayed increased VE-cadherin phosphorylation compared with control nondiabetic animals (Fig. 7).

DISCUSSION

Disruption of the blood-retinal barrier in patients with diabetes mellitus leads to leakage of fluid from the vasculature and subsequent retinal edema,²⁸ resulting in a significant visual impairment. The mechanisms by which hyperglycemia mediates the edema seen in diabetic retinopathy are not completely understood. Much of the previous work has focused on the role of the

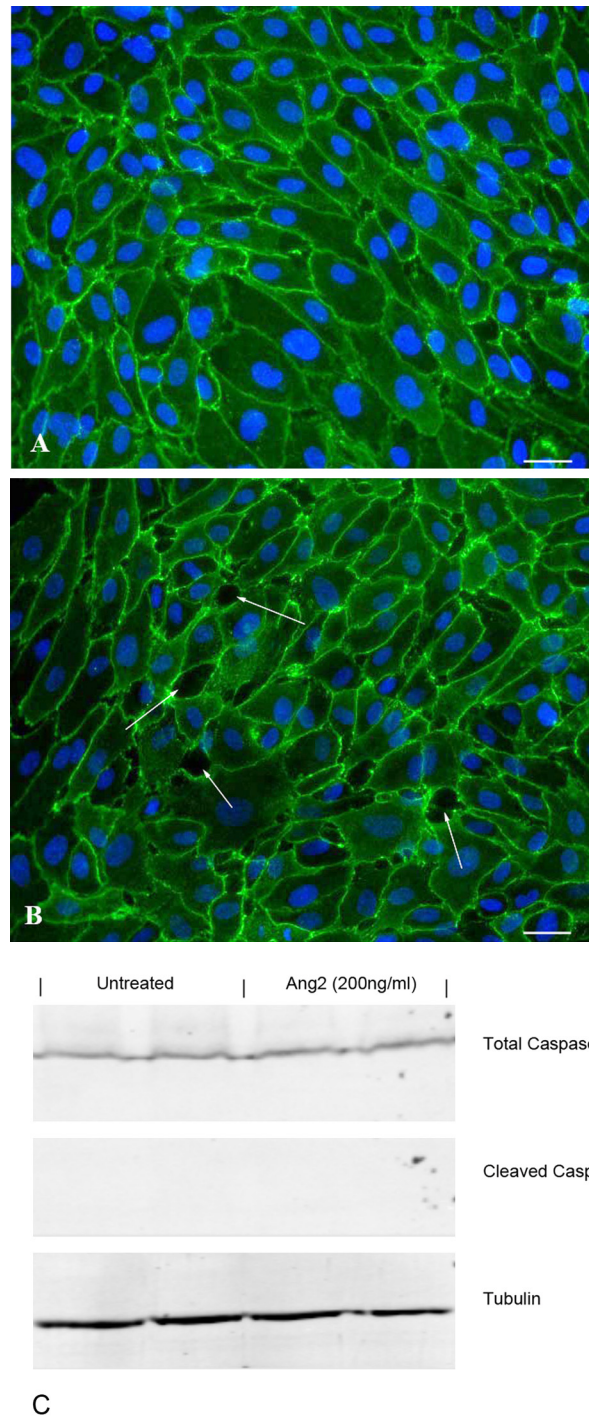


FIGURE 4. Ang-2 induces altered cell surface expression of VE-cadherin and the formation of intercellular gaps in an endothelial cell monolayer. Representative images of VE-cadherin staining in human retinal endothelial cells in untreated (A) or Ang-2-treated (200 ng/mL) (B) conditions. A disruption of the continuous pattern of VE-cadherin staining along cell borders and the presence of open gaps between adjacent cells (arrows) is seen in the Ang-2 treated cultures. (C) Representative Western blot of cleaved and full-length caspase-3 levels in control cells and cells treated with Ang-2. β -tubulin was used as a loading control. Bar, 10 μ m.

well-known permeability-inducing growth factor, VEGF^{29,30} in diabetic macular edema. The data suggest that hyperglycemia induces the overexpression of Ang-2 by cells of the retina which may also play a significant role in the alteration of the blood-

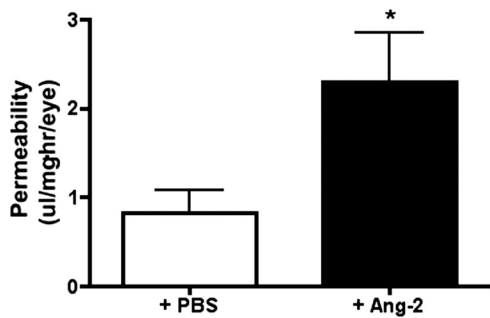


FIGURE 5. Intraocular injection of purified Ang-2 increased retinal vascular permeability. Normal nondiabetic rats receiving an intravitreal injection of purified Ang-2 (100 ng) show a nearly threefold increase in the extravascular accumulation of FITC-BSA in the retinal tissues compared to animals receiving an injection of PBS alone (2.291 ± 0.556 , $n = 4$ vs. 0.831 ± 0.254 , $n = 4$). *Significantly different, $P = 0.014$.

retinal barrier. Previous studies have demonstrated the combined action of VEGF and Ang-2 in endothelial cell permeability, although the mechanisms for this synergistic effect are not well understood.³¹ Since VEGF is also upregulated in the early stages of diabetic retinopathy, the combined action of VEGF and Ang-2 may have a profound effect on the regulation of vascular permeability in the progression of this disease.

In the present study, we observed a significant increase in the expression of Ang-2 mRNA and protein in the retina of rats with 8 weeks of diabetes. A similar finding for increased Ang-2 has been reported in the vitreous and plasma of patients with diabetic retinopathy although its role in mediating changes in

vascular permeability has not been fully addressed.^{11,32} Others have shown that Ang-2 may be important in mediating the loss of pericytes, a characteristic of the vascular changes seen in diabetic retinopathy.^{13,33} Since pericytes have been shown to be important in the maturation and maintenance of the microvasculature, the loss of pericytes, induced by Ang-2, may lead to alterations in endothelial cell behavior and subsequent permeability changes. In the present study, intravitreal injection of Ang-2 caused a significant increase in the accumulation of FITC-BSA in the retina. Although this may reflect changes in several vascular hemodynamic factors in addition to permeability, Ang-2 may have altered vascular permeability directly at the level of intercellular junctions, or it may have altered permeability via the loss of pericytes and alterations of vascular homeostasis. Analysis of the functional properties of the endothelial barrier by ECIS suggests that Ang-2 can modify the endothelial cells directly at the level of the intercellular junctions. Although not to the same degree as the classic endothelial permeability factor VEGF, Ang-2 was able to elicit a dose-dependent decrease in monolayer resistance. Others have reported that a change in resistance, as measured by ECIS, correlates with changes in junctional and cytoskeletal organization that could affect permeability.³⁴⁻³⁶ The effect of Ang-2 appeared to be an independent one, as no change in VEGF expression was noted in cells treated with Ang-2 for an extended period. Based on the results of the in vitro experiments in the present study, we propose that the increased expression of Ang-2 in diabetes has the potential to affect endothelial cells directly and alter their barrier properties.

Previous studies have suggested that the primary function of Ang-2 is as an antagonist to the Ang-1/Tie-2 interactions that

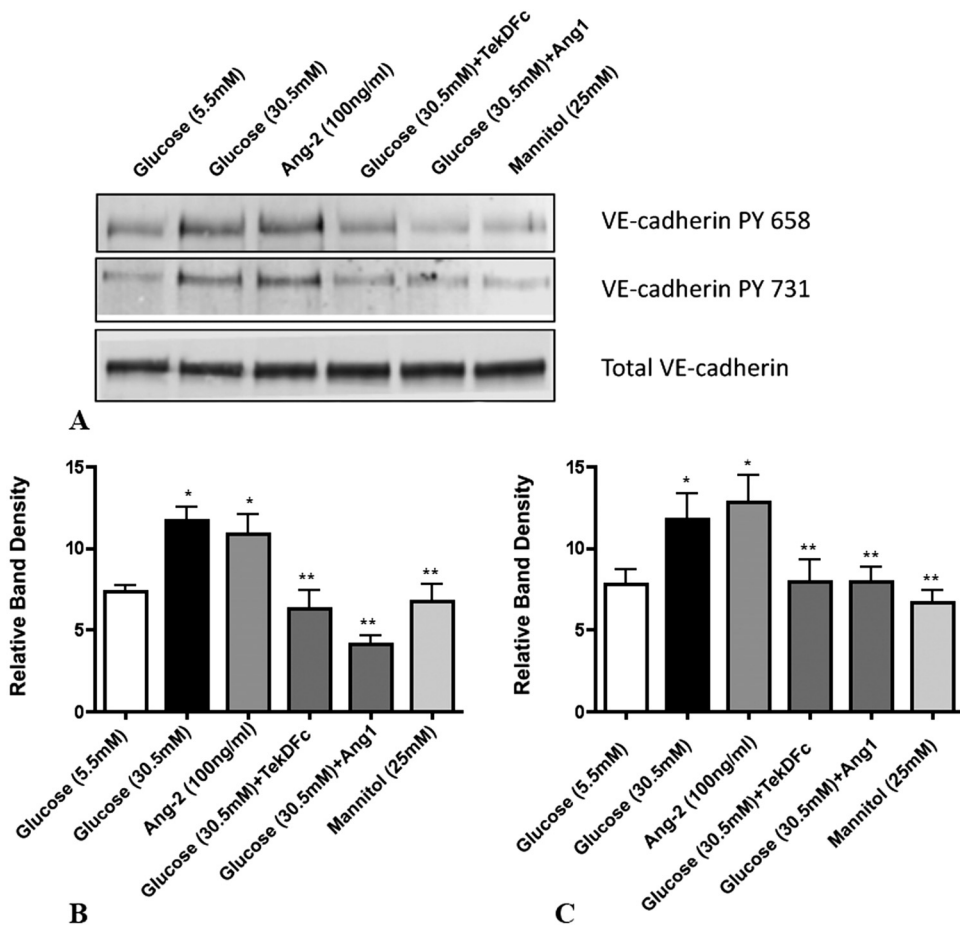


FIGURE 6. High glucose and Ang-2 induce VE-cadherin phosphorylation in vitro. (A) Representative Western blot image of phospho-VE-cadherin in human retinal endothelial cells. Quantitation of band density for P-Y658 (B) and P-Y731 (C) relative to total VE-cadherin for the indicated treatment conditions. *Significantly greater than 5.5 mM glucose. **Significantly less than 30.5 mM glucose alone.

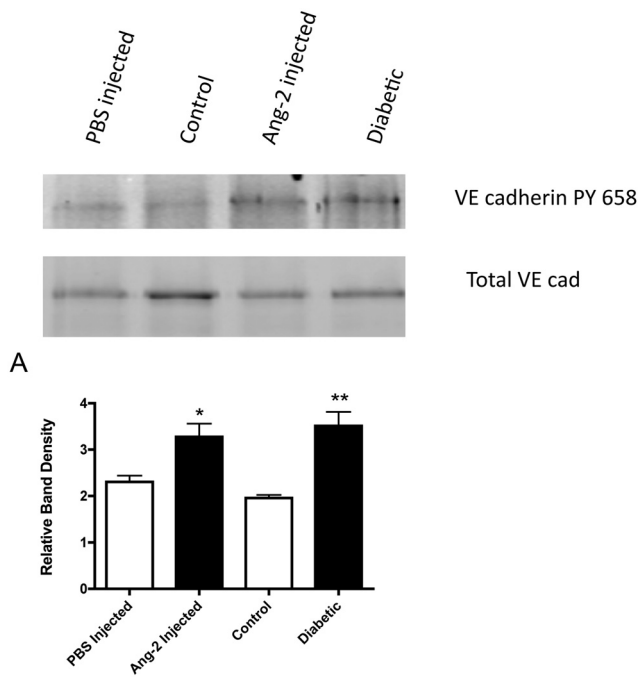


FIGURE 7. Increased VE-cadherin phosphorylation in the retina in response to Ang-2 and diabetes. (A) Representative Western blot image of phospho-VE-cadherin in rat retina extracts. Quantitation of band density for P-Y658 relative to total VE-cadherin for the indicated experimental conditions. *Significantly greater than in the PBS injected animals ($P = 0.04$). **Significantly greater than in the control nondiabetic animals ($P = 0.01$).

help to stabilize microvessels.⁸ The present study has demonstrated that Ang-2 can function as an agonist to retinal microvascular endothelial cells and modify cell behavior. Indeed other studies have suggested that Ang-2 could act as an antagonist or agonist, depending on the context and the concentrations of Ang-1 and -2.^{37,38} In an important finding, the expression of Ang-2 increased within the retina of diabetic animals, with a consistent low level of Ang-1 expression. This expression pattern correlated with a significant increase in the ratio of Ang-2 to Ang-1 protein. Because the affinity of the Tie-2 receptor for Ang-1 and -2 is similar,⁸ elevated levels of Ang-2 protein would be expected to successfully compete with Ang-1 for binding to this receptor and induce its effects, distinctly different from those resulting from Ang-1 stimulation.

Similar to the *in vivo* condition, Ang-2 expression is increased in isolated retinal microvascular endothelial cells in response to elevated glucose levels. The mechanism by which elevated glucose mediates increased angiopoietin expression in endothelial cells has not been fully elucidated. Studies have shown that glucose may influence gene expression in certain cell types by either direct binding of glucose to cell surface receptors or through a product of its metabolism within the cell that subsequently leads to intracellular signaling events.³⁹ Studies have also reported that the induction of endothelial cell dysfunction in response to hyperglycemia can be mediated by increased oxidative stress.⁴⁰ In addition to ROS, transcriptional regulators of Ang-2 have been shown to be modified by methylglyoxyl, a compound important in the formation of the advanced glycation endproducts common in diabetes.⁴¹ These reports all demonstrate the wide variety of possible mechanisms involved in the response of the microvasculature to hyperglycemic conditions leading to increased Ang-2 expression.

Most of the studies investigating Ang-1 and -2 in altered retinal vascular function have focused primarily on their roles in retinal angiogenesis.^{12,42} Although Ang-2 expression has been shown to be increased in diabetes and may be important in mediating pericyte dropout in the retina, no reports have provided a definitive role for Ang-2 in the regulation of retinal vascular permeability changes in diabetes. Several reports provide correlative evidence of a role for Ang-2 in mediating changes in cerebrovascular or pulmonary vascular permeability in conditions other than diabetes.⁴³ Peters et al.⁶ have proposed a role for Ang-2 in modification of the endothelial cell barrier *in vitro* through modulation of the tight junction protein ZO-1. In the present study, the incubation of isolated retinal vascular endothelial cells with increasing concentrations of Ang-2 resulted in decreased transendothelial resistance, breakdown of the retinal endothelial cell barrier, and the formation of gaps between adjacent cells. The formation of paracellular gaps within the endothelial cell monolayer has been described previously and relies on reversible interactions between the adherens junction complex and components of the actin cytoskeleton.^{20,44}

The Ang-2-induced changes were associated with increased phosphorylation of VE-cadherin, an adhesion molecule important in maintaining the function of the endothelial cell barrier.^{20,22,45} VE-cadherin associates intracellularly with several proteins that have signaling and regulatory functions, including p120, β -catenin, and plakoglobin. In addition, interaction of this complex with α -catenin provides a means for the adherens complex to interact with the actin-containing cytoskeleton.^{20,46} Modulation of VE-cadherin adhesion between endothelial cells induces vascular permeability and can be mediated by phosphorylation of VE-cadherin or components of the adherens complex,⁴⁷ by cleavage of the VE-cadherin extracellular domains,^{22,48} or by internalization of VE-cadherin.⁴⁹ VE-cadherin in retinal microvascular endothelial cells was phosphorylated by Ang-2 at tyrosine residues pY685 and pY731. The phosphorylation at these sites has been shown to be crucial for the VE-cadherin to stay in complex with VE-PTP, plakoglobin, and p120 and to the actin cytoskeleton that anchors the cell to the matrix.⁵⁰ Ang-2 could effect this phosphorylation through the downregulation of VE-PTP, a phosphatase that is associated with VE-cadherin and helps to maintain the phosphorylation status of VE-cadherin and its associated proteins.

Overall, the present study highlights the involvement of Ang-2 in the breakdown of the BRB through alteration of VE-cadherin at cell-cell contacts in endothelial cells. The Ang-2/Tie-2 system could serve as an alternative therapeutic target in diabetic macular edema. Perhaps a strategy of combination therapy, including the inhibition of both VEGF and Ang-2, could more effectively treat diabetic macular edema and prevent the recurrence of this condition in diabetic patients.

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