PhD Thesis

Molecular inflammatory factors in diabetic retinopathy: Role of azurocidin

Μοριακοί παράγοντες φλεγμονής στη Διαβητική Αμφιβληστροειδοπάθεια: Ο ρόλος της azurocidin

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TABLE OF CONTENTS:

1. ABSTRACT

PURPOSE. Azurocidin, released by neutrophils during leukocyte-endothelial interaction, is a main cause of leukocyte-evoked vascular leakage. Its role in the retina, however, is unknown.

METHODS. Brown Norway rats received intravitreal injections of azurocidin and vehicle control. Blood-retinal barrier (BRB) breakdown was quantified using the Evans blue (EB) dye technique 1, 3, and 24 hours after intravitreal injection. To block azurocidin, aprotinin was injected intravenously before the intravitreal injections. To investigate if azurocidin increases retinal leukostasis, number of adherent leukocytes in the retina 2 hours and 24 hours after azurocidin injection were quantified using the Concanavalin A perfusion technique. To investigate whether azurocidin plays a role in vascular endothelial growth factor (VEGF)-induced BRB breakdown, rats were treated intravenously with aprotinin, followed by intravitreal injection of $VEGF_{164}$. BRB breakdown was quantified 24 hours later. To investigate whether azurocidin may mediate BRB breakdown in early diabetes, aprotinin or vehicle was injected intravenously each day for 10 days to streptozotocin-induced diabetic rats, and BRB breakdown was quantified. To investigate whether azurocidin may mediate BRB breakdown in endotoxin induced uveitis (EIU) as another model of leukocyte mediated retinal vascular leakage , EIU rats were treated with aprotinin or vehicle and BRB breakdown was quantified 24 hours after the EIU induction.

RESULTS. Intravitreal injection of azurocidin (20 μg) induced a 6.8-fold increase in vascular permeability compared with control at 1–3 hours ($P < 0.05$), a 2.7-fold increase at 3-5 hours ($P < 0.01$), and a 1.7-fold increase at 24 hours ($P < 0.05$). Azurocidin did not increase static retinal leukostasis at 2 hours after the intravitreal injection or 24 hours after the intravitreal injection. Aprotinin inhibited azurocidin-induced BRB breakdown by 98% ($P < 0.05$). Furthermore, treatment with aprotinin significantly suppressed VEGF-induced BRB breakdown by 93% ($P < 0.05$) , BRB breakdown in early experimental diabetes by 40.6% ($P < 0.05$) and BRB breakdown in EIU by 73% ($P<0.05$)

CONCLUSIONS. Azurocidin increases retinal vascular permeability and is effectively blocked by aprotinin. The inhibition of VEGF-induced, early diabetic BRB breakdown and EIU retinal vascular leakage with aprotinin indicates that azurocidin may be an important mediator of leukocyte-dependent BRB breakdown especially in a disease model like diabetes that leukostasis is crucial in pathogenesis. Azurocidin may become a new therapeutic target in the treatment of retinal vascular leakage, diabetic retinopathy and diabetic macular edema.

1. ΠΕΡΙΛΗΨΗ

ΣΚΟΠΟΣ.Η Azurocidin, μετά την εκκρισή της από τα ουδετερόφιλα λευκοκύτταρα κατα την διάρκεια της αλληλεπίδρασης λευκοκυττάρων -αγγειακών ενδοθηλιακών κυτταρων είναι η κύρια αιτία αυξημένης αγγειακής διαπερατότητας σαν αποτέλεσμα της προσκόλλησης των λευκοκυττάρων. Ο ρόλος της στον αμφιβληστροειδή, όμως, παραμένει άγνωστος.

ΜΕΘΟΔΟΙ. Η azurocidin δόθηκε με ενδουαλοειδικές ενέσεις σε Brown Norway αρουραίους. Η ποσοτικοποίηση της ρήξης του αιματο-αμφιβληστροειδικού φραγμού (ΑΑΦ) έγινε με την τεχνική Evans Blue (EB) 1, 3, and 24 ώρες μετά την ενδουαλοειδική ένεση. Για να μελετήσουμε αν η azurocidin προκαλεί αυξημένη προσκόλληση λευκοκυττάρων στα αγγεία του αμφιβληστροειδή, ο αριθμός των προσκολλημένων λευκοκυττάρων μετρήθηκε με την μέθοδο της Concanavalin A 2 ώρες και 24 ώρες μετά την ενδουαλοειδική ένεση με azurocidin. Για την απενεργοποίηση της azurocidin, απροτινίνη δόθηκε ενδοφλέβια πριν την ενδουαλοειδική ένεση της azurocidin. Για να μελετήσουμε αν η azurocidin παίζει ρόλο στην ρήξη του ΑΑΦ που προκαλει ο αγγειακός αυξητικός ενδοθηλιακός παράγοντας (vascular endothelial growth factor-VEGF), οι αρουραίοι παρέλαβαν ενδοφλέβια θεραπεία με απροτινίνη και το VEGF δώθηκε ενδουαλοειδικά και η ρήξη του ΑΑΦ ποσοτικοποιήθηκε 24 ώρες αργότερα. Για να μελετήσουμε αν η azurocidin παίζει ρόλο στην ρήξη του ΑΑΦ στα αρχικά στάδια του διαβήτη, απροτινίνη δωθηκε ενδοφλέβια καθημερινά για 10 μέρες σε αρουραίους που

είχαν γίνει διαβητικοί με ενδοπεριτοναική ένεση streptozotocin και η ρήξη του ΑΑΦ μετρήθηκε 2 εβδομάδες μετά την έναρξη του διαβήτη. Για να μελετήσουμε αν η azurocidin παιζει ρόλο στην ρήξη του ΑΑΦ στην ραγοειδίτιδα λόγω ενδοτοξίνης (endotoxin induced uveitis, EIU) ως μοντέλο ρήξης του ΑΑΦ με την μεσολάβηση προσκόλλημένων λευκοκυττάρων, θεραπεία με απροτινίνη δωθηκε σε αρουραιους με πειραματική ραγοειδιτιδα EIU και η ρήξη του ΑΑΦ μετρήθηκε 24 ώρες μετά την έναρξη της ραγοειδίτιδας.

ΑΠΟΤΕΛΕΣΜΑΤΑ. Ενδουαλοειδική ένεση azurocidin (20 μg) αύξησε την αγγειακή διαπερατότητα του αμφιβληστροειδούς κατα 6.8 φορές σε σύγκριση με την ομάδα ελέγχου 1-3 ώρες μετά την ένεση ($P < 0.05$), κατά 2.7-φορές στις 3-5 ώρες ($P < 0.01$) και κατά 1.7 φορές στις 24 ώρες ($P < 0.05$). Η azurocidin δεν αύξησε τον αριθμό προσκολλημένων λευκοκυττάρων στα αγγεία του αμφιβληστροειδή 2 ώρες και 24 ώρες μετά την ενδουαολειδίκη ένεση. Η απροτινίνη ανέστειλε την αύξηση της διαπερατότητας μετά απο την ενδαυαλοειδική ένεση της azurocidin κατα 98% ($P < 0.05$). Επίσης , η απροτινίνη ανέστειλε την ρήξη του ΑΑΦ μετά απο την ενδουαλοειδική ένεση VEGF κατα 93% ($P < 0.05$), μείωσε την ρήξη του ΑΑΦ σε διαβητικούς αρουραίους κατά 40.6% ($P < 0.05$) και μειώσε την αγγειακή διαπερατότητα του αμφιβληστροειδούς στην πειραματική ραγοειδίτιδα κατα 73% ($P<0.05$).

ΣΥΜΠΕΡΑΣΜΑΤΑ. Η azurocidin αυξάνει την αγγειακή διαπερατότητα του αμφιβληστροειδή και η απροτινίνη ειναι αποτελεσματικός αναστολέας της δράσης

της azurocidin στην διαπερατότητα των αγγείων του αμφιβληστροειδή. Η αναστολή της ρήξης του ΑΑΦ με την απροτινίνη στο μοντέλο διαβητικής αμφιβληστροειδοπάθειας, μετά απο ένεση VEGF και στο μοντέλο ραγοειδίτιδας είναι ένδειξη πως η azurocidin είναι πιθανό να έχει σημαντικό ρόλο στην ρήξη του ΑΑΦ μέσω προσκόλλησης λευκοκυττάρων στα αγγειακά ενδοθηλιακά κύτταρα ειδικά στον διαβήτη όπου η προσκόλληση των λευκοκυττάρων ειναι κρίσιμη στην παθογένεση του διαβητικού οιδήματος της ωχράς. Η azurocidin μπορεί να αποτέλεσει ένα καινούριο θεραπευτικό στόχο για την διαβητική αμφιβληστροεδοπάθεια και το διαβητικό οίδημα της ωχράς.

2. INTRODUCTION

Diabetes mellitus (DM), also known as simply diabetes, is a group of metabolic diseases in which there are high blood sugar levels over a prolonged period.(1) This high blood sugar produces the symptoms of frequent urination, increased thirst, and increased hunger. Untreated, diabetes can cause many complications. (2) Acute complications include diabetic ketoacidosis and nonketotic hyperosmolar coma.(3) Serious long-term complications include heart disease, stroke, kidney failure, foot ulcers and damage to the eyes.(2)

Diabetes is due to either the pancreas not producing enough insulin, or the cells of the body not responding properly to the insulin produced.(4) There are three main types of diabetes mellitus:

- Type 1 DM results from the body's failure to produce enough insulin. This form was previously referred to as "insulin-dependent diabetes mellitus" (IDDM) or "juvenile diabetes". The cause is unknown.(2)
- Type 2 DM begins with insulin resistance, a condition in which cells fail to respond to insulin properly.(2) As the disease progresses a lack of insulin may also develop.(5) This form was previously referred to as "non insulin-dependent diabetes mellitus" (NIDDM) or "adult-onset diabetes".
- Gestational diabetes, is the third main form and occurs when pregnant women without a previous history of diabetes develop a high blood glucose level.(2)

Prevention and treatment involves a healthy diet, physical exercise, not using tobacco and being a normal body weight. Blood pressure control and proper foot care are also important for people with the disease. Type 1 diabetes must be managed with insulin injections.(2) Type 2 diabetes may be treated with medications with or without insulin.(6) Insulin and some oral medications can cause low blood sugar.(7) Weight loss surgery in those with obesity is an effective measure in those with type 2 DM.(8) Gestational diabetes usually resolves after the birth of the baby.(9)

2.1 EPIDEMIOLOGY

Globally, as of 2013, an estimated 382 million people have diabetes worldwide, with type 2 diabetes making up about 90% of the cases.(10)(11) This is equal to 8.3% of the adults population,(11) with equal rates in both women and men.(12) Worldwide in 2012 and 2013 diabetes resulted in 1.5 to 5.1 million deaths per year, making it the 8th leading cause of death.(6)(13) Diabetes overall at least doubles the risk of death.(2) The number of people with diabetes is expected to rise to 592 million by 2035.(14) The economic costs of diabetes globally was estimated in 2013 at $548 billion[13] and in the United States in 2012 $245 billion.(15)

Diabetes is a serious chronic condition, which increase the risk of cardiovascular diseases, kidney failure and nerve damage leading to amputation. Furthermore the ocular complications include diabetic retinopathy (DR) which is a microvascular complication of diabetes that primarily affects capillaries, which is the leading cause of blindness among adults in the industrialized countries, affecting from 2% to 5% of the entire population.(16,17) The causes of visual decrease include diabetic macular edema/diabetic maculopathy and proliferative diabetic retinopathy with formation of abnormal new vessels that can lead to bleeding and detachment.

Diabetic macular edema (DME) is swelling and thickening at the macula, central part of retina with highest concentration of photoreceptors and responsible for high definition vision and visual acuity, is the main cause of vision loss in patients in diabetic retinopathy . DME is the leading cause of blindness in young adults in developed countries, affecting 12% of type 1 and 28% of type 2 diabetic patients.(18) Attending the increase of DME at 2030, the DME prevalence can be increased to 100 million of patients. Despite of the fact that diabetic macular edema can have a spontaneous recovery occasionally especially in milder forms (it is important to recognize that about 33% to 35% of patients resolve DME spontaneously after six months without treatment (17) the treatment of patients who developed DME, has become the most important focus in the DM patient's treatment.

DME is the major cause of vision loss associated with DR. There are approximately 93 million people with DR, 17 million with proliferative DR, 21 million with DME, the overall prevalence of DME is 6.81% (6.74–6.89) in people with diabetes worldwide (19), accounting for 12% of new cases of blindness annually.(20) According to studies of the natural history of DME, 24% of eyes with DME will lose at least three lines of vision within 3 years. (21)

The prevalence of DME depends on the type and duration of diabetes. In patients with type I diabetes, DME occurred in the first 5 years following diagnosis of diabetes, with the prevalence gradually increasing to 40% over 30 years. The Diabetes Control and Complications Trial (DCCT) group reported that the incidence of DME in type I diabetes patients with a 9-year diabetic history was 27%.(22) Around 5% of type II diabetes patients had DME when diabetes was diagnosed, gradually increasing to 30% within 25–30 years. A Chinese population-based epidemiological study reported that the prevalence of DME in type II diabetes was 5.89% (23), while it was 4.3% in Beijing metropolitan areas (24)and 5.2% in rural areas (25).

Several systemic risk factors have been identified in population-based epidemiological studies. In patients <30 years old, independent risk factors for DME included duration of diabetes, proteinuria, gender, history of cardiovascular disease, use diuretics and elevated HbA1C. In patients >30 years old, the

incidence of DME is associated with longer duration of diabetes, elevated systolic blood pressure and elevated glycosylated hemoglobin. Proteinuria was positively associated in insulin-dependent patients but not in the group that were not using insulin. The prevalence of DME was also significantly associated with high serum cholesterol levels in patients with type I diabetes.(26) A sharp reduction (from 2.3% and 0.9%) in the prevalence of DME was noted in a Wisconsin population with better blood glucose control over two decades, confirming that chronic hyperglycemia is a critical factor in the pathogenesis of DME.(27) According to the new meta analysis data in 2013, all DR prevalence end points increased with diabetes duration, hemoglobin A1c, and blood pressure levels and were higher in people with type 1 compared with type 2 diabetes.(19)

2.2 PATHOPHYSIOLOGY OF DME

The pathogenesis of DME has not been fully elucidated since it is caused by complex multifactorial pathological process with many contributing factors. Dysfunction of the inner and outer retinal barriers leads to accumulation intra-retinal fluid in the inner- and outer-plexiform layers. Vascular endothelial growth factor (VEGF) has been widely investigated as one of the main factor that disrupt the inner blood-retinal barrier (BRB) function, making anti-VEGF agents the main pharmaceutical treatment for DME currenly used. (28). Despite the current advancement of DME treatment with the anti-VEGF agents intravitreal injections, DME does not improve in many patients and still lose significant vision

irreversibly causing severe visual impairment and significantly affecting quality of life in diabetic patients.

Breakdown of the retinal blood barrier is an early event in the pathogenesis of DME.(28) Hyperglycemia, hypoxia, ischemia, leukocyte adhesion, oxygen-free radicals and inflammatory mediators are all involved in the breakdown retinal blood barrier (BRB). Muller cell, pericyte and glial cell dysfunction combined with vitreous changes are involved in the occurrence and development of macular edema. Chronic hyperglycemia, hypertension and high cholesterol are also important systemic factors related to diabetic macular edema.(29)

The blood-retinal barrier (BRB)

The concept of the BRB, originating from the discovery of the blood–brain barrier, was first introduced by Ashton in 1965 based on the study of histamine-induced leakage from the ocular vessels.(30) In this study, significant vascular leakage was observed in many compartments of the eye, but retinal vessels were not affected. Shakib and Cunha-Vaz then confirmed the presence of “zonulae occludins” (tight junctions), epithelial cell-like structures between the endothelial cells of the retinal vessels, using electron microscopy.(31,32) The BRB is formed by extensive junctional complexes found between retinal pigment epithelial (RPE) and vascular endothelial cells. These complexes selectively prevent molecules from passing into the extracellular tissue of the retina.(33) The

breakdown of BRB results in accumulation of plasma proteins (e.g. albumin) which exert a high oncotic pressure leading to fluid extravasation and edema.

Tight junctions

Tight junction-associated proteins play a critical role in maintaining the normal biological function of the retina. The tight junctions of the BRB constitute a biological and mechanical barrier to solute flux between cells (para-cellular permeability), allowing the organism to control transport of nutrients and waste products through the cell (trans-cellular permeability).(34) Several reviews have outlined the molecular functions of tight junction proteins (33), the signaling cascade from and to the tight junction complex (35) and the modulation of tight junction function in retinal vascular diseases, especially in in vitro studies.(36,37)

Three integral proteins form tight junction complexes: occludin, claudins and junctional adhesion molecules (JAMs). Occludin and claudins are trans-membrane proteins, predicted to have four trans-membrane and two extra-cellular domains, which are the major structural components of tight junction strands.(33) Occludin, first discovered as a 65 kDa protein in chicken, has been shown to play an important role in regulating tight junction barrier function.(38) Claudins are a group of proteins that includes 27 members [23].(39) JAMs belong to the immunoglobulin super-family and are located close to tight junction strands.(33,40) There are also a group of proteins named membrane-associated

guanylate kinase homologs (MAGUKs) that are positioned in the cytoplasmic surface of junctional contacts. Zonula occludens (ZO-1) belongs to the MAGUKs family and is thought to interact with occludin.(41)

VEGF-A, a major regulator of blood retinal barrier breakdown in diabetic retinopathy

VEGF (also referred to as VEGF-A) was first identified as a 34–42 kDa protein in 1983 (42) and cloned in 1989.(43) On the basis of its ability to induce vascular leakage, measured by ^{125}I-labeled human serum albumin extravasation, VEGF-A was originally recognized as a 'vascular permeability factor' in guinea pigs.(42) On a molar basis, the effect of VEGF-A on vascular permeability is estimated to be 50,000 greater than that of histamine as evaluated by the Miles vessel permeability assay.(44)

VEGF-A belongs to the VEGF family that includes placenta growth factor, VEGF-B, VEGF-C, VEGF-D and VEGF-E. Among these VEGF members, VEGF-A has been studied most intensively so far.(45,46)

Human VEGF-A comprises at least five different isoforms: VEGF110, VEGF121, VEGF165, VEGF189 and VEGF206. An alternative distal splice acceptor site in exon 8, named $VEGF_{165b}$, which is an inhibitory splice variant of VEGF-A was identified by Bates et al.(47) The expression of $VEGF_{165b}$ was further evaluated in normal and diabetic human eyes, including the lens, sclera, retina, iris and

vitreous. $VEGF_{165b}$ was detected predominantly in normal but not in diabetic vitreous. About 65% of total VEGF-A in normal vitreous is $VEGF_{165b,}$ confirming that $VEGF_{165b}$ is regarded as the endogenous inhibitor of VEGFA .(48)

Three tyrosine kinase receptors have been identified with functionality that corresponds to the VEGF family members. VEGF-A receptor-1 (fms-like tyrosine kinase-1, FLT-1) and VEGF-A receptor −2 (fetal liver kinase-1, FLK-1) are activated by VEGF-A. FLK-1 has been also recognized as a vascular permeability factor, since one of the critical functions of FLK-1 is to regulate vessel permeability.(49) FLT-1 has been reported to be a negative regulator and a ‘decoy’ receptor of FLK-1 by several studies.(50) The third receptor, VEGF receptor-3 (fms-like tyrosine kinase-4, FLT-4) is thought to bind to VEGF-C and VEGF-D.(51) Additionally, two co-receptors for VEGF-A, neuropilin-1 (for VEGF165) and −2 (for VEGF145 and 165) have also been identified as the isoform-specific receptors in embryonic vessel formation.(52) It has been well accepted that FLK-1 is the principle mediator of VEGF-A’s effect on vascular permeability and angiogenesis. (53)

As a critical mediator of vascular leakage, VEGF-A and its receptors have been implicated in the pathogenesis of diabetic retinopathy. A close correlation between albumin leakage and increased expression of VEGF-A and FLK-1 was noted in diabetic rat retinas.(54) Increased paracellular permeability induced by VEGF-A, was detected as early as two weeks after induction of diabetes and

associated with the re-distribution of occludin in diabetic rat retinas.(55) Activation of protein kinase C and phosphorylation of occludin, both induced by VEGF-A, were also found in diabetic rat retinas.(56) VEGF-A levels were also found to be markedly elevated in patients (plasma, vitreous, excised proliferative membranes) with diabetic retinopathy.(57) VEGF-A antagonists significantly attenuated increased vascular permeability in retinal diseases.(53). VEGF-induced FLK-1 phosphorylation and activation of its downstream signalling cascade correlated with BRB breakdown both in vitro and in vivo and a direct relationship between diabetic BRB breakdown and over-expression of FLK-1 has been reported.(54) Several hypotheses have been adduced to account for the mechanism by which VEGF-A contributes to dysfunction of the BRB. Firstly, VEGF-A is a critical effector for leukocyte adhesion and several proinflammatory mediators in diabetic retinopathy, including cytokines, chemokines, and vascular cell adhesion molecules. Secondly, VEGF-A has direct effects on tight junction-associated proteins which are the fundamental components of BRB. The tight junction-associated proteins have been demonstrated to be phosphorylated in response to VEGF-A. Finally, induction of pericyte degeneration and depletion, a hallmark of early diabetic retinopathy, is mediated by the up-regulation of VEGF-A and FLK-1 in the retinal vascular wall.

Leukostasis and inflammatory cytokines

Leukostasis, the accumulation of leukocytes on the luminal surface of the retinal capillaries, is thought to be a major contributor and early event in BRB dysfunction.(58) Leukocyte adhesion causes endothelial dysfunction and capillary non-perfusion in several ways.

Firstly, it has been demonstrated that leukostasis contributes to DR through the up-regulation of intracellular adhesion molecule (ICAM)-1, a critical molecular player in leukostasis which mediates the adhesion of monocytes and neutrophils to vascular endothelium. ICAM-1 has been found to mediate retinal leukostasis, vascular permeability and BRB breakdown in diabetes. The expression of ICAM-1 is also significantly elevated in STZ-induced diabetic retinas (59) as well as in human diabetic retinas.(60) Furthermore, intravitreal treatment with glucocorticoids has been found to significantly attenuate the inflammatory responses concomitant with improved BRB function through the inhibition of ICAM-1 expression in STZ-rat retinas.(61)

Secondly, BRB breakdown resulting from leukostasis may be due to its interaction with VEGF-A. VEGF-A has been shown to up-regulate the expression of adhesive molecules in vitro, promoting inflammatory cell adhesion to endothelium.(62) In vivo, increased expression of neutrophil CD11a, b, and 18, together with endothelial nitric oxide synthase (eNOS), was induced by VEGF-A in diabetic rat retinas.(63) It has also been shown that the principle pro-

inflammatory cytokine, TNF is a mediator of VEGF-A induced BRB breakdown in vitro.(64) Elevated expression of ICAM-1 stimulated by VEGF-A was found to be attenuated by pigment epithelium-derived factor (PEDF) in a dose-dependent manner in STZ-diabetic rat retinas.(65) Furthermore, inflammation and BRB dysfunction have been demonstrated to be abrogated by anti-VEGF165 aptamer (EYE001) treatment of diabetic retinas, suggesting that the effect of VEGF-A on leukostasis is highly correlated with the pathogenesis of DR.(66)

Thirdly, leukostasis has been found to correlate with inter-endothelial tight junction complex dysfunction and disorganization. Leukostasis was found to induce elevated expression of β-catenin and plakoglobin as well as the disorganization of the vascular endothelial-cadherin/catenin complex, all of which were abrogated by a leukostasis inhibitor (an anti-integrin β monoclonal antibody) in vivo. (67)

Finally, leukocytes produce reactive oxygen species (ROS) and inflammatory cytokines following binding to the vascular endothelium, leading to increased vascular permeability.(68) There is evidence that the BRB can be preserved by non-steroidal anti-inflammatory drugs (aspirin, etanercept and meloxicam) by preventing retinal vascular leakage through the suppression of TNF.(69) The significance of leukostasis in the pathogenesis of DR could provide new insights for the treatment of DR.

There is also associative evidence that neutrophil adhesion may play a role in human DR. Song and co-workers compared the expression of CD18 on neutrophils from 38 DR patients and 10 controls and found it significantly elevated and increased with the severity of DR.(70) In a large retrospective study of almost 31,000 persons, Woo and colleagues found that blood neutrophil counts were increased by approximately 10% in diabetics and by 20% in patients with moderate nonproliferative DR or PDR. (71) The ratio of neutrophils to total white blood cell count (WBC) was also significantly increased in PDR. The neutrophil count was well correlated with DR severity score, and this parameter corresponded to a 2.7-fold odds ratio in DR patients within the highest quartile severity group.

Nitric oxide (NO)

In the late 1980s, Furochgott and Zawadzki (1980) found that vascular endothelial cells produce a substance which may induce vascular smooth muscle relaxation. In 1987, it was confirmed, and named the CM 17 endothelial cell-derived relaxing factor (EDRF).(72) Subsequently, it was discovered that eNOS is closely related to metabolic abnormalities and cardiovascular diseases and is an important neurotransmitter involved in a variety of cellular responses. eNOS is highly correlated with the retention of leukocytes in the microcirculation and destruction of the BRB .(73) Awata et al. also showed that polymorphisms of the eNOS gene are one of the most important factors in the pathogenesis of DME. eNOS gene polymorphisms not only play an important role in the occurrence and

development of the DME,(74) but are also highly correlated with the breakdown of the BRB. BRB breakdown is also accompanied by the up-regulation of ICAM-1 and decreased expression of tight cell junction protein ZO-1. In diabetic animals, vascular leakage and retinal leukostasis was significantly reduced and the BRB was protected by the NOS inhibitor L-NAME,(75) verifying the biological roles for eNOS in the pathogenesis of DME, including: (1) induction and retention of inflammatory cells in the microcirculation of the eye; (2) a direct effect on cell junction proteins, decreasing the expression of cell junction proteins; and (3) increasing the expression of VEGF-A which leads to the destruction of the BRB.

Hyperglycemia and its metabolic pathways

Whilst the etiology of DR is highly complex and not fully understood, hyperglycemia has been accepted as the major pathological factor contributing to the development of DR. Four distinct glucose metabolic pathways are activated by hyperglycemia :

1. Diacylglycerol (DAG)–protein kinase C (PKC) pathway. Hyperglycemia increases synthesis of DAG via the de novo pathway, which in turn activates PKC.(76) The pathogenic role of the DAG-PKC pathway in the pathogenesis of DR has been demonstrated in both human and animal studies.(77,78)

PKC, one of a family of serine/threonine protein kinases of which there are at least 12 known isoforms, has been implicated in the pathogenesis of diabetic

BRB breakdown both in vivo and in vitro through a variety of mechanisms. (79) Firstly, its effect is mediated via VEGF-A.(80) The regulation of VEGF-A gene expression has been shown to be controlled and enhanced by PKC-β in a transgenic mouse model.(81) The mitogenic effects of VEGF-A are also mediated by the activation of PKC-β in vitro. Secondly, PKC can be activated by oxidative stress through reactive oxygen species (ROS) produced by hyperglycemia or advanced glycation end-products (AGEs), shown to directly activate PKC.(82) Thirdly, PKC triggers phosphorylation of tight junction-associated proteins to induce BRB breakdown. Phosphorylation of occludin and ZO-1 was shown to correlate with the activation of PKC in diabetic BRB dysfunction in STZ rats.(83) On the other hand, increased vascular permeability was shown to be suppressed by a PKC-β selective inhibitor, ruboxistaurin mesylate (LY333531) in diabetic rat retinas.(84)

2. Advanced glycation end-products(AGEs). Intracellular elevated glucose reacts non-enzymatically with the amino group of proteins, lipids and nucleic acids to form a reversible Schiff base, which is subsequently converted to the stable Amadori product (glycation product) and further metabolized to AGEs.(85) AGEs modulate cellular function mediated through binding of their specific acceptor molecules. Receptor for AGE (RAGE) was identified and characterized as a 35 kDa, lactoferrin-like AGE binding receptor expressed on endothelial cells.(86) Binding of AGEs by RAGE leads to endothelial dysfunction and BRB breakdown in DR. In a RAGE transgenic mouse model, AGEs/RAGE interaction was shown

to induce leukostasis and BRB breakdown, which was attenuated by a soluble form of RAGE.(87) Dysfunction of endothelial progenitor cells was found to be induced by AGEs/RAGE through the p38MAPK pathway.(88) AGEs/RAGE interaction is also believed to trigger oxidative stress,(89) the release of pro-inflammatory cytokines (90) and increased expression of VEGF-A (91), leading to further diabetic BRB breakdown and neuronal degeneration in the retina.

AGEs are neurotoxic to retinal neurons. In vitro, retinal neuronal cell death induced by AGEs and hyperglycemia has been shown to occur in a time- and dose-dependent manner and be mediated through the activation of ROS, suggesting oxidative stress is a consequence of AGEs/RAGE interaction.(92) Both AGEs and ROS have been demonstrated to induce retinal ganglion cell degeneration, possibly mediated by PI3 kinase-dependent pathways.(89)

3. Polyol (sorbitol) pathway. Hyperglycemia leads to elevated levels of intracellular glucose, which is then converted to sorbitol by the enzyme aldose reductase. Sorbitol is subsequently metabolized to fructose, a step which is rate-limiting. Activation of the sorbitol pathway leads to DR. Activation of the enzyme aldose reductase and accumulation of sorbitol was found in retinal capillary pericytes of human diabetic and STZ-rat retinas.(93,94) Excess accumulation of sorbitol and fructose have been demonstrated to correlate strongly with diabetic micro-vascular dysfunction,(95) neuronal apoptosis (96), glial reactivity and complement deposition.(97) The selective aldose reductase inhibitors fidarestat

and aldose reductase inhibitor-809, have been demonstrated to significantly abrogate neuronal apoptosis by inhibition of oxidative-stress and glial cell activation in STZ-induced diabetic rat retinas.(97,98)

4. Hexosamine pathway. Hyperglycemia induces mitochondrial superoxide over-expression, and leads to the activation of the hexosamine pathway. (82) Activation of this pathway has been found to induce oxidative stress, (99) production of some pro-inflammatory cytokines such as TGF-α (82), −β (100) and plasminogen activator inhibitor (101), which subsequently induce diabetic retinal neuronal apoptosis (102) , leukocyte adhesion and endothelial dysfunction (103) and BRB breakdown.(104)

2.3 AZUROCIDIN

The polymorphonuclear (PMN) granule protein azurocidin/cationic antimicrobial protein of 37 kD (CAP37)/heparin-binding protein (HBP) was first identified and isolated by Shafer et al. in 1984.(105) Because of its potent antimicrobial activity, its cationicity, and hydrophobicity, it was considered a component of the oxygen-independent host defense. Its charge and its proposed size gave it the name CAP37. Somewhat later, Gabay et al. (106) characterized a PMN-derived bactericidal protein from the azurophilic granules of human PMN, which they

named azurocidin. In parallel, Flodgaard et al. (107) isolated a protein from human and porcine PMN that displayed strong binding capability for heparin, earning it the name HBP. Complete sequencing has shown that CAP37, azurocidin, and HBP are the same protein.

Azurocidin was viewed a member of the family of PMN-derived antimicrobial proteins, such as defensins and lysozyme. However, soon it became evident that azurocidin, like other antimicrobial proteins, not only exerts antimicrobial activity but also modulates immune function in a multifaceted manner. Azurocidin, however, possesses some features that make it unique among the PMN granule proteins: Azurocidin is the only PMN granule protein stored in two different compartments. As a result of its storage in secretory vesicles and primary granules, azurocidin is released at a very early stage of PMN extravasation as well as at a later stage when the PMN has reached the site of inflammation (108) , thereby allowing it to target cells in the bloodstream, the endothelial lining, and the extravascular environment. The amino acid sequence and the three-dimensional structure of azurocidin have been unveiled and show that azurocidin is a member of the serine protease superfamily. However, as a result of mutations in two of the three essential amino acids in the highly conserved catalytic triad seen in all serine proteases, azurocidin is devoid of significant protease activity (107, 109, 110, 111) . Azurocidin is released almost completely after granule mobilization. In contrast to, e.g., human neutrophlic peptides, which

are released mainly into the phagolysosome (112) , 90% of the azurocidin are released upon degranulation (108, 109) . These three distinct properties of azurocidin favor the promiscuous mode of action that this protein displays.

Azurocidin activates endothelial cells (EC)

EC is the first target of PMN-derived azurocidin released from secretory vesicles. As described above, this may induce endothelial cell adhesion molecules (CAM) expression and result in a more pronounced adhesion of immune cells. In addition, derangement of the endothelial barrier function, leading to plasma leakage and edema formation, is a characteristic feature of the inflammatory reaction. Previous studies clearly indicate that emigration of PMN is accompanied by efflux of plasma from the vasculature and that these cells are in a position to trigger permeability changes themselves (113, 114) . Of critical importance in a PMN-evoked permeability increase is the PMN adhesion and activation via β_2-integrins (115) . Adhesion of the PMN to the EC induces rapid intracellular Ca^{2+} mobilization in both cell types, leading to granule exocytosis in the PMN and rearrangement of the EC cytoskeleton. Blockage of β_2-integrin function abrogated these responses completely.(115) More recently, it was shown that azurocidin is released upon β_2-integrin ligation and that this protein has a central role in the PMN-evoked permeability change. Its location in rapidly mobilized secretory vesicles allows a rapid discharge upon PMN adhesion and

activation. PMN-derived azurocidin could be demonstrated to provoke a rapid rise in cytosolic-free Ca^{2+} in adjacent EC, formation of actin stress fibers, and increased paracellular permeability.(116) The responses to azurocidin stimulation are identical to those achieved by chemoattractant stimulation of PMN, and immunoneutralization of azurocidin in PMN-derived secretion inhibits the activity completely, substantiating the critical role of this protein in PMN-evoked alterations in vascular permeability. Besides the importance of the localization of azurocidin in secretory vesicles, which allows an almost instant permeability change upon PMN adhesion, another feature of azurocidin is at least equally important in this process. Azurocidin carries a large number of positively charged amino acid residues concentrated on one side of the protein, creating a strong dipole moment.(109) It is likely that the basic patch of azurocidin interacts with negatively charged proteoglycans on the EC surface by which EC conformational changes are induced. Yet, the exact mechanisms by which azurocidin activates signaling pathways in EC and stimulates reorganization of cytoskeletal and junctional complexes remain elusive.

Azurocidin recruits monocytes

Once the PMN senses a signal to extravasate at sites of injury or infection, it becomes activated and adheres to the endothelial lining.(117) Upon these initial events of PMN extravasation, the content of rapidly mobilizable, secretory

vesicles is discharged in the secluded compartment between the PMN and the endothelial cell. Azurocidin, a major component of secretory vesicles, is strongly, positively charged and may thus accumulate on the negatively charged EC surface. In this way, azurocidin becomes immobilized on the endothelium and thereby exposed to cells in the blood flow.(118) Interestingly, azurocidin is only deposited by adherent but not rolling PMN, indicating that PMN activation via β_2-integrins is an important signal for discharge of secretory vesicles. The accumulation of azurocidin on the endothelium is reduced by treatment with heparinase and chondroitinase, suggesting that negatively charged proteoglycans in the endothelial glycocalyx act as primary binding sites.(118,119) A specific receptor for azurocidin on EC has not been identified. In line with this, treatment of EC with inflammatory stimuli such as LPS or TNF-α does not enhance binding of azurocidin to EC. Azurocidin immobilized on the endothelium may interact with inflammatory cells in the bloodstream. In fact, it has been shown that azurocidin with preference binds to monocytes.(120,121) Once monocytes in flow recognize azurocidin presented on the endothelial surface, a mobilization of intracellular Ca^{2+} is initiated, which is crucial for the azurocidin-mediated adhesion of monocytes.(118) Similar to PMN-derived elastase and proteinase-3 (122,123) monocyte adhesion stimulated by azurocidin is mediated via β_2-intergins. The ability of azurocidin to enhance adhesion depends on a previous capturing of the monocyte from free flow. However, once the monocyte has slowed down, it is able to recognize azurocidin. The clinical relevance of

such a mechanism is suggested by the detection of azurocidin on the EC surface of specimens from chronic inflammatory diseases such as Alzheimer (124) and atherosclerotic plaques.(125)

Azurocidin activates monocytes and macrophages

Monocytes and macrophages are multifunctional cells contributing to bacterial clearance by phagocytosis and killing of bacteria. Moreover, mononuclear phagocytes are powerful in the control and fine-tuning of the immune response. They do so by presenting antigens and releasing a wide array of chemokines and cytokines.(126) Cytokines are mostly de novo-produced and generally act over short distances and bind to a specific membrane receptor, which then signals via second messengers, often tyrosine kinases, to alter the target cell's behavior.

Rasmussen et al. (127) were the first to describe an enhanced cytokine release from monocytes when treated with azurocidin. Interestingly, azurocidin alone had no effect on the release of TNF-α and IL-6. However, in the presence of LPS, azurocidin could enhance the release of these two cytokines several fold. TNF-α is a multifunctional cytokine that is involved in endothelial cells activation, activation of macrophages, and initiation of a local inflammatory response. IL-6 is a cytokine that is mainly involved in T and B cell growth and differentiation, initiating the acquired immune response.

Recently, azurocidin was identified as an activator of human macrophages, as demonstrated not only by intracellular Ca^{2+} mobilization but also by a change in the phenotype.(128) Treatment of macrophages with azurocidin enhanced the expression of HLA II, CD40, and CD86, in agreement with findings from microglial cells treated similarly.(129) Expression of these molecules is a sign of the classical macrophage activation being functionally related to enhanced antimicrobial effectiveness and a more powerful activation of the adaptive immune system.(130) Furthermore, macrophage receptors are up-regulated in response to treatment with azurocidin. This type of activation is typically mediated by a concerted action of the prototypic macrophage activators TNF-α and IFN-γ (130), both of which were found to be released from the macrophage in the presence of azurocidin.(128) More importantly, they were not only secreted from the macrophage in response to azurocidin, but they were also found to be responsible for the macrophage activation pattern. The autocrine activation of the macrophage by its own secretion products has been disputed.(130) However, the cytokine release in response to azurocidin and the subsequent macrophage activation seem to be an interesting contribution in favor of autocrine activation.

3. RATIONALE AND HYPOTHESIS

Leukocyte adhesion to the retinal vasculature is a very important common downstream step in multiple pathways involved in pathogenesis of diabetic retinopathy and retinal vascular leakage leading to diabetic macular edema. Multiple pathophysiological pathways in diabetic retinopathy promote retinal leukostasis either directly or indirectly through upregulation of mediators that promote leukocyte adhesion. The molecular pathways involved in BRB breakdown downstream of leukocyte adhesion though are not well understood and it is not known how leukocytes increase retinal vascular permeability and cause retinal vascular leakage.Therefore, elucidating the factors that compromise the BRB may improve our therapeutic approach to treat macular edema, the main cause of visual loss in patients with diabetes. Azurocidin has been shown to be missing link in leukocyte-induced endothelial permeability in non–central nervous system (CNS) vessels. However, whether azurocidin may have an effect on vessels of the CNS and retina vessels with their unique neurovascular barrier properties in particular is unknown.

Given the important role of azurocidin in mediating leukocyte-induced endothelial permeability in vascular systems outside the CNS and given that leukocyte adhesion is a crucial step in VEGF-induced and diabetic BRB breakdown, we hypothesize that azurocidin may also be an important mediator of BRB breakdown.

4. MATERIALS AND METHODS

Animals

All animal experiments adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, and protocols were approved by the Animal Care Committee of the Massachusetts Eye and Ear Infirmary. Male Brown Norway, Long-Evans and Lewis rats, weighing 200 to 300 g each, were used for the experiments. Animals were fed standard laboratory chow and allowed free access to water in an air-conditioned room with a 12-hour light/12-hour dark cycle until they were used for the experiments.

Injections

Animals were anesthetized with intramuscular injection of xylazine hydrochloride (6 mg/kg; Phoenix Pharmaceutical, St. Joseph, MO) and ketamine hydrochloride (40 mg/kg; Parke-Davis, Morris Plains, NJ). To perform the intravitreal injections, a 31-gauge needle (Hamilton) was inserted into the vitreous 1 mm posterior to the corneal limbus.20 21 Insertion and infusion were directly viewed through an operating microscope with a cover slip and goniosol to perform fundosccopy in order to prevent injury to the lens and retina. Eyes that exhibited signs of damage to these structures were excluded from the experiments. Intravenous injections

were performed through the tail vein with a 27-gauge butterfly needle under anesthesia.

Induction of Diabetes

Male Long-Evans rats weighing approximately 200 g each were used for these experiments. To induce diabetes, each animal received a single 60 mg/kg intraperitoneal injection of streptozotocin (Sigma, St. Louis, MO) in 10 mM sodium citrate buffer, pH 4.5, after an overnight fast. Control nondiabetic animals received citrate buffer alone. Animals with blood glucose levels higher than 250 mg/dL 24 hours after injections were considered diabetic. All experiments were performed 2 weeks after the induction of diabetes.

Induction of Endotoxin Induced Uvetis (EIU)

Eight-week-old male Lewis rats (180–220 g) were used. EIU was induced by footpad subcutaneous injection with 200 µg LPS from *Escherichia coli* (Sigma-Aldrich, St. Louis, MO) that had been diluted in 300 µl PBS. Control animals (no LPS) received footpad injection with PBS only . LPS injected animals were treated with 50,000 KIU Aprotinin (5ml of Trasylol) or the equivalent volume of saline q8 hrs for 24 hrs. Retinal vascular permeability was measured 24 hours after footpad injection using the Evans Blue technique.

Administration of Azurocidin and Aprotinin

Rats received intravitreal injections of 5 µL sterile phosphate-buffered saline (PBS) containing 1 or 20 µg or 50 µg of human neutrophil azurocidin (Athens Research and Technology, Atlanta, GA) in one eye and 5 µL of sterile PBS in the contralateral eye. Retinas were analyzed for BRB breakdown quantification by the Evans blue (EB) technique 1, 3, and 24 hours after azurocidin injection.

In a second group, the rats also received the azurocidin inhibitor aprotinin (10,000 or 30,000 kallikrein-inducing units [KIU]; equivalent to 1 or 3 mL Trasylol, respectively, Bayer Pharmaceuticals, Pittsburgh, PA) by intravenous injection. Aprotinin was administered through the tail vein 1 hour before intravitreal injection of azurocidin. Retinas were analyzed 1 hour after azurocidin administration for BRB breakdown quantification.

Administration of VEGF and Aprotinin

Rats received intravitreal injections of 5 µL sterile PBS containing 50 ng $VEGF_{164}$ (R&D Systems, Minneapolis, MN) in one eye and 5 µL sterile PBS in the contralateral eye. Retinas were analyzed 24 hours after VEGF injection for BRB breakdown quantification.

In a second group, rats also received the broad protease inhibitor aprotinin (50,000 KIU; equivalent to 5 mL Trasylol) by intravenous injection. Aprotinin was

administered through the tail vein 1 hour before and 8 and 16 hours after intravitreal injection of VEGF or PBS. Retinas were analyzed 24 hours after VEGF administration for BRB breakdown.

Blood-Retinal Barrier Breakdown Measurement with the Evans Blue Technique

Retinal vascular permeability was quantified with the Evans Blue (EB) technique. After the animals were deeply anesthetized, EB dye (30 mg/mL in saline; Sigma) was injected through the tail vein over 10 seconds at a dosage of 45 mg/kg. Blood samples were obtained from the left ventricle, just before perfusion, to obtain the time-averaged EB plasma concentration. Blood samples were centrifuged at 12,000 rpm for 15 minutes to separate the plasma from the cellular components. Plasma samples were diluted to 1/10,000 of their initial concentration in formamide (Sigma). Absorbance was measured with a spectrophotometer at 620 nm and 740 nm. After the dye had circulated for 2 hours, the chest cavity was opened, and the rats were perfused through the left ventricle with paraformaldehyde 1% in citrate buffer (0.05 M, pH 3.5) at a constant pressure of approximately 120 mm Hg. Retinas were then carefully dissected under an operating microscope. After measurement of the retinal weight, EB was extracted by incubation of each retina in 180 µL formamide for 18 hours at 70°C. The extract was ultracentrifuged at a speed of 14,000 rpm for 60 minutes at 25°C. Sixty microliters of the supernatant was used for

spectrophotometric measurement at 620 nm and 740 nm.22 Background-subtracted absorbance was determined by measuring each sample at 620 nm (absorbance maximum for EB in formamide) and 740 nm (absorbance minimum). BRB breakdown was calculated as previously described, and values were expressed as plasma (μL) × retinal weight (g^{-1}) × time ($hours^{-1}$).

Qualitative Evaluation and Visualization of Retinal Vascular Permeability

Retinal vascular permeability was also demonstrated in a histologic manner by intravenous injection of 20 kDa FITC-conjugated dextran (50 mg/kg; Sigma). Rats were killed 30 minutes later and perfused with 4% paraformaldehyde to fix the dextran conjugate in the tissues. Retinas were carefully dissected and flat mounted in an antifading medium (Vector Laboratories, Burlingame, CA). Flat-mounted retinas were examined by fluorescence microscopy. Digital color enhancement (green) was equally applied to all images to improve visualization of the fluorescence.

Ex Vivo Quantitation of Retinal Leukostasis

After the induction of deep anesthesia in the rat, the chest cavity was opened and a 14-gauge perfusion canula was introduced to the left ventricle. The right atrium was opened with a 12-gauge needle to achieve outflow. With the heart

providing the motive force, 250 mL/kg PBS was administered from the perfusion canula to remove erythrocytes and nonadherent leukocytes. Fixation was then achieved by perfusion with 1% paraformaldehyde and 0.5% glutaraldehyde at a pressure of 100 mm Hg. At this point, the heart stopped. A systemic blood pressure of 100 mm Hg was maintained by perfusing a total volume of 200 mL/kg over 3 minutes. The inhibition of nonspecific binding with 1% albumin in PBS (total volume 100 mL/kg) was followed by perfusion with FITC-coupled concanavalin A lectin (20 µg/mL in PBS [pH 7.4], total concentration, 5 mg/kg body weight; Vector Laboratories, Burlingame, CA). The latter stained adherent leukocytes and the vascular endothelium. Lectin staining was followed by PBS perfusion, to remove excess concanavalin A.The retinas were flatmounted in a water-based fluorescence anti-fading medium (Vector Laboratories, Burlingame, CA) and imaged by fluorescence microscopy. Only whole retinas in which the peripheral collecting vessels of the ora serrata were visible were used for analysis. The total number of adherent leukocytes per retina was counted. All experiments were performed in a masked fashion.

5. RESULTS

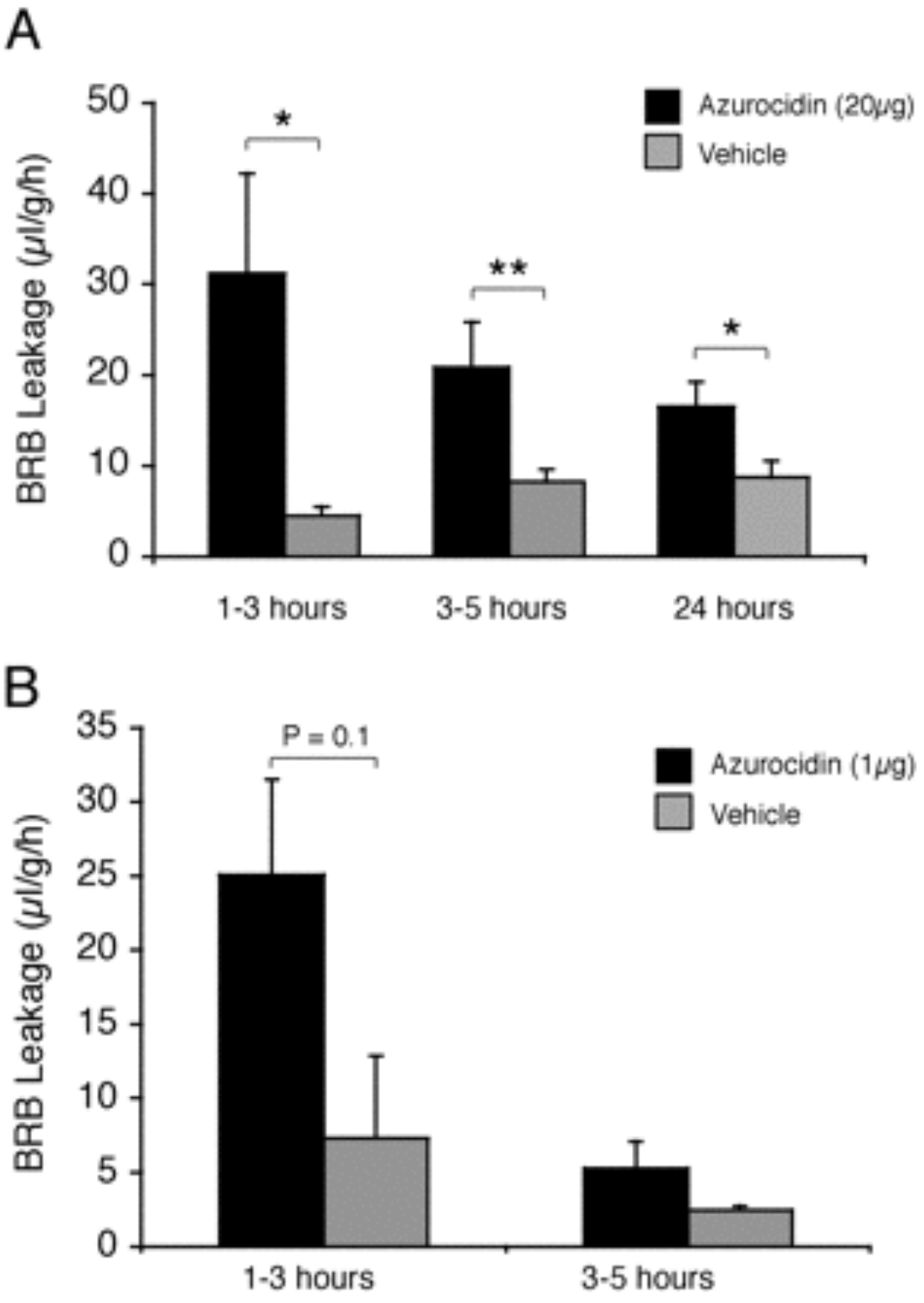
Azurocidin Increases Retinal Vascular Leakage In Vivo

To investigate whether exogenous azurocidin causes leakage in vessels with neurovascular barrier properties, we assessed the effect of intravitreal administration of azurocidin on retinal vascular leakage by the Evans blue technique. Azurocidin increased retinal vascular leakage in vivo in a time-dependent manner with a peak 1–3 hours after administration. Intravitreal injection of 20 μg azurocidin induced a 6.8-fold increase in the leakage of EB from retinal vessels compared with vehicle-injected control eyes, 1–3 hours after injection (31 ± 11 vs. 4.5 ± 1 μL/g/h, n = 12, P = 0.02), a 2.7-fold increase 3 to 5 hours after injection (21 ± 2.3 vs. 8.3 ± 1.4 μL/g/h, n = 11, P = 0.0006), and a 1.7-fold increase 24 hours after injection (16.5 ± 2.8 vs. 8.7 ± 1.8 μL/g/h, n = 6, P = 0.04; Fig. 1A).

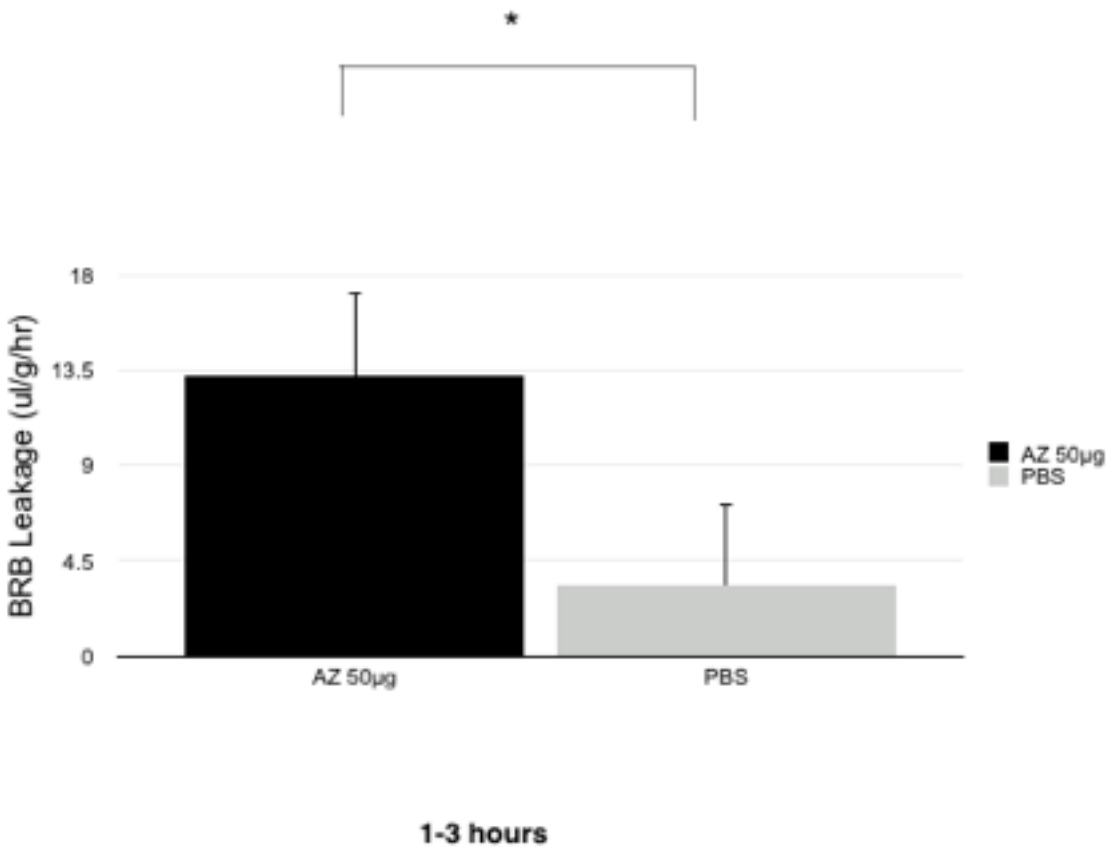
To investigate the dose-response of azurocidin on BRB leakage, 1 μg of purified protein was injected intravitreally, and retinal vascular leakage was compared with the vehicle-injected eyes. At the lower dose of 1 μg, azurocidin induced a 3.4-fold increase in BRB leakage compared with the vehicle-injected eyes of the same animals but it was not statistically significant. (25.1 ± 6.4 vs. 7.3 ± 5.5 μL/g/h, n = 3, P = 0.1). Three to five hours after the injection of 1 μg azurocidin, the difference in leakage between the injected eyes and the control eyes further

decreased and remained statistically nonsignificant. Our results suggest a concentration and time-dependent waning of the impact of azurocidin on BRB leakage in vivo (Fig. 1B) .

To further evaluate to dose response and see what dose can achieve the maximum increase of retinal vascular leakage , we also performed experiments with dose of 50 μg of Azurocidin per injection. 50 μg of Azurocidin were injected in one eye while the contralateral eye was injected with PBS and retinal vascular leakage was measured 1-3 hours later. Azurocidin induced a 3.96-fold increase in retinal vascular leakage 1-3 hours after injection compared to vehicle injected eyes of the same animals (13.2.1 ± 3.9 vs. 3.3 ± 0.4 μL/g/h, n = 12, $P < 0.05$). These data show that dose of Azurocidin of 20 μg has the maximum effect in vivo on retinal vascular leakage at 1-3 hours compared to later time points. (Fig 1C)


A
BRB Leakage (µl/g/h)
50
40
30
20
10
0
*
**
*
Azurocidin (20µg)
Vehicle
1-3 hours
3-5 hours
24 hours
B
BRB Leakage (µl/g/h)
35
30
25
20
15
10
5
0
P = 0.1
Azurocidin (1µg)
Vehicle
1-3 hours
3-5 hours

C



1-3 hours

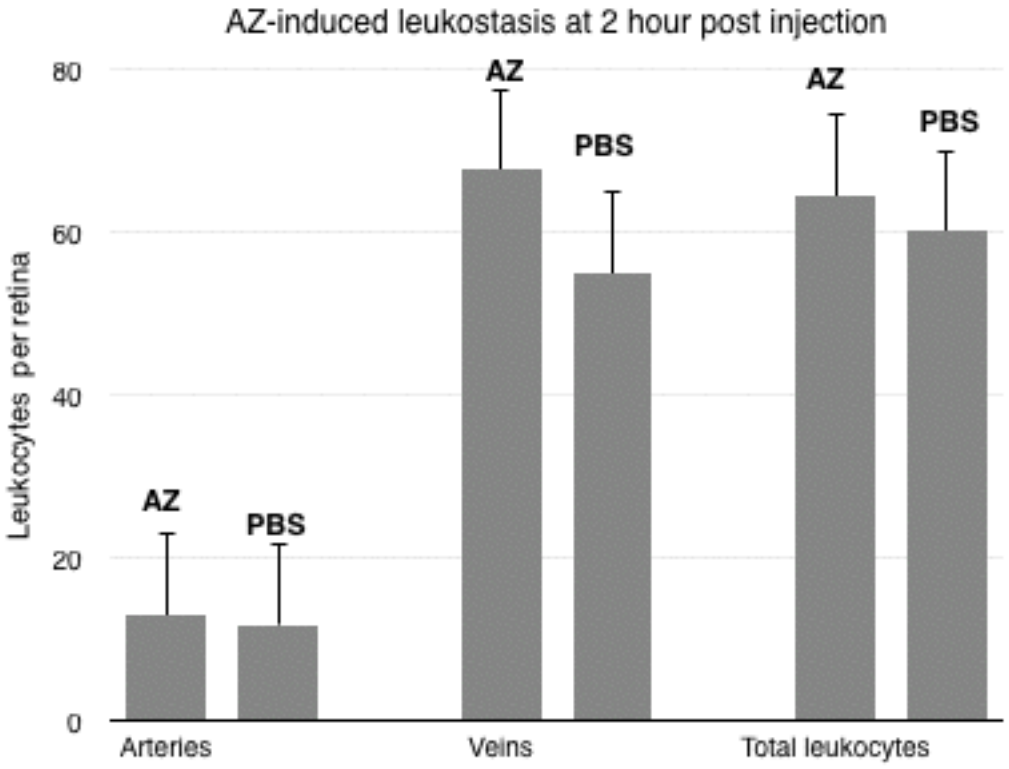
FIGURE 1

Azurocidin increases retinal vascular permeability in vivo in a time-dependent and dose-dependent manner. To assess whether azurocidin increases retinal vascular permeability in vivo, azurocidin was injected intravitreally at the doses of 20 µg (A) or 1 µg (B) or 50 µg (C) in one eye and the same volume of vehicle (5 µL PBS) in the contralateral eye. The BRB breakdown was quantified with the Evans blue technique at different time points after intravitreal injection. Intravitreal injection of 20 µg azurocidin induced a 6.8-fold increase in retinal vascular leakage compared with vehicle-injected control eyes, 1–3 hours after injection, a 2.7-fold increase 3 to 5 hours after injection and a 1.7-fold increase 24 hours after injection. Lower dose azurocidin of 1 µg per injection showed a trend towards increase in retinal vascular leakage at 1-3 hours compared with the vehicle-injected eyes of the same animals but it was not statistically significant. Three to five hours after the injection of 1 µg azurocidin, the difference in leakage between the injected eyes and the control eyes further decreased and remained statistically nonsignificant. Higher dose of azurocidin (50 µg per intravitreal injection) increased significantly retinal vascular leakage by 3.9-fold 1-3 hours after injection compared to vehicle injected eyes of the same animals showing a plateau of azurocidin permeability effect on retinal vessels in vivo with higher doses than 20 µg by injection. Bars represent mean ± SEM. *P < 0.05; **P < 0.01.

Azurocidin does not increase ex vivo static retinal leukostasis

To elucidate if azurocidin promotes retinal leukostasis, the static leukocyte adhesion after azurocidin injections in the larger retinal vessels, was evaluated with a perfusion technique using concanavalin A lectin. Twenty µg of azurocidin was injected in the one eye and the contralateral eye was injected with vehicle-PBS. Number of adherent leukocytes in arteries, veins and total number of adherent leukocytes 2 hours and 24 hours after intravitreal injections were measured in a masked fashion. Azurocidin intravitreal injection did not increase leukocyte adhesion in arteries, veins or total number of adherent leukocytes compared to control injection at the 2 hours time point which is the time pint that maximum retinal vascular leakage was noted. (64.2 ± 6.11 , n=9 vs. 60.2 ± 5.5; n = 9; P > 0.05) (Fig 2A). Azurocidin did not significantly increase static leukocyte adhesion in arteries, or, veins or total number 24 hours after intravitreal injection compared to control injections.(49.33 .2 ± 4.11, n=9 vs. 36.4 ± 5.3; n = 10; P > 0.05). (Fig 2B)

A


AZ-induced leukostasis at 2 hour post injection
Leukocytes per retina
80
60
40
20
0
AZ
PBS
AZ
PBS
AZ
PBS
Arteries
Veins
Total leukocytes

B

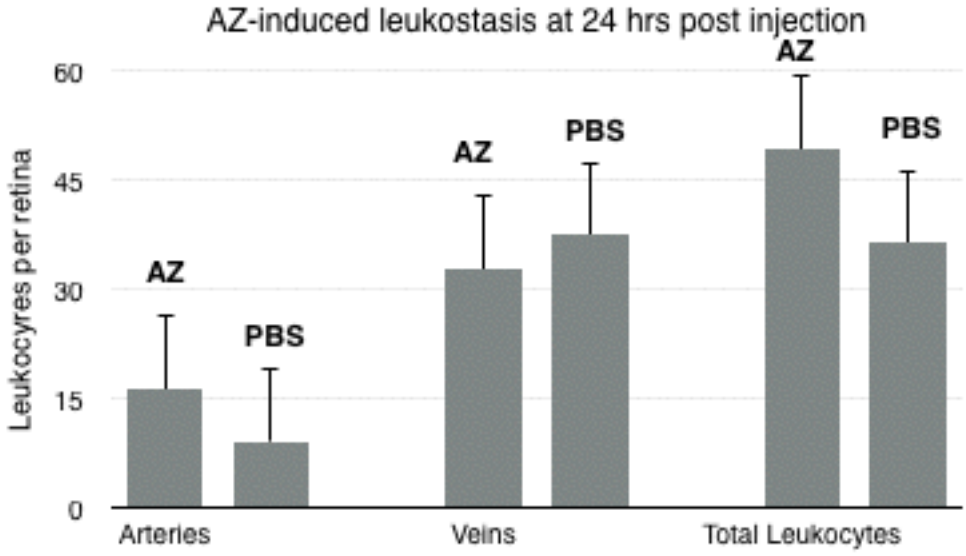
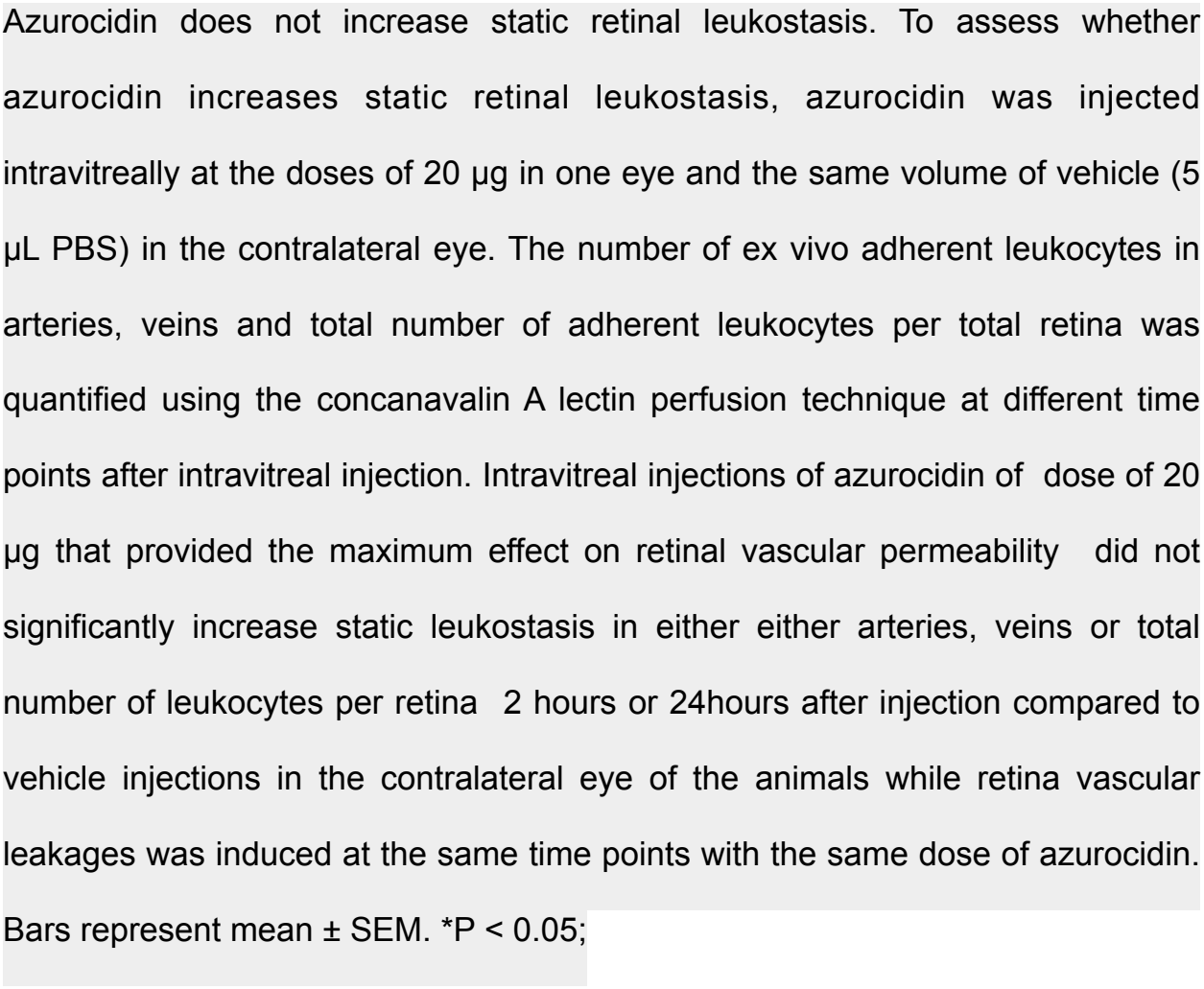

AZ-induced leukostasis at 24 hrs post injection
Leukocyres per retina
60
45
30
15
0
AZ
PBS
AZ
PBS
AZ
PBS
Arteries
Veins
Total Leukocytes

FIGURE 2



Azurocidin does not increase static retinal leukostasis. To assess whether azurocidin increases static retinal leukostasis, azurocidin was injected intravitreally at the doses of 20 µg in one eye and the same volume of vehicle (5 µL PBS) in the contralateral eye. The number of ex vivo adherent leukocytes in arteries, veins and total number of adherent leukocytes per total retina was quantified using the concanavalin A lectin perfusion technique at different time points after intravitreal injection. Intravitreal injections of azurocidin of dose of 20 µg that provided the maximum effect on retinal vascular permeability did not significantly increase static leukostasis in either either arteries, veins or total number of leukocytes per retina 2 hours or 24hours after injection compared to vehicle injections in the contralateral eye of the animals while retina vascular leakages was induced at the same time points with the same dose of azurocidin. Bars represent mean ± SEM. *P < 0.05;

Aprotinin Suppresses Azurocidin-Induced BRB Breakdown

To investigate whether the protease inhibitor, aprotinin, prevents azurocidin-induced BRB breakdown, rats were treated with a single intravenous injection of low- or high-dose aprotinin (10,000 or 30,000 KIU) 1 hour before intravitreal injection of azurocidin or vehicle control (PBS). Retinal vascular leakage was determined 1-3 hours later which is the time that azurocidin induced the maximum effect on retinal vascular permeability. Treatment of the animals with aprotinin (30,000 KIU) blocked azurocidin-induced leakage by >98% (45.5 ± 16.8 vs. 6.1 ± 0.64 μL/g/h, n = 8 and n = 6, respectively, P = 0.03), whereas aprotinin (30,000 KIU) treatment alone did not affect basal levels of retinal vascular leakage in vehicle-injected eyes (Fig. 2) . In comparison, the low-dose injection of aprotinin (10,000 KIU) also significantly blocked azurocidin-induced leakage (8.5 ± 3.4 μL/g/h, n = 8, P = 0.048), while systemic injection of lower dose of aprotinin alone did not impact the leakage of vehicle injected eyes (Fig. 3) .

Effect of Aprotinin on retinal vascular leakage 1-3 hours post intravitreal injection of AZ

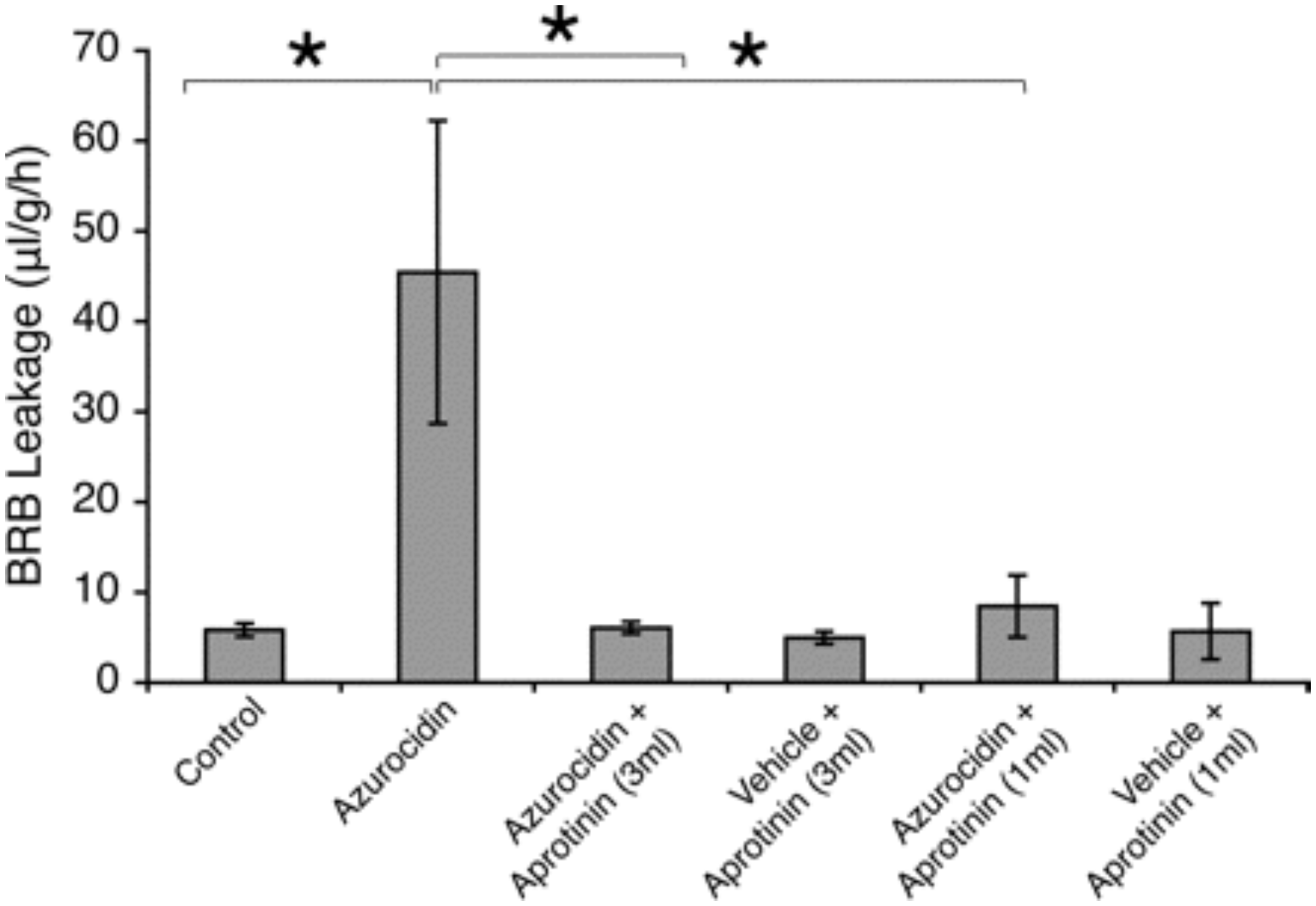


FIGURE 3.

Aprotinin suppresses azurocidin-induced BRB breakdown. To investigate whether the protease inhibitor, aprotinin, is effective in inhibiting azurocidin-induced BRB breakdown, rats were treated with a single intravenous injection of 1 or 3 mL aprotinin Trasylol (10,000 KIU or 30,000 KIU) 1 hour before intravitreal injection of azurocidin or vehicle. BRB breakdown was quantified with Evans blue technique 1-3 hours later. Aprotinin dramatically suppressed azurocidin-induced retinal vascular leakage at both doses of 3ml and 1 ml. The higher dose of 3ml had more significant effect on retinal vascular leakage suppression than the lower dose of 1 ml. Either dose did not affect basal levels of retinal vascular leakage in vehicle injected eyes. Data represent mean ± SEM. *P < 0.05.

Aprotinin Suppresses VEGF-Induced BRB Breakdown

To evaluate our hypothesis that azurocidin is a key factor in leukocyte-mediated retinal vascular leakage, we used the well established model of VEGF-induced BRB breakdown which has been shown to be leukocyte mediated and share many similarities with diabetic retinopathy.

Intravitreal injection of 50 ng VEGF induced a significant 3.2 ± 0.7-fold increase in retinal vascular leakage after 24 hours compared with vehicle (PBS)-injected eyes (29.7 ± 7.5 vs. 9.6 ± 1.4 μL/g/h, n = 8, P = 0.02; Fig. 4). To assess whether the protease inhibitor, aprotinin, reduces VEGF-induced BRB breakdown, rats were treated with intravenous injections of aprotinin 1 hour before and 8 and 16 hours after intravitreal injections of VEGF and vehicle control. BRB breakdown was quantified with the EB technique 24 hours after the intravitreal injections. VEGF-induced BRB breakdown was suppressed by 93% with intravenous administration of aprotinin (29.7 ± 7.5 vs. 6.2 ± 1.6 μL/g/h, n = 8 and n = 6, respectively, P = 0.02; Fig. 4).

Effect of Aprotinin on VEGF induced retinal vascular leakage

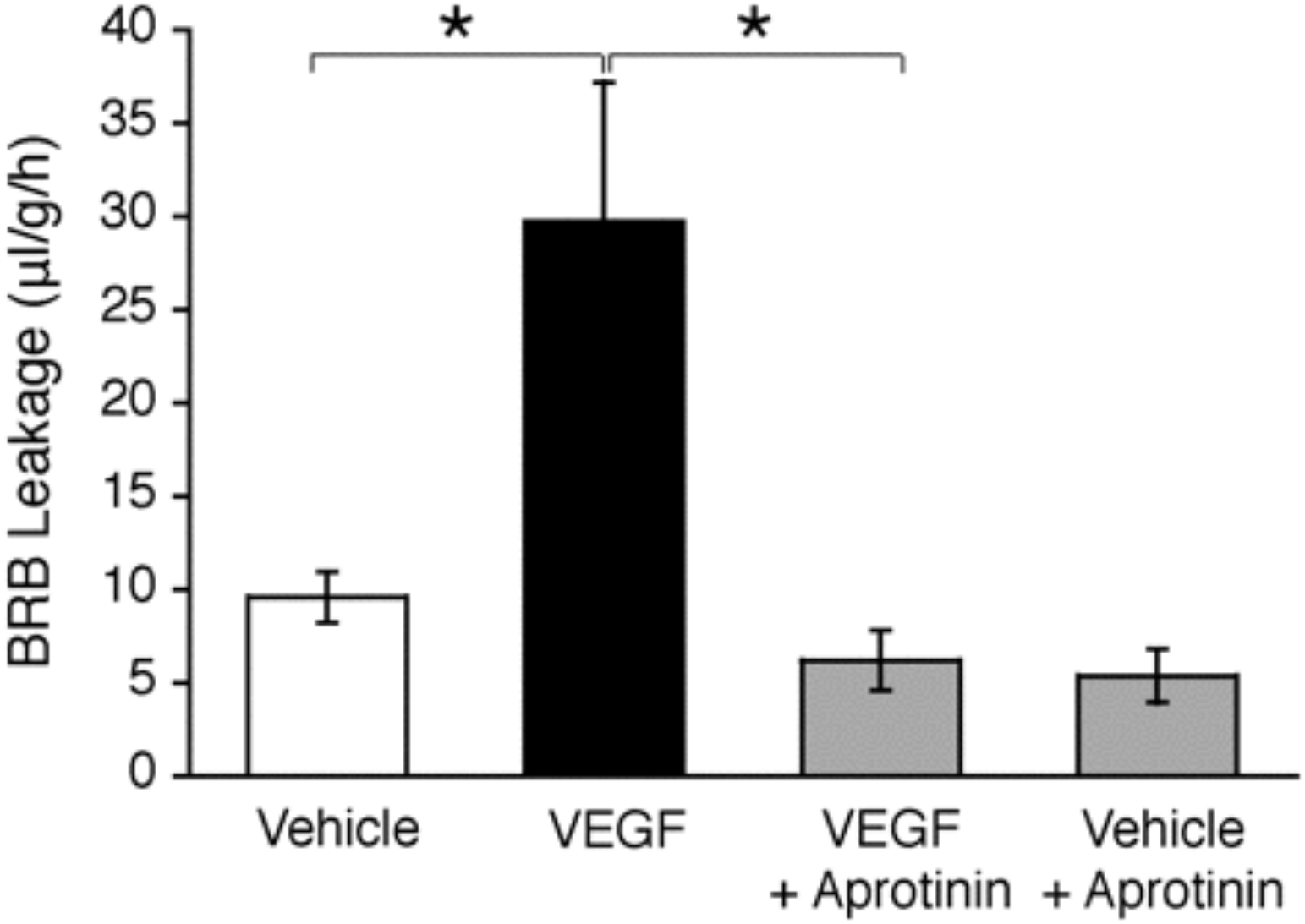
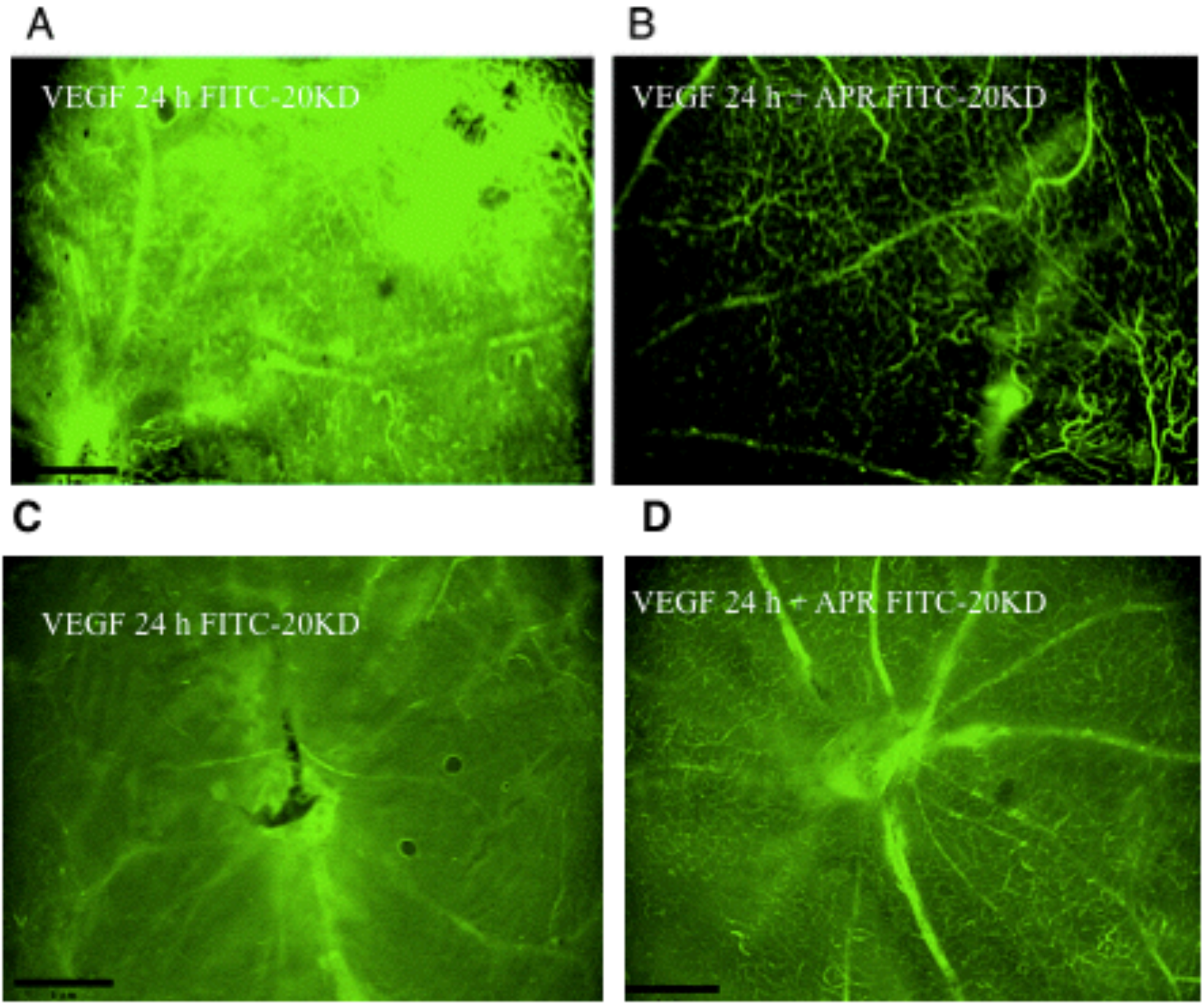


FIGURE 4

Aprotinin suppresses VEGF-induced BRB breakdown. $VEGF_{164}$ (50 ng in 5 µL) was injected intravitreally in one eye, and the same volume of vehicle control was injected in the other eye of the same animal. BRB breakdown was measured using the Evans blue technique 24 hours after intravitreal injection. In the treated group, aprotinin (50,000 KIU) was administered by tail vein injection every 8 hours. Intravitreal injection of 50 ng VEGF induced a significant 3.2 -fold increase in retinal vascular leakage after 24 hours compared with vehicle (PBS)-injected eyes. Treatment with aprotinin dramatically decreased VEGF-induced retinal vascular leakage by 93%. Data represent mean ± SEM. *P < 0.05.

To visualize the difference in leakage in animals receiving VEGF injections when treated with aprotinin, the retinal distribution of intravenously injected 20 kDa FITC-conjugated dextran was performed. Fluorescence microscopy of flat-mounted retinas from VEGF-injected eyes showed diffusely distributed fluorescence throughout the retinal tissues in intraluminal and extravascular locations indicating breakdown of the blood retina barrier and retinal vascular leakage consistent with increased leakage seen with Evans Blue technique. However, when animals that received VEGF intravitreal injections were treated with intravenous aprotinin, fluorescence was mainly confined to the intraluminal space of the retinal vessels with very little extravascular fluorescence showing that aprotinin ameliorated the VEGF-induced BRB breakdown and dramatically decreased retinal vascular leakage (Fig. 5) .


A
VEGF 24 h FITC-20KD
B
VEGF 24 h + APR FITC-20KD
C
VEGF 24 h FITC-20KD
D
VEGF 24 h + APR FITC-20KD

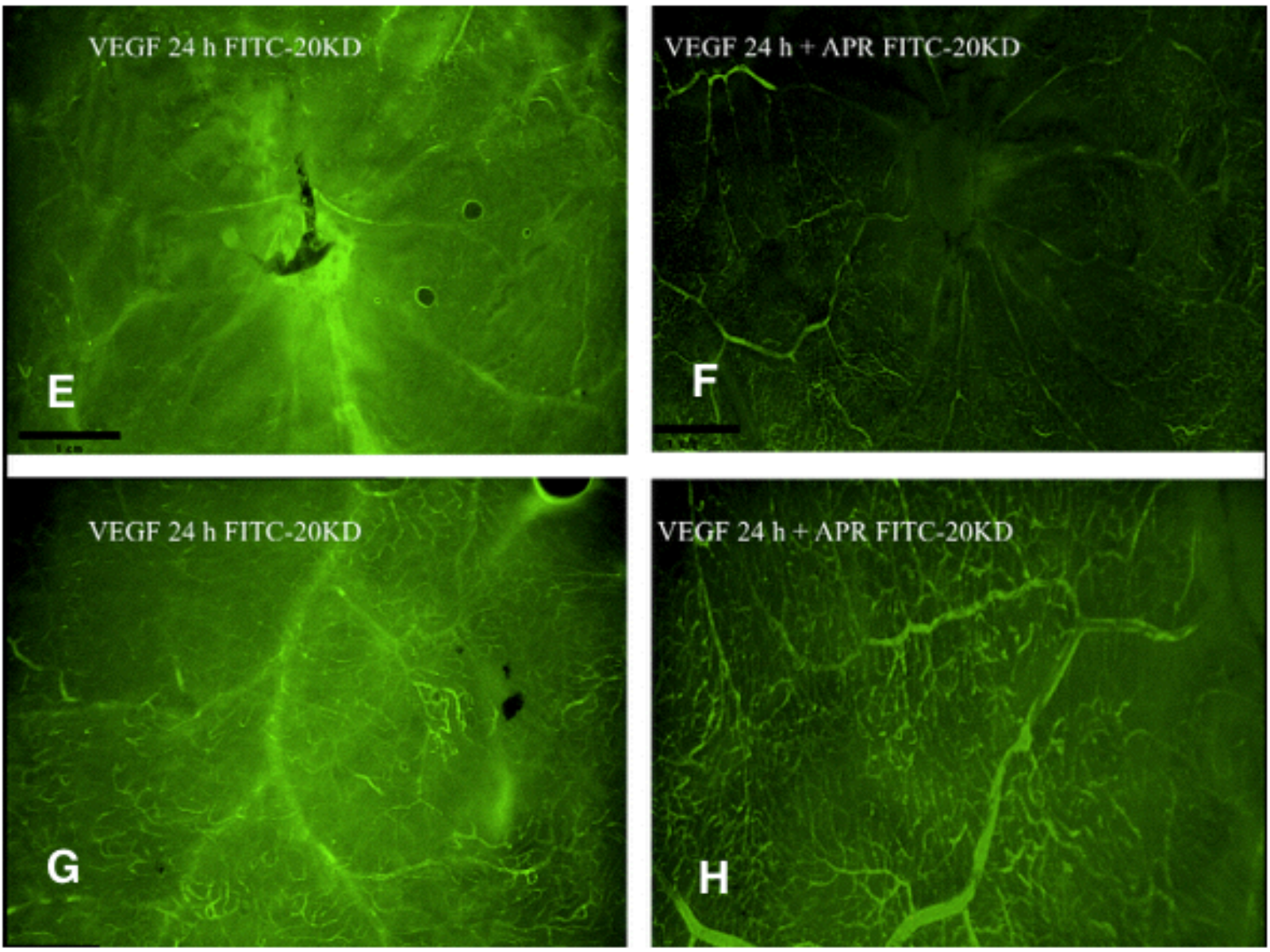

VEGF 24 h FITC-20KD
E
VEGF 24 h + APR FITC-20KD
F
VEGF 24 h FITC-20KD
G
VEGF 24 h + APR FITC-20KD
H

FIGURE 5

Qualitative evaluation of VEGF-induced retinal vascular permeability by fluorescein-dextran perfusion. Fluorescence photomicrographs of retinal flat mounts 24 hours after intravitreal injection of $VEGF_{164}$ (50 ng) alone (A,C,E,G) and intravitreal injection of VEGF in an animal treated with intravenous aprotinin every 8hrs after VEGF injection (B,D,F,H). In control animals (A,C.E.G) there is significant hypefluorscent signal outside the vascular lumen consistent with significant vascular leakage of dextran in extravascular space consistent with BRB breakdown noted after intravitreal injection of VEGF. When animals were treated with aprotinin (B,D,E,H) there is a dramatic decrease in hyperlfuorescent signal in the extravascular space and within retinal tissue and dextran was seen mostly within the vascular space indicating that aprotinin ameliorated the VEGF-induced BRB breakdown and retinal vascular leakage.

Aprotinin Reduces BRB breakdown in the Diabetic Retina

To further evaluate our hypothesis that azurocidin is a mediator in diabetic BRB breakdown and assess whether aprotinin reduces BRB breakdown in the STZ-induced diabetic model, diabetic rats were treated with aprotinin (1.5 mL/d bolus tail vein injection) for 10 days, and BRB breakdown was measured at 14 days using the Evans blue leakage assay and compared to diabetic animals that received treatment with vehicle only. Retinal vascular permeability was also evaluated in normal non diabetic animals and compared to diabetic animals that did not receive any treatment to demonstrate that diabetes induces BRB breakdown in 2 weeks after induction that can be measured with the standardized Evans Blue technique in our laboratory. BRB breakdown was increased 2.4-fold in the diabetic animals compared with age-matched non diabetic animals at 2 weeks. (18.2 ± 3.3 vs. 7.2 ± 1.6 μL/g/h, n = 8 and n = 9, respectively, P = 0.01; Fig. 6). Treatment with aprotinin after diabetes was confirmed resulted in a significant reduction of diabetic BRB breakdown by 40.6% when compared to diabetic animals that received treatment with vehicle only. (18.9 ± 2.97 vs. 11.2 ± 1.43 μL/g/h, n = 9 in each group, P = 0.03; Fig. 6).

Effect of Aprotinin on diabetic retinal vascular leakage

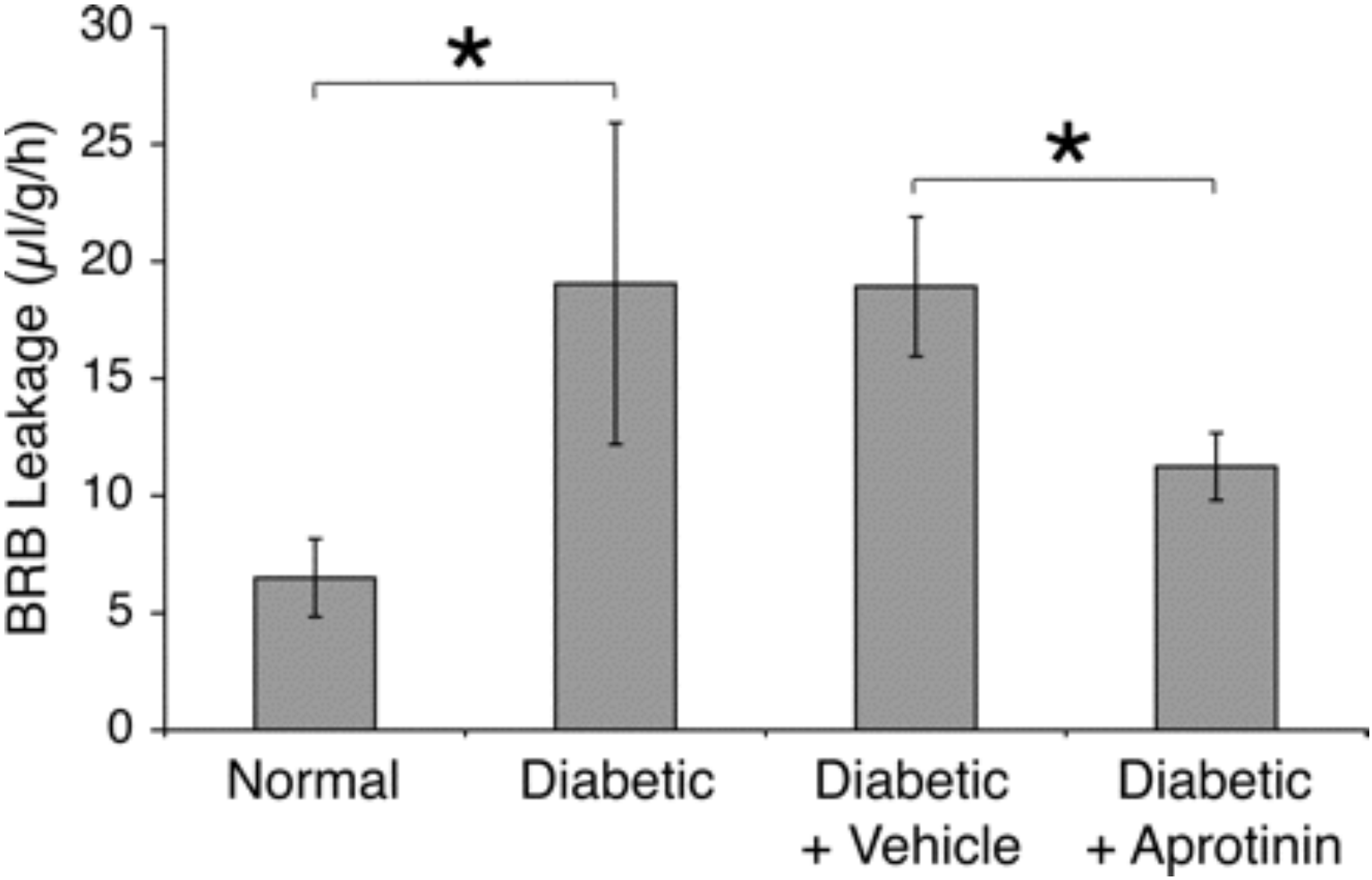


FIGURE 6

Aprotinin reduces BRB breakdown in the diabetic retina. BRB breakdown was evaluated with the Evans blue technique 2 weeks after diabetes induction and was compared with non diabetic control animals. Diabetic animals demonstrated a 2.4 fold increase of retinal vascular leakage at 2 weeks after diabetes induction compared to non diabetic control animals 18.2 ± 3.3 vs. 7.2 ± 1.6 μL/g/h, n = 8 and n = 9, respectively, P = 0.01). One group of diabetic animals received daily injections of aprotinin (15,000 KIU) for 10 days or the same volume of vehicle control through the tail vein. Aprotinin treatment significantly decreased retinal vascular leakage by 40.6% at 2 weeks compared with diabetic animals that received vehicle only treatments (18.9 ± 2.97 vs. 11.2 ± 1.43 μL/g/h, n = 9 in each group, P = 0.03). Data represent mean ± SEM. *P < 0.05.

Aprotinin Reduces vascular leakage in EIU

To further support our hypothesis that azurocidin maybe a key mediator of leukocyte mediated retinal vascular permeability, we decided to assess whether aprotinin reduces retinal vascular leakage in another animal model of leukocyte-mediated retinal vascular leakage and we used the rat model of EIU, a well established model characterized by leukostasis leading to retinal vascular vascular leakage. EIU was induced by hind footpad injections of LPS (200μg) in male Lewis rats. Animals were treated with 50,000 KIU Aprotinin (5ml of Trasylol) or the equivalent volume of saline, q8 hrs for 24 hrs. Retinal vascular permeability was measured with the Evans Blue (EB) technique 24 hours after the LPS footpad injection and also in control non uveitic animals that received vehicle (no LPS) footpad injection.

Footpad injection of LPS induced a significant 3.8-fold increase in retinal vascular leakage after 24 hours compared with vehicle (PBS)-injected rats (26.97 ± 16.5 vs. 7.17 ± 3.47 μL/g/h, n = 13, P = 0.006; Fig. 7). Treatment with aprotinin after LPS footpad injection resulted in a significant reduction of BRB breakdown by 73% when compared to LPS injected animals that received treatment with vehicle only. (26.97 ± 16.5 vs. 12.6.2 ± 2.8 μL/g/h, n = 13 in each group, P = 0.003; Fig. 7).

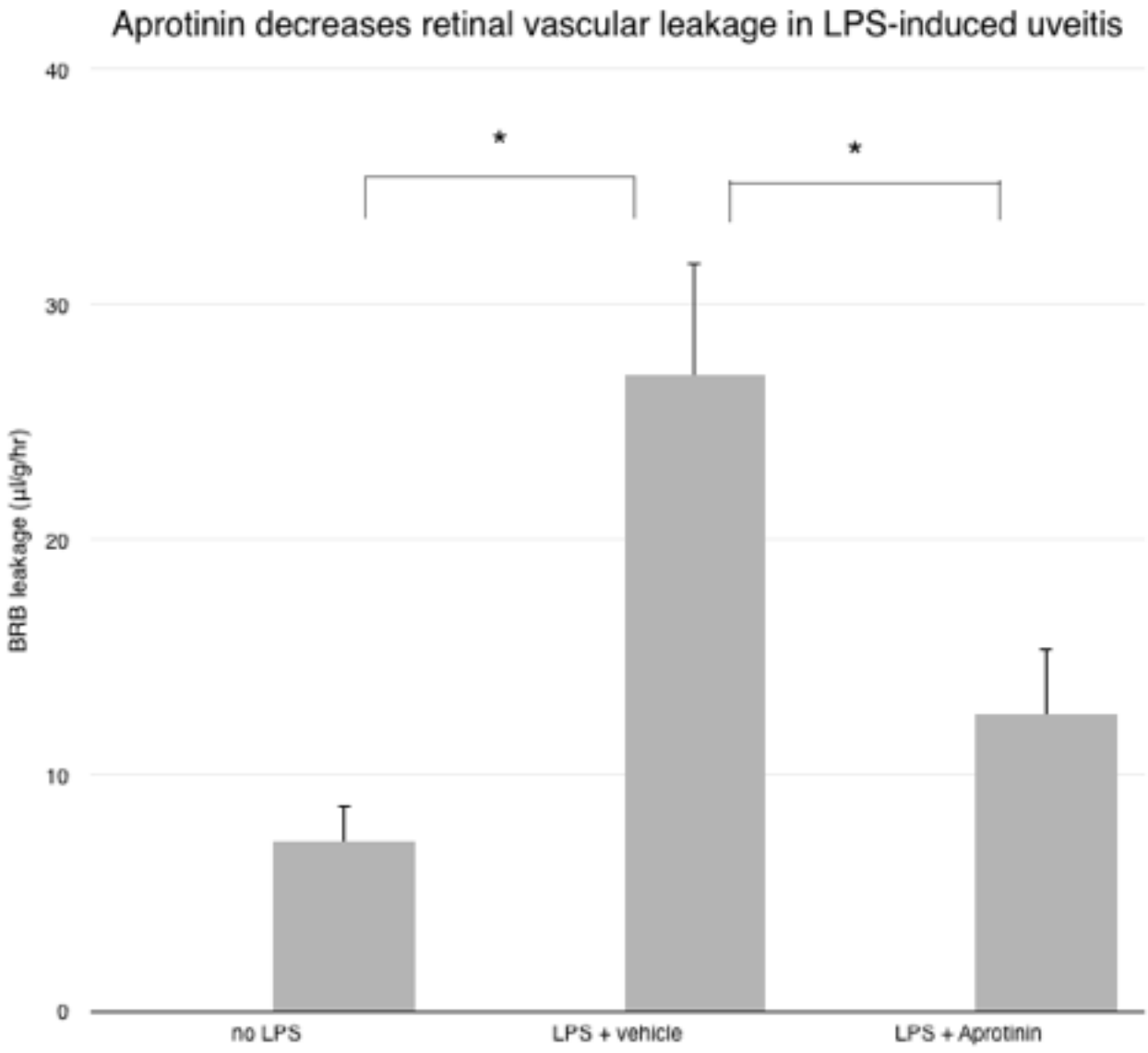

Aprotinin decreases retinal vascular leakage in LPS-induced uveitis
BRB leakage (µl/g/hr)
40
30
20
10
0
*
*
no LPS
LPS + vehicle
LPS + Aprotinin

FIGURE 7

Aprotinin reduces BRB breakdown in EIU uveitis . BRB breakdown was evaluated with the Evans blue technique 24 hours after EIU induction and was compared with non LPS control animals. LPS injected animals demonstrated a 3.8-fold increase in retinal vascular leakage after 24 hours compared with vehicle (PBS)-injected rats (26.97 ± 16.5 vs. 7.17 ± 3.47 μL/g/h, n = 13, P = 0.006). One group of EIU animals received treatment with aprotinin and another group of EIU animals received the same volume of vehicle. Aprotinin treatment significantly decreased retinal vascular leakage by 73% at 24 hours compared with uveitis animals that received vehicle only treatments (26.97 ± 16.5 vs. 12.6.2 ± 2.8 μL/g/h, n = 13 in each group, P = 0.003). Data represent mean ± SEM. *P < 0.05.

6. DISCUSSION

In this thesis, we investigated the role of the protease azurocidin, in BRB breakdown. We show for the first time that azurocidin increased retinal vascular leakage in vivo and that aprotinin was an effective inhibitor of the azurocidin-induced leakage in the retina. Furthermore, we have shown for the first time that the protease inhibitor aprotinin significantly decreased BRB breakdown after intravitreal VEGF administration, in a model of experimentally induced diabetes as well as another animal model of leukocyte-mediated BRB breakdown as seen in LPS induced uveitis.

Azurocidin is an important mediator of leukocyte-induced leakage during firm adhesion to the endothelium.(116) However, its effect as a permeability factor on vessels with neurovascular barrier properties is unknown. In our study, intravitreal administration of azurocidin rapidly increased albumin leakage in the retinal vessels, suggesting that azurocidin may be a key mediator of BRB breakdown.

To our knowledge, it has never been demonstrated before that azurocidin increases permeability in vessels with neurovascular barrier properties in vivo. Our results are in line with those of previous in vitro reports showing that the administration of azurocidin in vitro decreases transendothelial electrical resistance in aortic endothelial cell monolayers within 30 minutes of application. (116) The slightly different response time between our experiments and the

previous reports may be attributed to different experimental models. In our experiments, azurocidin was administered into the vitreous cavity but required diffusion into the inner retina to exert its effects on retinal vessels. In contrast, in the in vitro studies, azurocidin was applied directly to the endothelial cells, allowing immediate contact of azurocidin with its putative endothelial receptors. Furthermore, the focus of our study was barrier-privileged retinal vessels, which may have different dose-response and time-response to azurocidin than non-CNS endothelial cells. Our results indicate that exogenous azurocidin was a potent cause of leakage in retinal vessels in vivo. Our laboratory has also shown that azurocidin increases transendothelial resistance in vitro using CNS endothelial cells with barrier properties and that in vitro direct application of exogenous azurocidin on CNV endothelial cell cultures causes barrier breakdown and increases permeability and affects occludin ’s integrity in tight junctions connecting the cells (unpublished data). The in vitro data are in line with our vivo findings that azurocidin causes retinal vascular leakage and BRB breakdown in retinal endothelial cells, a hallmark of diabetic macular edema suggesting that it maybe a key mediator in diabetic BRB breakdown.

However, it is important to note that intravitreal azurocidin administration may induce the expression of VEGF or other inflammatory cytokines in the retinal microenviroment and can also recruit and activate leukocytes, monocytes and macrophages which can also compromise BRB function. Our data did not show

significant increase in number of static adherent leukocytes in 2 hours or 24 hours after azurocidin intravitreal injection, time points we have shown that azurocidin increases retinal vascular leakage. This may be due to the the fact that activated and recruited leukocytes may have already transmigrated through the compromised blood retinal barrier. Also the ex vivo concanavalin A perfusion technique evaluates only static adherent leukocytes but it does not provide insight about dynamic leukocyte adhesion parameters and the status of rolling leukocytes and activation status with up-regulation of adhesion molecules at the surface of the leukocytes and endothelial cells that promote leukocyte rolling, adhesion and activation and further secretion of cytokines.

Although endothelial cells, monocytes, and macrophages seem the main targets for azurocidin, additional cell types are also activated. Indeed, one of the earliest studies about pleiotropic effects of azurocidin reports the contraction of fibroblasts in response to azurocidin .(131) More recently, Pereira and colleagues (132) demonstrated that azurocidin induces migration of corneal epithelial cells, which is an important step during healing after bacterial cornea injury. Interestingly, in this model, azurocidin was detected before infiltration of PMN, indicating a local expression of this protein.(133) Healing mechanisms in the cornea may also be supported by azurocidin-induced corneal epithelial cell proliferation and corneal epithelial cell adhesion molecule expression. (132) Similar results were also found with regard to the effects of azurocidin on smooth

muscle cells. There, Lee et al (134) demonstrate the presence of azurocidin in smooth muscle cells of atherosclerotic vessels. Functionally, azurocidin was found to stimulate proliferation, migration, and expression of E-selectin and ICAM-1 in aortic smooth muscle cells. (135)

Even though our results show intravitreal azurocidin causes retinal vascular leakage, the mechanistic details, such as changes in tight endothelial junctions or paracellular transport, remain to be investigated in detail. Unpublished data from our team have demonstrated direct effect of azurocidin on tight junctions protein expression in vitro when using CNV endothelial cell lines. The exact mechanisms by which azurocidin activates signaling pathways in endothelial cells and stimulates reorganization of cytoskeletal and junctional complexes remain elusive since no specific azurocidin receptor has not been recognized yet . It has been proposed that due to azurocidin ’s bipolar charge status, its positive charged part interacts strongly with the negative charged proteoglycans on endothelial cells surface and this interaction initiates the changes in endothelial cells that lead to increased permeability. (109)

Regarding how azurocidin promotes BRB breakdown, a possible mechanism would be through activation of PKC pathway in retinal endothelial cells which is one of the major pathways involved in diabetic retinopathy pathogenesis, endothelial cell changes and diabetic BRB breakdown. Azurocidin related activation of PKC could be further promoting chemotaxis and leukocyte activation

, leukocyte adhesion and more azurocidin secretion amplifying the initial stimulation in a multiplying cascade manner. It has been recently shown that azurocidin activates the PKC signaling cascade in corneal endothelial cells leading to azurocidin-directed human corneal endothelial cells chemotaxis. (136) Azurocidin could be one of the key mediators upstream the PKC pathway activation and the missing link connecting diabetic status, leukocyte activation and PKC activation leading to the molecular sequelae noted in diabetic retina and BRB breakdown. Future studies could be directed to further evaluate the interaction between azurocidin and PKC pathway activation in retinal endothelial cells in diabetes.

Interestingly, more recently it has been shown that azurocidin actually contains a protease activity which cleaves insulin growth factor binding protein (IGFBP) -1, IGFBP-2 and IGFBP-4.*(*137) In catabolic states, high levels of IGFBP-1 predict poor survival, diabetes complications and may contribute to wasting in catabolism through inhibition of insulin grown factor (IGF)-mediated protein synthesis (138, 139, 140). IGFBP-2 may be important for metabolic control and IGFBP-4 for diabetic macroangiopathy, thus it is of interest that azurocidin may regulate the cleavage of these proteins and by this their action in diabetes. (141, 142). Several distinct insulin-like growth factor binding proteins (IGFBPs) are present in tissues and fluids of the developing and adult eye. However, the mechanism(s) involved in the regulation of ocular IGFBP levels is unknown.

About 2 decades ago, Moshyedi et al identified an endogenous factor in vitreous and aqueous humors that interacts with specific low molecular weight IGFBPs. Incubation in the presence of the serine-proteinase inhibitor aprotinin result in marked inhibition of this endogenous factor activity. Preliminary characterization then suggested that this factor most likely a serine proteinase, suggesting a mechanism for regulating the levels of these IGFBPs and thus the functional activities of IGFs in ocular fluids under normal and/or pathological conditions. Given the recent discovery of the pleotropic functions of azurocidin as a serine protease which is inhibited by aprotinin and its interactions with IGFP's, it is possible that the factor identified in ocular fluids regulating aqueous and vitreous IGFBPs two decades ago may represent azurocidin.(143) It has been shown that IGF play a pathogenic role in diabetic disease and diabetic retinopathy. (144, 145) Azurocidin's role in diabetic retinopathy may be partially also mediated by IGFBPs affecting IGF levels due to increased release of azurocidin from activated adherent leukocytes in retina. The involvement of azurocidin as a mediator in diabetic BRB breakdown and its proteolytic effects on IGFBP-1, IGFBP-2 and IGFBP-4 in diabetic retinopathy are of interest and further studies are needed to investigate their association.

Pereira et al have shown that in vitro azurocidin is a chemoattractant for microglia, a tissue resident innate immune /neuroglial cell type and azurocidin-

treated microglia are activated and produce proinflammatory cytokines and chemokines suggesting that azurocidin has the potential to serve as a neuroinflammatory molecule.(129) Given microglial activation resulting to release of inflammatory mediators, reactive oxygen species and nitric oxide is a key mechanism by which neuroinflammation is instigated in diabetic retinopathy, azurocidin released at the diabetic retinal microenviroment could promote microglial activation and neuroinflammation causing BRB breakdown.(146)

That release of azurocidin and activation of endothelial cells are of clinical importance has been demonstrated in a model of septic acute lung injury. Streptococcus pyogenes infections may lead to the streptococcal toxic shock syndrome, which is characterized by hypotension, multiple organ failure, and lung edema. In the course of the infection, S. pyogenes shed M1 protein, which forms complexes with fibrinogen.(147) These activate PMN to degranulate in the circulation (148), releasing proteins from all granule subsets, including azurocidin.(147) Degranulation of PMN was found to be causative of the subsequent lung damage and edema formation and injection of antibodies to azurocidin abrogated the lung injury pointing at the central position of this protein in the pathogenesis of M1 protein-induced lung damage. (148)

Interestingly, azurocidin expression has been demonstrated with immunocytochemical studies in the cerebral microvasculature in Alzheimer's disease, while not detected in brain vessels of normal controls or patients with other non- inflammatory neuropathologic conditions. Treatment of cerebral endothelial cultures with inflammatory mediators, cytokines or β-amyloid in vitro results in the induction of azurocidin expression without the presence of leukocytes indicating localization of azurocidin in endothelial cells with neurovascular properties independently of neutrophil adhesion. These data showing endothelial-azurocidin expression after inflammatory stimulation in CNS endothelial cells with could represent an additional mechanism that azurodicin could contribute to microvascular injury in diabetic retinopathy similarly to another neuroinflammatory process like Alzheimer's disease. (124)

Azurocidin has recently emerged as an important mediator in pathogenesis and biomarker in several clinical diseases. Serum levels of azurocidin have been found to be significantly elevated in patients during acute coronary syndrome since infiltrating neutrophils and macrophages are inversely associated with intraplaque collagen content and azurocidin may influence plaque vulnerability. (149) This association was improved with treatment with heparin in acute coronary syndrome as heparin may release azurocidin from cell surfaces and effects of azurocidin on endothelial permeability and monocyte recruitment could be affected by the yet undefined activities of the azurocidin- heparin-complexes. (150) Elevated azurocidin levels is an early indicator of organ failure and poor

neurological outcome after cardiac arrest , independent of microbial infection and is currently considered for prospective trials. The temporal profile of azurocidin is suggestive of a role in the pathogenesis of critical illness after cardiac arrest.(151) Azurocidin is a potential biomarker for early detection of acute respiratory distress syndrome (ARDS) development after trauma with possible causal role in the pathogenesis of this severe complication of trauma which is associated with increased permeability and neutrophil activation.(152) Azurocidin, has multiple functions in the inflammatory process during severe infections causing vascular leakage leading to extravascular efflux, which is an important pathophysiologic event in the development of septic shock. Not surprisingly, high azurocidin plasma levels are found in severe sepsis patients and in septic shock patients as well as in serious infections associated with endothelial damage. Linder and colleagues have recently demonstrated new aspects of azurocidin daily monitoring in intensive care unit (ICU) patients showing that that high azurocidin plasma levels are associated with an increased mortality rate in both septic and nonseptic critically ill patients, indicating that azurocidin may be a reliable prognostic biomarker.(153)

Our study was the first one to show a direct effect of azurocidin on retina vascular permeability and our published data was the first report ever to propose that azurocidin may be a key mediator in diabetic retinopathy, diabetic macular edema pathogenesis and diabetic microvascular complications. Interestingly ,

after our data report, it was recently shown that azurocidin could be one of the biofluid biomarkers to be used for retinal and microvascular complications in type one diabetes patients. In this study, in order to screen for biomarkers present in the most abundant human biofluids and to compare their proteolytic profile, the gelatinolytic protease activity of serum, saliva and urine from healthy individuals was evaluated and compared to the protease profiling of type 1 diabetics for at least 10 years with diabetic retinopathy only, diabetic retinopathy and diabetic nephropathy and also diabetics without any complications. Interestingly, the most outstanding disease-related proteolytic alterations were detected in urine and saliva. Among these with more activity, azurocidin was found in type 1 diabetic patients with retinopathy and/or nephropathy. These data are in line with our hypothesis and findings that azurocidin may have a role in pathogenesis of diabetic retinopathy and also it could be used as a useful screening biomarker for microvascular complications in patients with diabetes as well as therapeutic target. Future prospective studies are essential for the early predictive value of this potential biomarker for diabetes diagnosis, prognosis and the role of azurocidin in diabetic retinopathy in humans remains to be further investigated.(154)

Our results show that aprotinin is an effective in vivo inhibitor of the azurocidin-induced leakage in retinal vessels suppressing 98% of azurocidin induced BRB breakdown. Aprotinin treatment also significantly decreased VEGF-induced

leakage and BRB breakdown in experimentally induced diabetes and EIU, suggesting a possible role for azurocidin in these leukocyte mediated processes. Aprotinin is a serine protease inhibitor and it has been demonstrated in vitro that azurocidin-induced increases in the permeability of endothelial cells can be completely attenuated by aprotinin.(116) Unfortunately rat and murine antibodies against azurocidin as well specific azurocidin inhibitors are not commercially available. Based on previous reports that aprotinin in vitro is a an strong azurocidin inhibitor we decided to use aprotinin as a potential azurocidin inhibitor in the animals models of VEGF induced, diabetic BRB breakdown and EIU. Our data suggest that azurocidin could be a new therapeutic target for treatment of diabetic macular edema and aprotinin/azurocidin inhibitors could potentially be a new treatment for diabetic macular edema creating a new approach for pharmaceutical strategy of patients with diabetic macular edema that has never been investigated before.

Aprotinin eye drops have been shown to decrease corneal haze in an experimental rabbit mode of of photorefractive keratectomy.(155) Systemic aprotinin supplement has been shown to prevent light-induced retinal damage in an experimental animal model of guinea pigs.(156) The same group has also studied the effect of aprotinin in experimental uveitis in guinea pings and have shown decreased histopathological changes and inner plexiform layer thickness in aprotinin treated animals.(157) Interestingly the same group have investigated

the effect of aprotinin of ischemia reperfusion injury in the retina in guinea pigs showing decreased inner plexiform retinal thickness on histology.(158) Ischemia reperfusion and uveitis share some important characteristics with VEGF induced and diabetic retinal changes. Leukocyte activation, adhesion and its correlation with the BRB breakdown noted are hallmarks of all of these processes. Our team has also shown that aprotinin dramatically decreases blood-aqueous barrier by 77% and and suppresses BRB breakdown by 73% in rat model of uveitis model by dramatically suppressing anterior chamber protein leakage and retinal vascular leakage.(159)

As a broad serine protease inhibitor, aprotinin also blocks other serine proteases, such as neutrophil-derived elastase, cathepsin G, proteinase 3, and some proteases in coagulation and fibrinolysis pathways, including plasmin and kallikrein.(160, 161, 162) Some of these proteases may be involved in retinal vascular leakage.(163) Because aprotinin does not exclusively block azurocidin, our results do not exclude the potential involvement of other proteases in the VEGF-induced retinal vascular leakage or the BRB breakdown seen in early diabetes. Aprotinin could also protect BRB function in diabetes through reduction of oxidative stress in retina tissue similarly to what has been demonstrated in experimental light induced retinal damage, retina ischemia reperfusion injury and experimental uveitis.(156,157,158) Furthermore, aprotinin may inhibit leukocyte recruitment as well through inhibition of azurocidin and potentially other factors,

so its protective function on BRB breakdown could in part be due to its anti-inflammatory properties. Taken together, these results suggest that aprotinin may be useful in the treatment of retinal vascular leakage, such as that caused by diabetic retinopathy or uveitis potentially suppressing multiple pathogenetic pathways simultaneously.

Aprotinin (Trasylol, Bayer Pharmaceuticals) is an FDA-approved medicine readily available in clinical use for patients undergoing extensive cardiothoracic and orthopedic surgery, who often experience neutrophil sequestration in organs and massive leakage of fluid from the vasculature. Aprotinin has shown to significantly reduce blood loss and blood transfusion requirements after surgery. (164) Gautam et al. (118) proposed the inhibition of azurocidin as a possible mechanism of action of aprotinin for these patients given the crucial role of azurocidin in neutrophil-evoked permeability. Some published articles reported increased risk for renal (165) and cardiovascular toxicity, including myocardial infarction and stroke, (166) after aprotinin administration in major surgeries leading to temporary suspension of medicine in Europe and USA but recent meta analysis of multiple studies did not support this evidence of increased risks. Based on multiple clinical data analysis , aprotinin effectively reduces blood loss and the need for transfusion associated with heart surgery and currently in Canada and in Europe, health committees believe the accumulated evidence of the benefits of aprotinin outweigh its risks in isolated CABG surgery and the risks

with aprotinin are not higher compared to other antifibrinolytic agents used in the same surgical setting and its use has been re-approved. (167) However, if used in the treatment of diabetic retinopathy and ocular inflammatory conditions, aprotinin could be delivered intravitreally or locally/periocularly with low risk of systemic side effects. Further pharmacological studies to evaluate the pharmakinetics, the intravitreal bioavailibity, pH changes in vitreous cavity, intravitreal dose studies and potential retinal toxicity with direct intravitreal injection of aprotinin would be needed. Our studies show for the first time that aprotinin significantly decreased retinal vascular leakage in VEGF induced retinal vascular leakage , diabetic retinopathy and uveitis and could be a new treatment widening the horizon for potential new innovative intravitreal therapies for macular edema, a major cause of vision loss.

In summary, our results show that azurocidin is a newly characterized potent retinal vascular permeability factor and seems to play a role in leukocyte mediated BRB breakdown induced by VEGF and in experimental diabetes. Azurocidin release from neutrophils may be the final common pathway for a variety of upstream factors and pathways, which during diabetic retinopathy promote leukocyte adhesion and cause BRB breakdown. These findings indicate that targeting azurocidin and/or potential clinical trials of aprotinin (which is already in clinical use) may prove beneficial in the treatment of retinal vascular

leakage caused by ocular diseases such as diabetic retinopathy, uveitis/ intraocular inflammation and other causes of macular edema.

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Characterization of Azurocidin as a Permeability Factor in the Retina: Involvement in VEGF-Induced and Early Diabetic Blood-Retinal Barrier Breakdown

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Purpose. Azurocidin, released by neutrophils during leukocyte-endothelial interaction, is a main cause of neutrophil-evoked vascular leakage. Its role in the retina, however, is unknown.

Methods. Brown Norway rats received intravitreal injections of azurocidin and vehicle control. Blood-retinal barrier (BRB) breakdown was quantified using the Evans blue (EB) dye technique 1, 3, and 24 hours after intravitreal injection. To block azurocidin, aprotinin was injected intravenously before the intravitreal injections. To investigate whether azurocidin plays a role in vascular endothelial growth factor (VEGF)-induced BRB breakdown, rats were treated intravenously with aprotinin, followed by intravitreal injection of $VEGF_{164}$. BRB breakdown was quantified 24 hours later. To investigate whether azurocidin may mediate BRB breakdown in early diabetes, aprotinin or vehicle was injected intravenously each day for 2 weeks to streptozotocin-induced diabetic rats, and BRB breakdown was quantified.

Results. Intravitreal injection of azurocidin (20 μg) induced a 6.8-fold increase in vascular permeability compared with control at 1–3 hours ($P < 0.05$), a 2.7-fold increase at 3 to 5 hours ($P < 0.01$), and a 1.7-fold increase at 24 hours ($P < 0.05$). Aprotinin inhibited azurocidin-induced BRB breakdown by more than 95% ($P < 0.05$). Furthermore, treatment with aprotinin significantly suppressed VEGF-induced BRB breakdown by 93% ($P < 0.05$) and BRB breakdown in early experimental diabetes by 40.6% ($P < 0.05$).

Conclusions. Azurocidin increases retinal vascular permeability and is effectively blocked by aprotinin. The inhibition of VEGF-induced and early diabetic BRB breakdown with aprotinin indicates that azurocidin may be an important mediator of leukocyte-dependent BRB breakdown secondary to VEGF. Azurocidin may become a new therapeutic target in the treatment of retinal vascular leakage, such as during diabetic retinopathy. (*Invest Ophthalmol Vis Sci.* 2008;49:726–731) DOI:10.1167/iovs.07-0405

Text

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Firm adhesion of neutrophils to the inflamed endothelium is a cause of vascular leakage.[1–3] Vascular endothelial growth factor (VEGF) is a hypoxia-induced angiogenic factor[4,5] and a major vasopermeability factor.[6,7] VEGF is causally linked to the pathogenesis of diabetic retinopathy, playing an important role in leukocyte-mediated breakdown of the blood-retinal barrier (BRB) and retinal neovascularization.[8] Within the first 2 weeks of experimental diabetes in rats, retinal VEGF levels increase with associated upregulation of intracellular adhesion molecule (ICAM)-1 in retinal endothelial cells and its ligands, the β_2-integrins, on the surfaces of peripheral blood neutrophils.[9–11] These molecular events result in an increased adhesion of leukocytes, predominantly neutrophils, and a concomitant increase in retinal vascular permeability.[9–11]

Intravitreal injection of VEGF induces the retinal vascular changes seen in experimental diabetes, including retinal leukostasis and concomitant BRB breakdown,[8] whereas blockade of VEGF abolishes retinal leukostasis and vascular leakage in experimentally induced diabetes.[8,9,12] Furthermore, when leukocyte adhesion is inhibited, through the blockade of ICAM-1 or β_2-integrins, VEGF-induced and diabetic BRB breakdown is suppressed, indicating a link between leukocyte adhesion and increased retinal vascular leakage.[11,13] However, the molecular pathways involved in BRB breakdown downstream of leukocyte adhesion are not well understood.

Azurocidin (heparin-binding protein or CAP37) is a 28-kDa inactive serine protease stored in the azurophilic granules of neutrophils.[14,15] It is a multifunctional protein with antimicrobial and chemotactic properties, especially for monocytes.[16] Recently, azurocidin has been shown to be the missing link in neutrophil-induced endothelial permeability in non–central nervous system (CNS) vessels.[17] During firm adhesion of neutrophils to activated endothelium, β_2-integrin ligation with endothelial intercellular adhesion molecule-1 (ICAM-1) causes azurocidin release.[17] Azurocidin induces Ca^{2+}-dependent cytoskeletal rearrangement and intercellular gap formation in endothelial cell monolayers in vitro and increases macromolecular permeability in peripheral non-CNS vessels in vivo.[17] Moreover, azurocidin blockade prevents neutrophil-induced endothelial hyperpermeability, emphasizing the crucial role of azurocidin in vascular responses during inflammation.[17]

However, whether azurocidin may have an effect on vessels of the CNS is unknown. A unique property of these vessels is their neurovascular barrier function, known as the blood-brain barrier (BBB) or, in the case of the retina, the BRB. The BBB and the BRB act as regulatory interfaces between the blood and the nervous system[18] and are essential for the protection of neu-

rons from blood-borne molecules and cells.[18,19] In diabetic retinopathy, BRB breakdown leads to macular edema, which results in visual loss. Therefore, elucidating the factors that compromise the BRB may improve our therapeutic approach to macular edema, the main cause of visual loss in patients with diabetes.

Given the important role of azurocidin in mediating neutrophil-induced endothelial permeability in vascular systems outside the CNS and given that neutrophil adhesion occurs in VEGF-induced and diabetic BRB breakdown, we hypothesize that azurocidin may also be an important mediator of BRB breakdown in these two scenarios.

Materials and Methods

Animals

All animal experiments adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, and protocols were approved by the Animal Care Committee of the Massachusetts Eye and Ear Infirmary. Male Brown Norway and Long-Evans rats, weighing 200 to 300 g each, were used for the experiments. Animals were fed standard laboratory chow and allowed free access to water in an air-conditioned room with a 12-hour light/12-hour dark cycle until they were used for the experiments.

Injections

Animals were anesthetized with intramuscular injection of xylazine hydrochloride (6 mg/kg; Phoenix Pharmaceutical, St. Joseph, MO) and ketamine hydrochloride (40 mg/kg; Parke-Davis, Morris Plains, NJ). To perform the intravitreal injections, a 31-gauge needle (Hamilton) was inserted into the vitreous 1 mm posterior to the corneal limbus.[20,21] Insertion and infusion were directly viewed through an operating microscope to prevent injury to the lens and retina. Eyes that exhibited signs of damage to these structures were excluded from the experiments. Intravenous injections were performed through the tail vein with a 27-gauge butterfly needle under anesthesia.

Induction of Diabetes

Male Long-Evans rats weighing approximately 200 g each were used for these experiments. To induce diabetes, each animal received a single 60 mg/kg intraperitoneal injection of streptozotocin (Sigma, St. Louis, MO) in 10 mM sodium citrate buffer, pH 4.5, after an overnight fast. Control nondiabetic animals received citrate buffer alone. Animals with blood glucose levels higher than 250 mg/dL 24 hours after injections were considered diabetic. All experiments were performed 2 weeks after the induction of diabetes.

Administration of Azurocidin and Aprotinin

Rats received intravitreal injections of 5 μL sterile phosphate-buffered saline (PBS) containing 1 or 20 μg human neutrophil azurocidin (Athens Research and Technology, Atlanta, GA) in one eye and 5 μL of sterile PBS in the contralateral eye. Retinas were analyzed for BRB breakdown quantification by the Evans blue (EB) technique[22] 1, 3, and 24 hours after azurocidin injection.

In a second group, the rats also received the azurocidin inhibitor aprotinin (10,000 or 30,000 kallikrein-inducing units [KIU]; equivalent to 1 or 3 mL Trasylol, respectively, Bayer Pharmaceuticals, Pittsburgh, PA) by intravenous injection. Aprotinin was administered through the tail vein 1 hour before intravitreal injection of azurocidin. Retinas were analyzed 1 hour after azurocidin administration for BRB breakdown quantification.

Administration of VEGF and Aprotinin

Rats received intravitreal injections of 5 μL sterile PBS containing 50 ng $VEGF_{164}$ (R&D Systems, Minneapolis, MN) in one eye and 5 μL sterile PBS in the contralateral eye. Retinas were analyzed 24 hours after VEGF injection for BRB breakdown quantification.

In a second group, rats also received the broad protease inhibitor aprotinin (50,000 KIU; equivalent to 5 mL Trasylol) by intravenous injection. Aprotinin was administered through the tail vein 1 hour before and 8 and 16 hours after intravitreal injection of VEGF or PBS. Retinas were analyzed 24 hours after VEGF administration for BRB breakdown.

Blood-Retinal Barrier Breakdown Measurement with the Evans Blue Technique

Retinal vascular permeability was quantified as previously described.[9,22] After the animals were deeply anesthetized, EB dye (30 mg/mL in saline; Sigma) was injected through the tail vein over 10 seconds at a dosage of 45 mg/kg. Blood samples were obtained from the left ventricle, just before perfusion, to obtain the time-averaged EB plasma concentration. Blood samples were centrifuged at 12,000 rpm for 15 minutes to separate the plasma from the cellular components. Plasma samples were diluted to 1/10,000 of their initial concentration in formamide (Sigma). Absorbance was measured with a spectrophotometer at 620 nm and 740 nm. After the dye had circulated for 2 hours, the chest cavity was opened, and the rats were perfused through the left ventricle with paraformaldehyde 1% in citrate buffer (0.05 M, pH 3.5) at a constant pressure of approximately 120 mm Hg. Retinas were then carefully dissected under an operating microscope. After measurement of the retinal weight, EB was extracted by incubation of each retina in 180 μL formamide for 18 hours at 70°C. The extract was ultracentrifuged at a speed of 14,000 rpm for 60 minutes at 25°C. Sixty microliters of the supernatant was used for spectrophotometric measurement at 620 nm and 740 nm.[22] Background-subtracted absorbance was determined by measuring each sample at 620 nm (absorbance maximum for EB in formamide) and 740 nm (absorbance minimum). BRB breakdown was calculated as previously described, and values were expressed as plasma (μL) $\times$ retinal weight (g^{-1}) $\times$ time ($hours^{-1}$).[9,22]

Qualitative Evaluation and Visualization of Retinal Vascular Permeability

Retinal vascular permeability was also demonstrated in a histologic manner by intravenous injection of 20 kDa FITC-conjugated dextran (50 mg/kg; Sigma). Rats were killed 30 minutes later and perfused with 4% paraformaldehyde to fix the dextran conjugate in the tissues. Retinas were carefully dissected and flat mounted in an antifading medium (Vector Laboratories, Burlingame, CA). Flat-mounted retinas were examined by fluorescence microscopy. Digital color enhancement (green) was equally applied to all images to improve visualization of the fluorescence.

Results

Azurocidin Increases Retinal Vascular Leakage In Vivo

To investigate whether exogenous azurocidin causes leakage in vessels with neurovascular barrier properties, we assessed the effect of intravitreal administration of azurocidin on retinal vascular leakage by the Evans blue technique. Azurocidin increased retinal vascular leakage in vivo in a time-dependent manner with a peak 1–3 hours after administration. Intravitreal injection of 20 μg azurocidin induced a 6.8-fold increase in the leakage of EB from retinal vessels compared with vehicle-injected control eyes, 1–3 hours after injection (31 $\pm$ 11 vs. 4.5 $\pm$ 1 μL/g/h, $n = 12$, $P = 0.02$), a 2.7-fold increase 3 to 5 hours after injection (21 $\pm$ 2.3 vs. 8.3 $\pm$ 1.4 μL/g/h, $n = 11$, $P = 0.0006$), and a 1.7-fold increase 24 hours after injection (16.5 $\pm$ 2.8 vs. 8.7 $\pm$ 1.8 μL/g/h, $n = 6$, $P = 0.04$; Fig. 1A).

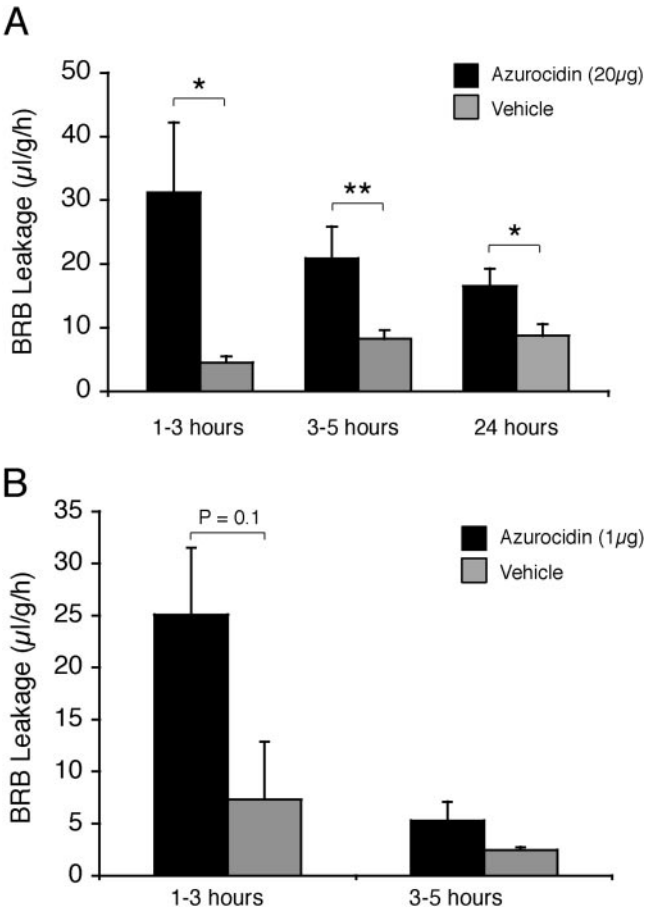

FIGURE 1. Azurocidin increases retinal vascular permeability in vivo in a time-dependent manner. To assess whether azurocidin increases retinal vascular permeability in vivo, azurocidin was injected intravitreally at the doses of 20 μg (**A**) or 1 μg (**B**) in one eye and the same volume of vehicle (5 μL PBS) in the contralateral eye. The BRB breakdown was quantified with the Evans blue technique at different time points after intravitreal injection. Bars represent mean ± SEM. $^{*}P < 0.05$; $^{**}P < 0.01$.

To investigate the dose-response of azurocidin on BRB leakage, 1 μg of purified protein was injected intravitreally, and ocular leakage was compared with the vehicle-injected eyes. At the lower dose of 1 μg, azurocidin induced a 3.4-fold increase in BRB leakage compared with the vehicle-injected eyes of the same animals (25.1 ± 6.4 vs. 7.3 ± 5.5 μL/g/h, $n = 3$, $P = 0.1$). Three to five hours after the injection of 1 μg azurocidin, the difference in leakage between the injected eyes and the control eyes further decreased and remained statistically nonsignificant. Our results suggest a concentration and time-dependent waning of the impact of azurocidin on BRB leakage in vivo (Fig. 1B).

Aprotinin Suppresses Azurocidin-Induced BRB Breakdown

To investigate whether the protease inhibitor, aprotinin, prevents azurocidin-induced BRB breakdown, rats were treated with a single intravenous injection of low- or high-dose aprotinin (10,000 or 30,000 KIU) 1 hour before intravitreal injection of azurocidin or vehicle control (PBS). Retinal vascular leakage was determined 1 hour later. Treatment of the animals with aprotinin (30,000 KIU) blocked azurocidin-induced leakage by >98% (45.5 ± 16.8 vs. 6.1 ± 0.64 μL/g/h, $n = 8$ and $n = 6$, respectively, $P = 0.03$), whereas aprotinin (30,000 KIU) treatment alone did not affect basal levels of retinal vascular

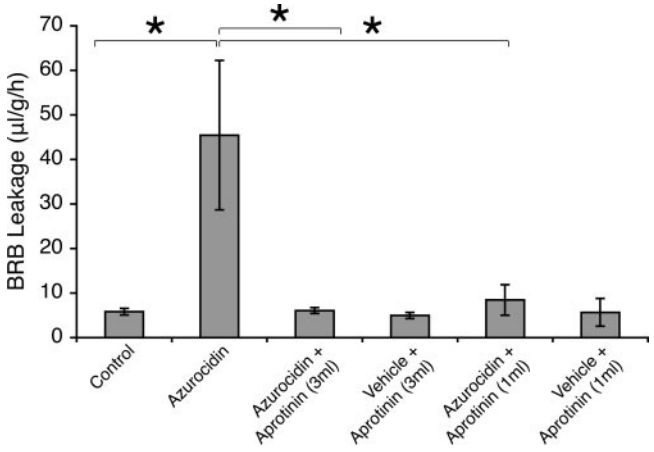

FIGURE 2. Aprotinin suppresses azurocidin-induced BRB breakdown. To investigate whether the protease inhibitor, aprotinin, is effective in inhibiting azurocidin-induced BRB breakdown, rats were treated with a single intravenous injection of 1 or 3 mL aprotinin (10,000 KIU or 30,000 KIU) 1 hour before intravitreal injection of azurocidin or vehicle. BRB breakdown was quantified with Evans blue technique 1 hour later. Data represent mean ± SEM. $^{*}P < 0.05$.

leakage in vehicle-injected eyes (Fig. 2). In comparison, the low-dose injection of aprotinin (10,000 KIU) also significantly blocked azurocidin-induced leakage (8.5 ± 3.4 μL/g/h, $n = 8$, $P = 0.048$), while systemic injection of aprotinin alone did not impact the leakage of vehicle injected eyes (Fig. 2).

Aprotinin Suppresses VEGF-Induced BRB Breakdown

Intravitreal injection of 50 ng VEGF induced a significant 3.2 ± 0.7-fold increase in retinal vascular leakage after 24 hours compared with vehicle (PBS)-injected eyes (29.7 ± 7.5 vs. 9.6 ± 1.4 μL/g/h, $n = 8$, $P = 0.02$; Fig. 3). To assess whether the protease inhibitor, aprotinin, reduces VEGF-induced BRB breakdown, rats were treated in one eye with intravenous injections of aprotinin 1 hour before and 8 and 16 hours after

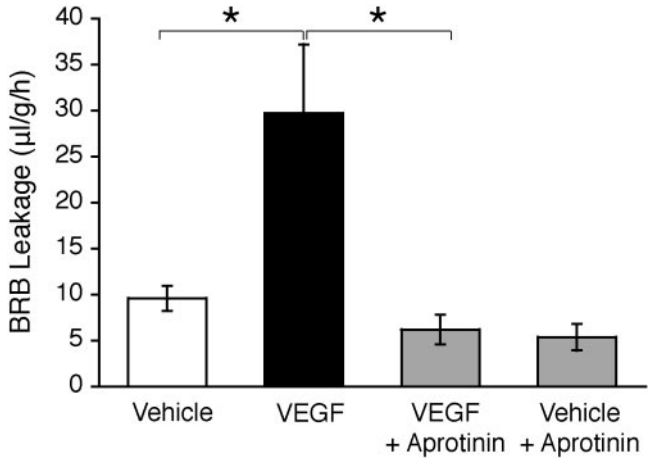

FIGURE 3. Aprotinin suppresses VEGF-induced BRB breakdown. $VEGF_{164}$ (50 ng in 5 μL) was injected intravitreally in one eye, and the same volume of vehicle control was injected in the other eye of the same animal. BRB breakdown was measured using the Evans blue technique 24 hours after intravitreal injection. In some animals aprotinin (50,000 KIU) was administered by tail vein injection. Data represent the comparison of the leakage between the $VEGF_{164}$ and vehicle-treated eyes ± SEM. $^{*}P < 0.05$.

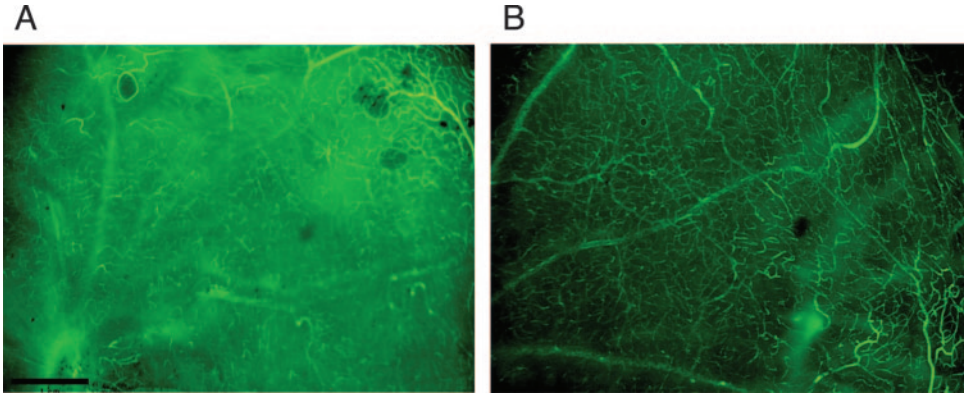

FIGURE 4. Qualitative evaluation of VEGF-induced retinal vascular permeability by fluorescein-dextran perfusion. Fluorescence photomicrographs of retinal flat mounts 24 hours after (**A**) intravitreal injection of $VEGF_{164}$ (50 ng) alone and (**B**) intravitreal injection of VEGF in an animal treated with intravenous aprotinin 1 hour before and 8 and 16 hours after injection.

intravitreal injection of VEGF or vehicle control in the contralateral eye. BRB breakdown was quantified with the EB technique 24 hours after the intravitreal injections. VEGF-induced BRB breakdown was suppressed by 93% with intravenous administration of aprotinin (29.7 ± 7.5 vs. 6.2 ± 1.6 μL/g/h, $n = 8$ and $n = 6$, respectively, $P = 0.02$; Fig. 3).

To visualize the differences in leakage, the retinal distribution of intravenously injected 20 kDa FITC-conjugated dextran was performed. Fluorescence microscopy of flat-mounted retinas from VEGF-injected eyes showed diffusely distributed fluorescence throughout the retinal tissues in intraluminal and extravascular locations. However, when animals were additionally treated with aprotinin, fluorescence was mainly confined to the intraluminal space of the retinal vessels with very little extravascular fluorescence (Fig. 4).

Aprotinin Reduces BRB breakdown in the Diabetic Retina

To assess whether aprotinin reduces BRB breakdown in the STZ-induced model, diabetic rats were treated with aprotinin (1.5 mL/d bolus tail vein injection) for 10 days, and BRB breakdown was measured at 14 days using the Evans blue leakage assay. BRB breakdown was increased 2.4-fold in the diabetic animals compared with age-matched nondiabetic animals (18.2 ± 3.3 vs. 7.2 ± 1.6 μL/g/h, $n = 8$ and $n = 9$, respectively, $P = 0.01$; Fig. 5). Treatment with aprotinin resulted in a significant reduction of diabetic BRB breakdown by 40.6% (18.9 ± 2.97 vs. 11.2 ± 1.43 μL/g/h, $n = 9$ in each group, $P = 0.03$; Fig. 5).

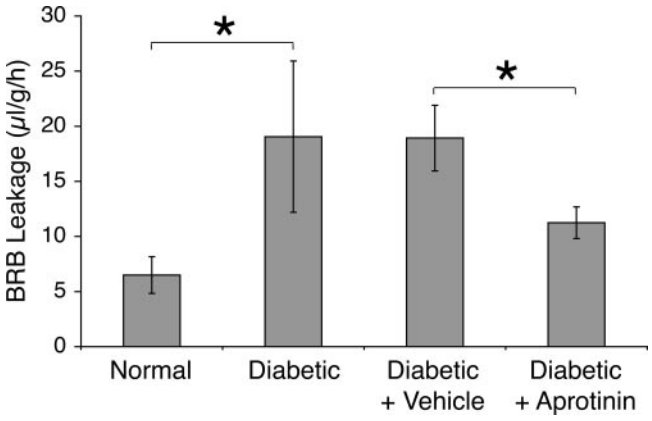

FIGURE 5. Aprotinin reduces BRB breakdown in the diabetic retina. BRB breakdown was evaluated with the Evans blue technique 2 weeks after diabetes induction and was compared with nondiabetic control animals. One group of diabetic animals received daily injections of aprotinin (15,000 KIU) or the same volume of vehicle control through the tail vein. Data represent mean ± SEM. $^*P < 0.05$.

DISCUSSION

This study investigated the role of the inflammatory mediator, azurocidin, in BRB leakage. We show that azurocidin increased retinal vascular leakage in vivo and that aprotinin was an effective inhibitor of the azurocidin-induced leakage in the retina. Furthermore, aprotinin significantly decreased BRB breakdown after intravitreal VEGF administration and in a model of experimentally induced diabetes.

Azurocidin is an important mediator of neutrophil-induced leakage during firm adhesion to the endothelium.[17] However, its effect as a permeability factor on vessels with neurovascular barrier properties is unknown. In our study, intravitreal administration of azurocidin rapidly increased albumin leakage in the retinal vessels, suggesting that azurocidin may be a key mediator of BRB breakdown. Our results are in line with those of previous reports showing that the administration of azurocidin in vitro decreases transendothelial electrical resistance in aortic endothelial cell monolayers within 30 minutes of application.[17] The slightly different response time between our experiments and the previous reports may be attributed to different experimental models. In our experiments, azurocidin was administered into the vitreous cavity but required diffusion into the inner retina to exert its effects on retinal vessels. In contrast, in the in vitro studies, azurocidin was applied directly to the endothelial cells, allowing immediate contact of azurocidin with its putative endothelial receptors. Furthermore, the focus of our study was barrier-privileged retinal vessels, which may have different dose-response and response-time to azurocidin than non-CNS endothelial cells. Our results indicate that exogenous azurocidin was a potent cause of leakage in retinal vessels in vivo.

However, it is important to note that intravitreal azurocidin administration may induce the expression of VEGF or other cytokines, which can also compromise BRB function. Therefore, indirect effects of azurocidin on BRB in vivo cannot be excluded. In addition, even though our results indicate intravitreal azurocidin causes retinal vascular leakage, the mechanistic details, such as changes in tight endothelial junctions or paracellular transport, remain to be investigated.

Our results show that aprotinin is an effective inhibitor of the azurocidin-induced leakage in retinal vessels in vivo. Aprotinin treatment also significantly decreases VEGF-induced leakage and BRB breakdown in experimentally induced diabetes, suggesting a possible role for azurocidin in these events. The role of azurocidin in diabetic retinopathy in humans remains to be investigated. As a broad serine

protease inhibitor, aprotinin also blocks other serine proteases, such as neutrophil-derived elastase, cathepsin G, proteinase 3, and some proteases in coagulation and fibrinolysis pathways, including plasmin and kallikrein.[23–25] Some of these proteases may be involved in retinal vascular leakage.[26] Because aprotinin does not exclusively block azurocidin, our results do not exclude the potential involvement of other proteases in the VEGF-induced retinal vascular leakage or the BRB breakdown seen in early diabetes. Furthermore, because aprotinin is known to inhibit leukocyte recruitment, its protective function on BRB breakdown could in part be due to its anti-inflammatory properties. Taken together, these results suggest that aprotinin may be useful in the treatment of retinal vascular leakage, such as that caused by diabetic retinopathy.

Aprotinin is in clinical use for patients undergoing extensive cardiothoracic and orthopedic surgery, who often experience neutrophil sequestration in organs and massive leakage of fluid from the vasculature. Aprotinin can help to reduce blood loss and blood transfusion requirements after surgery.[24,27] Gautam et al.[17] proposed the inhibition of azurocidin as a possible mechanism of action of aprotinin for these patients given the crucial role of azurocidin in neutrophil-evoked permeability. Two recently published articles report increased risk for renal[28] and cardiovascular toxicity, including myocardial infarction and stroke,[29] after aprotinin administration in major surgeries. These systemic side effects of aprotinin may be due in part to its limited specificity in vivo. However, if used in the treatment of diabetic retinopathy and ocular inflammatory conditions, aprotinin could be delivered intravitreally with low risk of systemic side effects.

In summary, our results suggest that azurocidin plays a role in BRB breakdown induced by VEGF or in experimental diabetes. Azurocidin release from neutrophils may be the final common pathway for a variety of upstream factors, which during diabetic retinopathy promote neutrophil adhesion and cause BRB breakdown.[30–34] These findings indicate that targeting azurocidin may prove beneficial in the treatment of retinal vascular leakage caused by ocular diseases such as diabetic retinopathy.

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