

The RAGE Axis in Early Diabetic Retinopathy

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PURPOSE. The receptor for advanced glycation end products (AGEs) has been implicated in the pathogenesis of diabetic complications. This study was conducted to characterize the role of the RAGE axis in a murine model of nonproliferative diabetic retinopathy (NPDR).

METHODS. The retinas of hyperglycemic, hyperlipidemic (HGHL, apolipoprotein E^{-/-} db/db) mice were examined for the development of early retinal vascular lesions of NPDR and compared to littermates at 6 months of age. Neural function was assessed with electroretinography. Immunohistochemistry, real-time RT-PCR, autofluorescence, and ELISA studies were used to localize and quantify the AGE/RAGE axis. Soluble RAGE, a competitor of cellular RAGE for its ligands, was administered to assess the impact of RAGE blockade.

RESULTS. Early inner retinal neuronal dysfunction, manifested by prolonged latencies of the oscillatory potentials and b-wave, was detected in hyperglycemic mice. HGHL mice exhibited accelerated development of acellular capillaries and pericyte ghosts compared with littermate control animals. AGEs were localized primarily to the vitreous cavity and internal limiting membrane (ILM) of the retina, where they were intimately associated with the footplates of RAGE-expressing Müller cells. AGE accumulation measured by ELISA was increased within the retinal extracellular matrix of hyperglycemic mice. AGE fluorescence and upregulation of RAGE transcripts was highest in the retinas of HGHL mice, and attenuation of the RAGE axis with soluble RAGE ameliorated neuronal dysfunction and reduced the development of capillary lesions in these mice.

CONCLUSIONS. In early diabetic retinopathy, the RAGE axis, comprising the cellular receptor and its AGE ligands, is amplified within the retina and is accentuated along the vitreoretinal interface. Antagonism of the RAGE axis in NPDR reduces neurovascular perturbations, providing an important therapeutic

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Diabetic retinopathy, the leading cause of irreversible blindness in the working population in the Western world, encompasses both vascular and neural dysfunction.¹ Diabetes mellitus leads to alterations in the perfusion and permeability of the retinal vasculature, resulting in retinal ischemia and/or edema, with loss of reading vision when these events occur in the central macular region.² Diabetic retinopathy is also a degenerative disease of the neural retina, associated with alterations in neuronal function before the onset of clinical vascular disease.³ In advanced, proliferative diabetic retinopathy, an angiogenic, VEGF-mediated response with retinal neovascularization ensues, placing the eye at further risk of severe visual loss due to the development of vitreous hemorrhage or traction retinal detachment.⁴ Although many cases of diabetic retinopathy may be amenable to treatment with laser photocoagulation or vitrectomy, such efforts may not prevent irreversible vascular or neuronal damage, thereby underscoring the need for early intervention.

The duration and severity of hyperglycemia is the single most important factor linked to the development of diabetic retinopathy. The degree of hyperglycemia is the major alterable risk factor for both the development and progression of diabetic retinopathy, in both types 1 and 2 diabetes, as seen in the Diabetes Control and Complications Trial (DCCT)⁵ and in the UK Prospective Diabetes Study (UKPDS),⁶ respectively. Additional established risk factors for the acceleration of diabetic retinopathy include hypertension and hyperlipidemia, with several clinical studies demonstrating benefit in the treatment of diabetic retinopathy with intensive blood pressure control and lipid-lowering therapy.^{7–13} One metabolic consequence of chronic hyperglycemia is the accelerated formation of advanced glycation end products (AGEs), the accumulation of which in diabetic tissues is enhanced not only by elevated glucose but also by oxidant stress and inflammatory stimuli.¹⁴ In the setting of diabetic retinopathy, AGEs, especially N^ε-(carboxymethyl)lysine (CML) adducts, have been detected within retinal vasculature and neurosensory tissue of diabetic eyes.¹⁵ Multiple consequences of AGE accumulation in the retina have been demonstrated, including upregulation of VEGF, upregulation of NF-κB, and increased leukocyte adhesion in retinal microvascular endothelial cells.^{16–18} In diabetic patients, AGEs also accumulate within the vitreous cavity and may result in characteristic structural alterations sometimes referred to as diabetic vitreopathy.^{19,20} Support for a role for AGEs as a contributing factor in the pathogenesis of diabetic retinopathy has been drawn from studies in animals with inhibitors of AGE formation.^{21,22} In a 5-year study in diabetic dogs, administration of aminoguanidine prevented retinopathy. Similar beneficial effects in the retinal vasculature of diabetic rats have been observed with other inhibitors of AGE formation, including pyridoxamine and benfotiamine.^{23,24}

AGEs exert cell-mediated effects via RAGE, a multiligand signal-transduction receptor of the immunoglobulin superfamily.²⁵ Coinciding with pathologic changes in tissues, RAGE

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expression increases dramatically, with AGE ligands further upregulating receptor expression to magnify local cellular responses.²⁶ RAGE also binds the proinflammatory mediators, the S100/calgranulins, and amphoterin,^{27,28} and is an endothelial cell adhesion receptor capable of promoting leukocyte recruitment through interaction with the integrin Mac-1.²⁹ Consequences of ligand-RAGE interaction include increased expression of vascular cell adhesion molecule (VCAM)-1, vascular hyperpermeability, enhanced thrombogenicity, induction of oxidant stress and abnormal expression of eNOS, all pathogenetic mechanisms that potentially contribute to the ischemic and vasopermeability events of diabetic retinopathy.^{30,31}

Based on these considerations, we examined the RAGE axis in a newly characterized murine model of nonproliferative diabetic retinopathy. We first bred hyperlipidemic apoE^{-/-} mice into the hyperglycemic *db/db* background, observing that hyperlipidemia accelerates structural vascular changes in diabetic retinas that exhibit neuronal dysfunction. We localized and quantified the RAGE axis—specifically, AGE ligands and their cellular receptor RAGE, in the eyes of these mice. The findings provide new insights into the role of the RAGE axis in the pathogenesis of early diabetic retinopathy.

METHODS

Generation of the Mouse Colony

To generate the apoE^{-/-} *db/db* mice, apoE^{-/-} mice were first backcrossed six generations into mice heterozygous for the diabetes spontaneous mutation (*Lepr^{db}*). As the homozygous *db/db* mouse is sterile, we ultimately bred apoE^{-/-} *db/m* offspring to generate apoE^{-/-} *db/db* mice. Initially, male mice heterozygous for the diabetes spontaneous mutation (*Lepr^{db}*) in the leptin receptor gene on chromosome 4 (BKS.Cg-m^{+/+} *Lepr^{db}*, former name C57BLK/J-m^{+/+} *Lepr^{db}*, Type JAX GEMM TM Strain; Spontaneous Mutation Congenic, stock no. 000642; Jackson Laboratory, Bar Harbor, ME) were crossed with female mice homozygous for the *ApoE^{tm1Unc}* mutation in chromosome 7 (B6.129P2-*ApoE^{tm1Unc}*, former name C57BL/6J-*ApoE^{tm1Unc}*, Type JAX GEMM TM Strain; Targeted Mutation Congenic, stock no. 002052; Jackson Laboratory) at approximately 8 weeks of age. All mice were fed normal rodent chow (5053; PMI Nutrition International, Inc., St. Louis, MO) and exposed to a 12-hour light-dark cycle. All offspring were heterozygous for the apoE mutation. The genotype of their offspring was identified by PCR with primers from Invitrogen Corp. (Carlsbad, CA). The heterozygous mice from different parents were again crossed at 8 weeks of age. Mice homozygous for the *ApoE^{tm1Unc}* mutation and heterozygous for the *Lepr^{db}* mutation (apoE^{-/-} *db/m*) were used as breeders and were crossed with one another to breed the double-knock-out apoE^{-/-} *db/db* mice. Control mice were littermates obtained from the same colony: apoE^{+/+} *db/m* mice (homozygous for the wild type allele *ApoE^{tm1Unc}* and heterozygous for the *db* mutation) which are normoglycemic, nonobese littermates; apoE^{+/+} *db/db* mice (homozygous for the wild-type allele *ApoE^{tm1Unc}* and homozygous for the *Lepr^{db}* mutation) which are hyperglycemic, normolipidemic littermates. Glucose measurements were performed during the course of generation of the colony with a glucometer (Freestyle; Therasense, Alameda, CA). Cholesterol measurements were then performed (Infinity Cholesterol Liquid Stable Reagent kit; Thermo Electron Corp., Waltham, MA). The generation of the colony and all experiments were performed in agreement with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and were approved by the Institutional Animal Care and Use Committee at Columbia University.

Elastase Retinal Digest

An elastase digest with histopathologic vascular analysis was performed on 35 mice at age 6 months, including analysis of the following phenotypes: apoE^{+/+} *db/m* (*n* = 7; normoglycemic, normolipidemic [NGNL]); apoE^{-/-} *db/m* (*n* = 8; normoglycemic, hyperlipidemic

[NGHL]); apoE^{+/+} *db/db* (*n* = 7; hyperglycemic, normolipidemic [HGHL]); and apoE^{-/-} *db/db* (*n* = 13; hyperglycemic, hyperlipidemic [HGHL]). At the time of death, the eyes were enucleated and placed in 10% formalin for 2 days. After fixation, the retina was gently dissected from the neurosensory retina under microscopic observation. The neurosensory retina was placed in distilled water overnight to remove fixative. The elastase digestion method described by Laver et al.³² was then performed. After the vascular specimen was mounted on a slide, periodic acid-Schiff and hematoxylin staining of the vascular network and nuclei was performed. The specimens were then analyzed by microscope with digital capture (Axioskop 2 Plus; Carl Zeiss MicroImaging Inc., Thornwood, NY) for the presence of acellular capillaries and pericyte ghosts. Acellular capillaries were at least one-third thickness of normal capillary width, and intercapillary bridges were excluded from analysis.³³ The examiner was masked to the nature of the specimen during the assessment of pathology. As vascular lesions may be distributed nonuniformly, the entire retina was scanned during this process, and images were pasted into a single image (Photoshop, ver. 7.0; Adobe Systems Inc., San Jose, CA) to obtain an image of whole-mounted retina for area calculations. The virtual area of each prepared retina was measured (OphthaVision Imaging System, ver. 3.25; MRP Group Inc., Lawrence, MA). The number of acellular capillaries and pericyte ghosts for each digest was divided by the area scanned. The data obtained were analyzed with frequency and descriptive statistics, as described below.

Electrophysiology

Electroretinograms (ERGs) were performed on the following age-matched, 6-month-old littermates: NGNL wild-type (apoE^{+/+} *db/m*; *n* = 18), NGHL (apoE^{-/-} *db/m*; *n* = 11), HGHL (apoE^{+/+} *db/db*; *n* = 8), and HGHL (apoE^{-/-} *db/db*; *n* = 14) mice. The mice were dark adapted overnight before each experiment, and the ensuing procedures were performed under dim red light in a darkroom. The mice were anesthetized with a mixture of 50 mg/kg ketamine and 5 mg/kg xylazine administered intraperitoneally. The right eye pupil was dilated with drops of 2.5% phenylephrine hydrochloride and 0.5% tropicamide. The electroretinogram (ERG) responses were amplified and averaged by a computerized data-acquisition system (PowerLab; ADInstruments, Colorado Springs, CO). Once anesthetized, the mouse was placed on a heated block, and body temperature was maintained near 37°C. The mouse was placed in a centered position at the edge of a Ganzfeld dome. A rectal thermometer was placed in the mouse and checked throughout the recording. A ground electrode was inserted in the right leg, and the reference electrode was inserted in the forehead. The data collected and analyzed included all the above and temperature of the animal during the experiment, a- and b-wave latency and amplitude, oscillatory potential (OP)1, OP2, and OP3 implicit times and amplitudes, as previously described.^{34,35} The data obtained were analyzed with frequency and descriptive statistics, as described later.

Immunochemical Staining

Eyes of 6-month-old mice were fixed overnight in 4% phosphate-buffered paraformaldehyde and embedded in paraffin. The 4-mm paraffin sections were deparaffinized and heated in citrate buffer with a microwave for 15 minutes. After pretreatment with PBS containing 5% normal goat serum (Jackson ImmunoResearch Laboratories Inc., West Grove, PA), 0.5% BSA, and 0.1% Triton X-100 for 30 minutes at room temperature (RT), sections were incubated with anti-mouse RAGE antibody³⁶ (1:100), anti-AGE antibody³⁶ (1:100), anti-vimentin antibody (1:200, Santa Cruz Biotechnology Inc., Santa Cruz, CA), anti-glial fibrillary acidic protein (GFAP) antibody (1:100, Chemicon International, Inc., Temecula, CA), or anti-CD31 antibody (1:200, BD-Pharmingen, San Diego, CA) for 1 hour at RT and then overnight at 4°C. After they were rinsed with PBS, the sections were incubated for 1 hour at RT with secondary antibody conjugated to Alexa Fluor 488 (Molecular Probes Inc., Eugene, OR) or Alexa Fluor 546 (Molecular Probes Inc.). All antibodies were diluted in PBS containing 0.5% goat serum, 0.5% BSA, and 0.1% Triton X-100. Rabbit or chicken serum was used instead

TABLE 1. Glucose and Cholesterol Level at Euthanizing*

	NGNL	NGHL	HGNL	HGHL
Glucose (mg/dL)	121.3 ± 28.5 (15)	113.8 ± 24.2 (18)	452.6 ± 109.4 (13)	455.5 ± 68.4 (10)
Cholesterol (mg/dL)	62.5 ± 14.4 (5)	471.2 ± 72.4 (15)	201.3 ± 30.4 (5)	955.6 ± 149.1 (15)

Data are expressed as the mean ± SD (*n*).

* Mice were euthanatized at six months.

of primary antibody for a negative control. The retina was examined with a microscope (Eclipse E800; Nikon Instruments Inc., Meville, NY) equipped with a confocal laser scanning system (Radiance2000; Bio-Rad Laboratories, Hercules, CA). Images were captured and processed (LaserSharp 2000 software; Bio-Rad Laboratories).

Autofluorescence and ELISA of Retinal AGEs

Five mice from each group were euthanatized. Whole retinas were homogenized in 0.1 mL of PBS with 0.1% Triton X-100 at 0°C. Samples were centrifuged at 20,000g for 5 minutes at 4°C. Protein concentration was determined with BSA used as a standard. The protein level in the supernatant was adjusted to 1.6 mg/mL and used for a cellular protein autofluorescence assay. The pellet, mostly extracellular matrix (ECM), was washed with 20 mM phosphate buffer (pH 7.0), with 10 mM EDTA, and digested with 20 μ L of 25 U/mL papain (P5306, Sigma-Aldrich, St. Louis, MO) in 20 mM phosphate buffer (pH 7.0) 10 mM EDTA, and 20 mM cysteine at 37°C. After 24 hours, another 20 μ L of papain solution was added, and the incubation was continued for 24 hours. The supernatant was used for the measurement of ECM autofluorescence and ELISA of AGEs after appropriate dilution. Fluorescence intensities were measured on a multiwell plate reader (Cyto-Fluor 4000; Applied Biosystems [ABI], Foster City, CA) using 360 ± 40/460 ± 40-nm excitation/emission wavelengths. These excitation/emission wavelengths allow for detection of well-defined AGEs.^{37,38} Fluorescence was expressed in fluorescence intensity per 0.1 mg cellular protein or its equivalent retinal size for ECM. For immunochemical measurement of AGEs in ECM, a noncompetitive ELISA was used. The wells (96-well Nunc-Immuno Plate; Nalge Nunc International, Rochester, NY) were coated with BSA control, AGE-BSA standard,³⁶ and biological samples in 0.1 mL of 50 mM carbonate buffer (pH 9.6) at 4°C overnight. The wells were then washed with PBS containing 0.05% Tween-20 (washing buffer) and blocked at room temperature with 0.3 mL of 1% BSA and 5% rabbit serum in PBS (blocking buffer) for 1 hour. After they were washed, the wells were incubated with anti-AGE antibody³⁶ in blocking buffer for 3 hours at room temperature, followed by washing and secondary antibody (rabbit anti-chicken IgY-HRP; Biomedica Corp, Foster City, CA) for 1 hour at room temperature. The wells were then washed again and developed with 0.1 mL of peroxidase substrates (*o*-phenylenediamine tablets; Sigma-Aldrich) in the dark at room temperature. The absorbance at 490 nm was measured after adding 0.05 mL of blocking solution (2 M H₂SO₄) at 10 minutes.

Quantitative Real-Time PCR

At least five mice of each group were euthanatized. Retinas were isolated and stored in pairs at -80°C in preservative (RNAlater; Ambion, Inc., Austin, TX). Total RNA was then prepared (RNeasy Minikit; Qiagen, Inc., Valencia, CA). After quantification at OD₂₆₀, total RNA was analyzed (RNA Nano LabChips; 2100 Bioanalyzer; Agilent Technologies, Palo Alto, CA), to assess RNA quality. Only samples showing minimal degradation were used. cDNA was synthesized (TaqMan Reverse Transcription Reagents Kit; ABI) according to the manufacturer's instructions. Primers and probes for β -actin and RAGE were designed on computer (Primer Express; ABI). To confirm specific amplification of the target mRNA, an aliquot of the PCR product was analyzed by gel electrophoresis. The sequences of the primers and probe were as follows: for β -actin, 5'-ACG GCC AGG TCA TCA CTA TTG-3' (forward), 5'-TGG ATG CCA CAG GAT TCC AT-3' (reverse), and 5'-6FAM-ACG TCT ACC AGC GAA GCT ACT GCC GTC-TAMRA-3' (probe); and for

RAGE, 5'-GGA CCC TTA GCT GGC ACT TAG A-3' (forward), 5'-GAG TCC CGT CTC AGG GTG TCT-3' (reverse), and 5'-6FAM-ATT CCC GAT GGC AAA GAA ACA CTC GTG-TAMRA-3' (probe) (ABI). Real-time PCR was conducted on a sequence-detection system (Prism 7900HTI ABI), and results were analyzed by the 2^{- $\Delta\Delta C_T$} method.³⁹ Experiments were repeated three times, and statistical analysis was performed as described later.

Administration of Soluble RAGE

Soluble (s)RAGE, the extracellular two-thirds of the receptor, binds AGEs and interferes with their ability to bind and activate cellular RAGE. Preparation, characterization, and purification of sRAGE was performed with a baculovirus expression system using Sf9 cells (BD-Clontech, Palo Alto, CA; Invitrogen Corp.) as previously described.³⁶ Purified murine sRAGE (a single-band of ~40 kDa, by Coomassie-stained SDS-PAGE) was dialyzed against PBS; made free of detectable endotoxin, based on the Limulus amoebocyte assay (E-Toxate; Sigma) after passage onto gel columns (Detoxi-Gel; Pierce Chemical Co., Rockford, IL); and sterile-filtered (0.2 μ m). We administered daily doses of 100 μ g sRAGE based on previous dose-response studies.²⁷

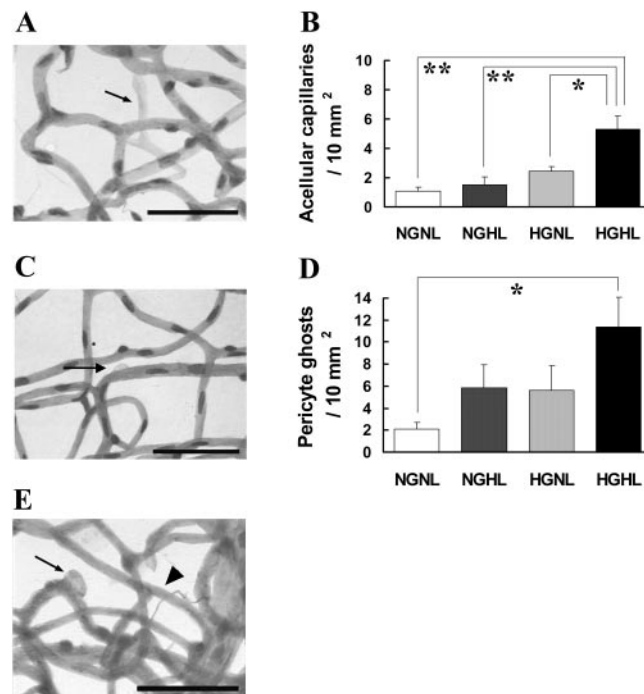


FIGURE 1. Retinal elastase digest results among diabetic, hyperlipidemic, and littermate control mice at age 6 months. The development of acellular capillaries (A, arrow; B) was accelerated in the retinas of HGHL mice, with significantly more acellular capillaries present per unit area than in NGNL or NGHL and HGHL mice. Pericyte ghosts (C, arrow; D) were also increased in the retinas of HGHL mice compared with NGNL littermates at age 6 months. Capillary outpouching (E, arrow), suggesting early microaneurysm formation, was observed in the retinal vasculature of HGHL mice. An intercapillary bridge, a normal feature of retinal digests not included in analysis, was also visible (arrowhead). Results are expressed as the mean ± SEM. **P* < 0.05; ***P* < 0.01. Scale bar, 50 μ m.

TABLE 2. ERG Latencies in Mice at Age 6 Months

	NGNL (<i>n</i> = 18)	NGHL (<i>n</i> = 10)	HGNL (<i>n</i> = 8)	HGHL (<i>n</i> = 14)
b-Wave	32.0 ± 2.0	32.4 ± 4.0	35.3 ± 3.5	34.5 ± 2.9
OP1	23.4 ± 1.4	23.0 ± 2.1	25.4 ± 1.9	24.6 ± 1.7
OP2	32.0 ± 2.0	31.7 ± 3.2	34.8 ± 2.5	33.8 ± 2.2
OP3	42.6 ± 2.9	42.9 ± 5.4	45.4 ± 3.2	44.9 ± 3.1
ΣOPs	98.0 ± 6.2	97.5 ± 10.6	105.6 ± 7.3	103.3 ± 6.8

Data are expressed as the mean latency ± SD (ms).

Statistical Analysis

To analyze the vascular, neuronal, and experimental data among the four groups, we used a two-factor analysis of variance (ANOVA) model. The two factors considered were glucose (normal/high) and lipid (normal/high). Interactions were tested for all analyses, but none was found. A one-way ANOVA was also used to compare the four groups in analyzing the AGE ELISA and autofluorescence data and the RAGE q-PCR data. For the experiment involving treatment with sRAGE, a one-way ANOVA was used to compare the three groups: NGNL, HGHL, and sRAGE. If a difference was found among the groups ($P < 0.05$), a post hoc analysis using the Duncan test was performed. All data were analyzed on computer (SAS system software; SAS Institute Inc., Cary, NC).

RESULTS

Effect of Hyperlipidemia on the Development of Vascular Lesions of Early Diabetic Retinopathy in Hyperglycemic Mice

The serum levels of glucose and cholesterol for each of the four groups are presented in Table 1. We first examined the impact of the introduction of hyperlipidemia into the hyperglycemic *db/db* background on vascular properties in the retina. At age 6 months, the retinas of HGHL (*apoE*^{-/-} *db/db*) mice displayed the most significant capillary lesions of NPDR (Fig. 1). Whereas the eyes of HGNL mice exhibited some development of acellular capillaries within the retina, only the eyes of HGHL mice had a significantly higher number of acellular capillaries than all other groups (Fig. 1B). The development of pericyte ghosts was detectable in both the HGNL and NGHL phenotypes, but only in the HGHL mice was there a significant difference from the NGNL control animals (Fig. 1D). Only in HGHL mice was there evidence of capillary outpouching consistent with early microaneurysm formation (Fig. 1E).

Electrophysiologic Neural Dysfunction of the Inner Retina of Hyperglycemic Mice

Electrophysiologic testing at age 6 months revealed that hyperglycemia resulted in early inner retinal dysfunction of the retina detected by prolongation in the latencies of the b-wave and the OPs (Table 2). Specifically, there were significant hyperglycemia-induced delays in the implicit time of the b-wave and OP1, OP2, and OP3 (see Table 4). The ERG amplitudes were not significantly affected in this study, with hyper-

glycemic mice demonstrating a statistically significant decline only in the amplitude of OP1 (Tables 3, 4). Hyperlipidemia alone did not induce statistically significant differences in any of the parameters recorded and studied (Table 4).

The RAGE Axis at the Vitreoretinal Interface

RAGE expression was predominantly localized to glial cells of the inner retina. Most of the RAGE-expressing cells within the neural retina were consistent with the distribution of Müller cells—particularly their internal footplates. In merged images, RAGE-positive cells of the inner retina colocalized with vimentin expression, confirming Müller cell expression (Fig. 2). GFAP expression in astrocytes of the inner retina revealed no evidence of colocalization with adjacent RAGE expression of Müller cell processes and footplates (Figs. 3A–C). Expression of RAGE was also detected adjacent to the microvasculature, suggesting intimate neurovascular localization for RAGE in the circulation of the inner retina (Figs. 3D, 3E). AGEs were prominently detected within the vitreous cavity of the eye and particularly along the vitreoretinal interface including the internal limiting membrane (Figs. 4B, 4F). AGEs were consistently detected within the lens capsule and Bruch's membrane and occasionally within the basement membrane of the microvasculature (not shown). In AGE and RAGE merged images, AGE was localized to vitreous fibrils and the internal limiting membrane, where there was close apposition to the footplates of RAGE-expressing Müller cells (Fig. 4).

RAGE and Its AGE Ligands in NPDR

We next quantified the RAGE axis in this murine model of NPDR. As AGEs can accumulate within cellular protein as well as within the proteins of ECM, we assayed the autofluorescence of AGEs independently. As shown in Table 5, there was not a significant difference among groups with regard to AGE autofluorescence in the cellular protein. In contrast, AGE autofluorescence increased in ECM in the setting of hyperglycemia, but only the retinas of HGHL mice had a significant difference in fluorescence when compared with NGNL mice. To quantify AGEs further in the ECM, we performed a non-competitive ELISA. This study revealed significantly increased AGE formation in the retinal ECM of hyperglycemic mice, both HGNL and HGHL (Fig. 5A). As RAGE expression may be amplified in the setting of its ligands,⁴⁰ RAGE mRNA expression from whole retina was then examined by quantitative real-time PCR for each group. RAGE mRNA expression was increased in

TABLE 3. ERG Amplitudes in Mice at Age 6 Months

	NGNL (<i>n</i> = 18)	NGHL (<i>n</i> = 10)	HGNL (<i>n</i> = 8)	HGHL (<i>n</i> = 14)
b-Wave	568.4 ± 163.2	517.0 ± 141.0	462.5 ± 138.9	489.3 ± 186.5
OP1	234.6 ± 62.9	210.5 ± 84.4	173.5 ± 52.6	175.6 ± 67.2
OP2	244.5 ± 89.8	206.8 ± 96.6	219.4 ± 54.7	202.9 ± 68.2
OP3	96.2 ± 50.6	80.0 ± 39.8	109.9 ± 32.6	96.0 ± 50.0
ΣOPs	575.3 ± 191.8	497.3 ± 212.1	502.8 ± 109.7	474.5 ± 168.2

Data are expressed as the mean amplitude ± SD (mV)

TABLE 4. Two-Factor ANOVA Analysis of ERG Data from Tables 2 and 3

	Glucose Effect	Lipid Effect	Interaction
b-Wave			
Latency	0.004	0.805	0.516
Amplitude	0.174	0.799	0.422
OP1			
Latency	0.001	0.216	0.649
Amplitude	0.021	0.588	0.516
OP2			
Latency	0.001	0.376	0.675
Amplitude	0.550	0.225	0.661
OP3			
Latency	0.031	0.933	0.744
Amplitude	0.283	0.271	0.934
ΣOPs			
Latency	0.004	0.545	0.685
Amplitude	0.376	0.324	0.643

Data are probabilities.

the retinas of hyperglycemic mice (glucose effect for two-factor ANOVA: $P < 0.01$); a significant increase was observed in HGHL mice compared with each group of normoglycemic mice (Fig. 5B). These studies demonstrate that the RAGE axis comprising the cellular receptor and its AGE ligands is amplified in the diabetic retina, particularly in eyes with significant capillary lesions of NPDR (HGHL mice).

Effect of Antagonism of RAGE on the Vascular Lesions of Diabetic Retinopathy and Neuronal Dysfunction at 6 Months of Age

Based on the upregulation of AGEs and RAGE in the HGHL group, we tested the potential contribution of RAGE to the pathogenesis of vascular and neuronal perturbation. Murine sRAGE was administered to 10 HGHL mice from age 8 weeks to age 6 months. The number of acellular capillaries per 10 mm² in the retinal digest of treated mice was significantly less than those observed in nontreated mice (Fig. 6A). In addition, there were significantly fewer pericyte ghosts in the retinas of treated mice than in those of nontreated mice (Fig. 6B). Electrophysiologic studies demonstrated that prophylactic treatment with sRAGE reduced retinal neuronal dysfunction, with a statistically significant ($P < 0.05$) reduction in the hyperglycemia-induced latency delays observed in OP2, OP3, and ΣOPs (summation of OPs) at 6 months of age (Table 6). Treatment with sRAGE had no significant effect on the amplitudes of the b-wave and OPs (data not shown).

DISCUSSION

The pathogenesis of diabetic retinopathy remains complex, but prolonged hyperglycemia is essential in the development of anatomic retinal vascular lesions in human diabetic retinopathy and most animal models of diabetic retinopathy.⁴¹ In this context, we investigated the *db/db* mouse, a well-characterized murine model of hereditary, insulin-resistant diabetes first detected in the progeny of the C57BLKS/J strain at the Jackson Laboratory and later characterized as being deficient in leptin receptor signaling.⁴² While the *db/db* mouse develops neuropathy and nephropathy, the anatomic retinal vascular findings, apart from basement membrane thickening, are less dramatic. Our previous anatomic studies revealed acellular capillaries and pericyte ghosts at age 8 months in *db/db* mice, but these anatomic findings were variable and inconsistently present (Barile GR, et al. *IOVS* 2000;41:ARVO Abstract 2156). Hyperlipidemia is associated with the severity of diabetic retinopathy,⁸⁻¹⁰ and successful treatment of hyperlipidemia in diabetic

patients may retard the progression of retinopathy or improve it.¹¹⁻¹³ For these reasons, we investigated the influence of hyperlipidemia on the retinal findings of the *db/db* mouse model of diabetes mellitus, ultimately crossing it with mice carrying a mutation in the apoE gene that leaves them devoid of functioning apoE protein. We observed that the classic anatomic retinal lesions of nonproliferative diabetic retinopathy developed at the highest rate in HGHL mice, when compared with the other groups, consistent with the burgeoning notion that hyperlipidemia accelerates the retinal vascular disease of diabetes mellitus. These results further support increasing evidence that dyslipidemia in diabetes mellitus independently contributes to the pathogenesis and severity of diabetic retinopathy, possibly through amplification of inflammatory mechanisms.^{43,44}

Whereas diabetic retinopathy is classically a microvascular disease of the retinal capillaries, diabetes may impair retinal neuronal function before the onset of visible vascular lesions. Numerous psychophysical and electrophysiological studies demonstrate early retinal neuronal dysfunction in diabetes mellitus, before the onset of the classic microvascular lesions of diabetic retinopathy.^{45,46} In particular, Bresnick and Palta⁴⁷ and Bresnick⁴⁸ have emphasized that alterations in the OPs of the electroretinogram better predict the development of high-risk proliferative retinopathy than do clinical fundus photographs. Pathologic quantification of neural loss by Barber et al.⁴⁹ showed apoptosis of retinal neurons and retinal atrophy, with loss of inner retinal thickness and cell bodies, in both diabetic rats and humans. Several investigators have noted other retinal neuronal alterations in early diabetes, including GFAP activation and glutamate transporter dysfunction in Müller cells.^{50,51} In our study, chronic hyperglycemia caused significant implicit time delays of OPs at 6 months that are comparable to those in previous studies of diabetes,⁵² whereas hyperlipidemia did not influence these electrophysiologic parameters. In conjunction with the histopathologic vascular changes observed, this study supports the concept of early diabetic retinopathy as a neurovascular disease of the retina, with physiologic disturbances of neuronal function accompanying traditional microvascular capillary pathologic disease.

It was in these contexts that we examined the RAGE axis in this newly characterized murine model of NPDR. Not surprisingly, we observed prominent AGE localization within the vitreous cavity. The increased AGE formation in the vitreous cavity of diabetic eyes has been postulated to increase collagen cross-linking and cause vitreous changes characteristic of diabetic eyes, well-recognized phenomena sometimes referred to as diabetic vitreoschisis or vitreopathy.^{19,20} An additional finding of our study was prominent AGE accumulation along the vitreoretinal interface, specifically posterior vitreous cortex and the internal limiting membrane. Similar to the vitreous cavity, the accumulation of AGEs at the vitreoretinal interface may result in structural alterations that promote mechanical traction in this region. Vitrectomy procedures are sometimes performed to remove tractional effects that promote diabetic macular edema. The localization of AGEs along the vitreoretinal interface is consistent with the concept of a structurally altered posterior hyaloid and internal limiting membrane capable of promoting subclinical vitreomacular disease in early diabetic retinopathy. AGEs may also exert nontractional, receptor-mediated effects through the RAGE axis. In this regard, an intriguing finding of our study is the localization of RAGE primarily to the Müller cells that extend from the internal limiting membrane to the external limiting membrane of the retina. The anatomic close apposition of an AGE-laden internal limiting membrane with the RAGE-expressing footplates suggests that a possible physiologic benefit of diabetic vitrectomy is the removal of AGE ligands from the posterior vitreous

FIGURE 2. RAGE expression in the retina of NGNL and HGHL mice. RAGE immunofluorescence (A, D, green) colocalizes with vimentin (B, E, red), a marker of Müller cells (arrows) in both NLNG and HGHL mice (C, F). The extension of Müller cells from the internal to the external limiting membranes of the neurosensory was highlighted with RAGE's expression (A, D). ILM, internal limiting membrane; IPL, inner plexiform layer; INL, inner nuclear layer; ONL, outer nuclear layer; ELM, external limiting membrane. Scale bar, 50 μ m.

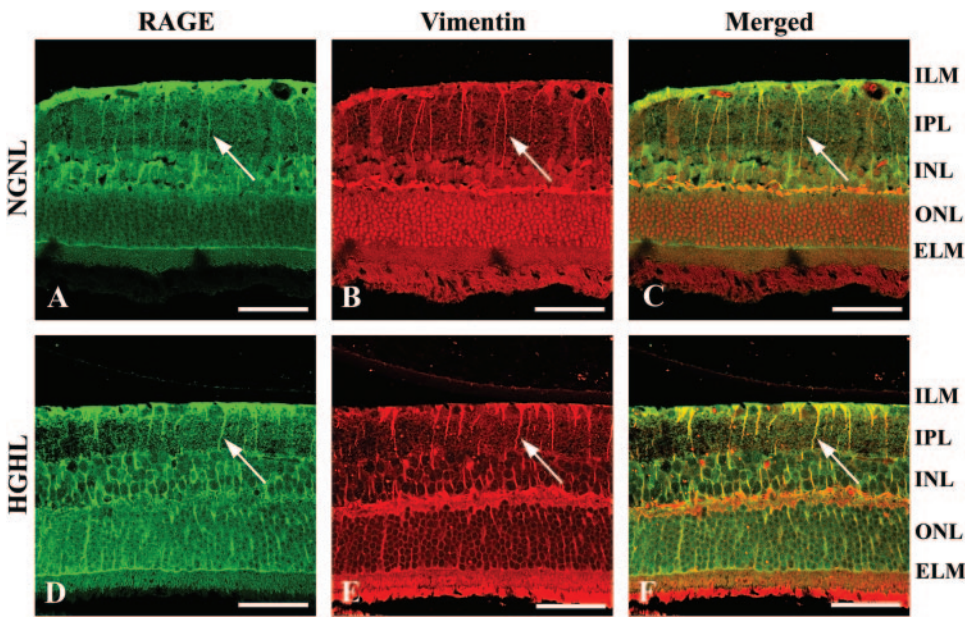


FIGURE 3. RAGE, GFAP, and CD31 immunohistochemistry of the retina of HGHL mice. RAGE expression was prominent in Müller cell processes, particularly their internal footplates (A, D; green, arrowheads) and was not observed in adjacent astrocytes (B, C; red, arrows). The intimate vasoglia relationship of the RAGE-expressing Müller cell (D, green) with the vascular endothelium of a retinal capillary (E, red) is observed in (F). ILM, internal limiting membrane; IPL, inner plexiform layer; INL, inner nuclear layer. Scale bar, 25 μ m.

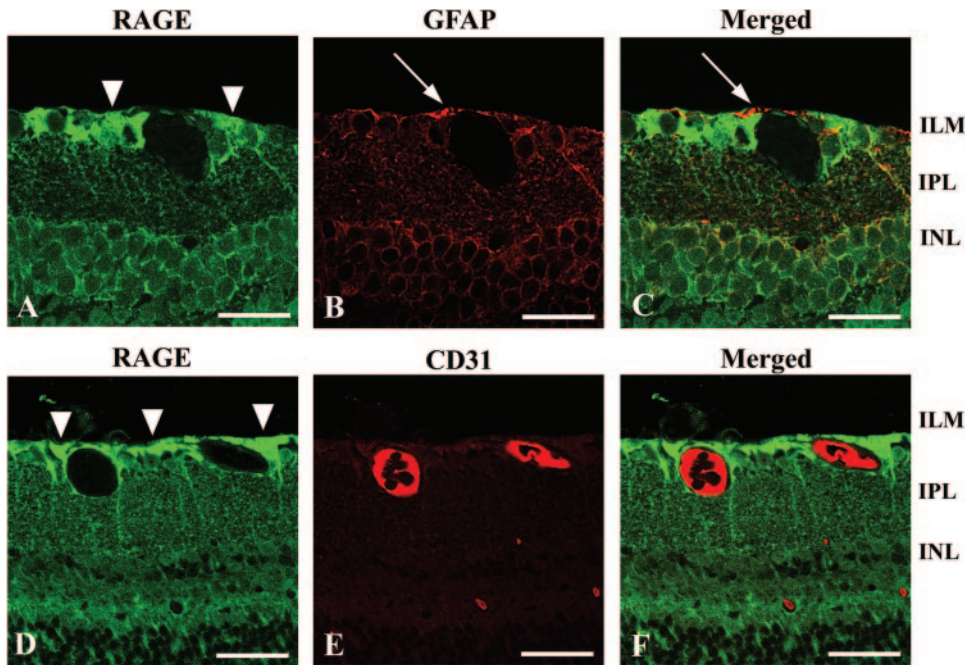


FIGURE 4. RAGE (green) and AGE (red) immunohistochemistry of the vitreoretinal interface in NGNL mice (A-C) and HGHL mice (E-G). AGES are detected within the vitreous cavity, posterior vitreous cortex, and internal limiting membrane of the retina (B, F, red). The internal footplates of RAGE-expressing Müller cells (A, E, green) are immediately adjacent to AGES in the internal limiting membrane (C, G). Controls (D, H). Vit, vitreous cavity; ILM, internal limiting membrane; GCL, ganglion cell layer; IPL, inner plexiform layer; INL, inner nuclear layer. Scale bar, 25 μ m.

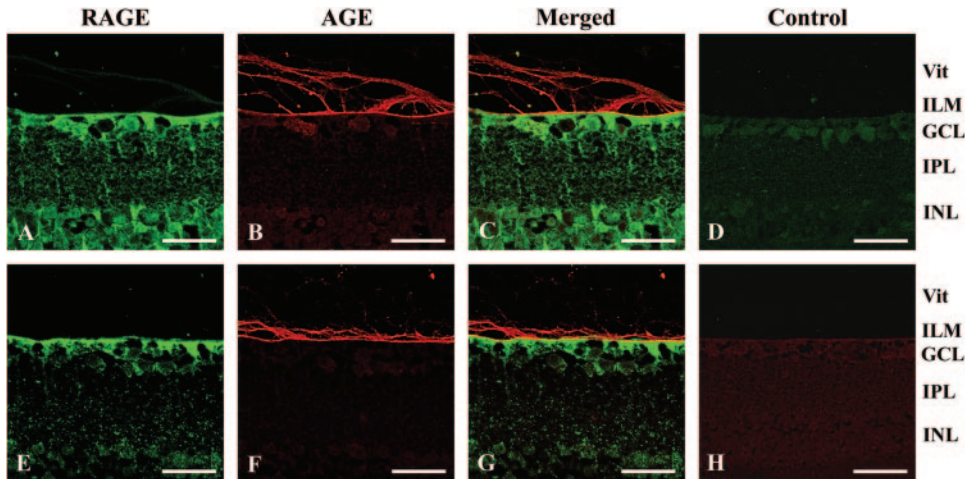


TABLE 5. Retinal AGE Fluorescence Intensities

	NGNL	NGHL	HGNL	HGHL
Cellular protein	1357 ± 149	1666 ± 182	1122 ± 194	1181 ± 161
ECM	2801 ± 673	2342 ± 531	3713 ± 1229	5259 ± 715*

Data are expressed as the mean autofluorescence ± SE.

* Significant difference ($P < 0.05$) compared to the NGNL group.

cortex and internal limiting membrane, downregulating the proinflammatory RAGE axis in adjacent Müller cells.

The localization of RAGE to Müller cells raises exciting possibilities for novel roles of these cells in the pathogenesis of diabetic retinopathy. The specific RAGE-dependent mechanisms by which AGEs may alter Müller cell structure and function are the subject of future study. These cells are well known to display a varied repertoire of structural and physiologic properties in the retina. The contact of vasoglia neuronal tissue and especially Müller cells with underlying capillaries in the retina suggests a potential pathophysiologic relationship in diabetic retinopathy, once suggested by Ashton in his Bowman lecture and supported by several recent studies.⁵³ In the setting of diabetes, alteration of the glutamate transporter, in part, it is speculated, by oxidation; increased expression of GFAP suggestive of reactive gliosis; and striking upregulation of VEGF all have been detected in Müller cells.⁵⁴ Indeed, *in vitro* analyses suggests that incubation of cultured Müller cells with AGEs upregulate expression of VEGF.⁵⁵ Müller cell ischemia

induces phosphorylation of extracellular signal-regulated kinase (ERK) MAPKs in these cells,⁵⁶ again suggesting that a wide array of changes in gene expression may ensue in these cells when perturbed. The possible RAGE-dependence of these phenomena remains to be determined, but the intimate relationship of RAGE-expressing Müller cells with underlying vascular endothelium suggests a potential role for Müller cell RAGE in neurovascular dysfunction.

In addition to the specific localization of RAGE and its AGE ligands in our study, we observed that AGEs accumulate in the neurosensory retina with associated amplification of cellular RAGE in the setting of hyperglycemia and early diabetic retinopathy. The diversity by which AGEs may form on the amino groups of proteins, lipids, and DNA is reflected in the variety of locations that these products may accumulate during hyperglycemia, including the serum, ECM, and intracellular cytoplasm.¹⁹ In this regard, it is noteworthy that we did not detect significantly different AGE levels by fluorescence studies within cellular proteins among the hyperlipidemic and hyperglycemic phenotypes. Instead, the retinas with the most severe capillary disease had the highest levels of AGEs detected within the ECM, both by fluorescence and ELISA studies. Hyperglyce-

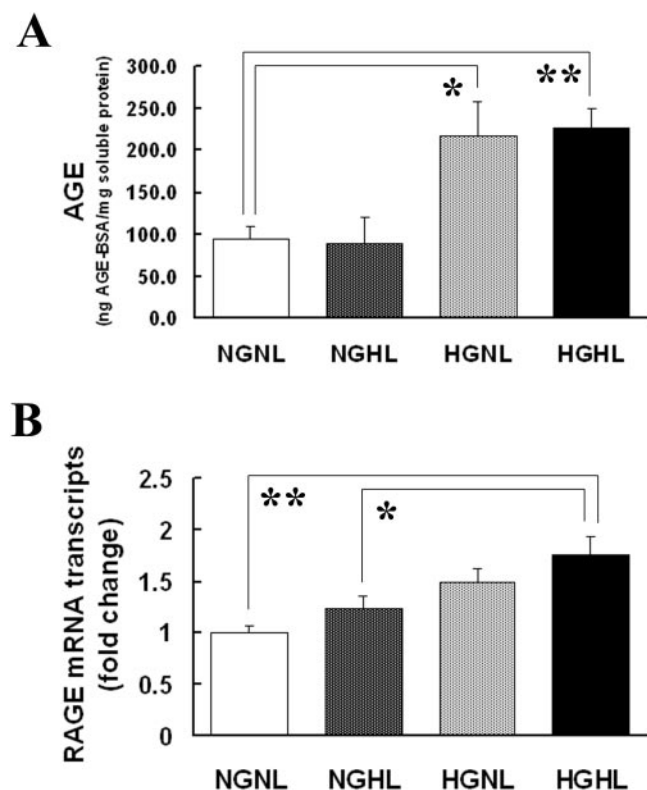


FIGURE 5. Retinal AGE ELISA and RAGE mRNA transcripts. Retinal AGEs accumulated in the retinas of hyperglycemic mice: Both HGNL mice and HGHL mice had significantly increased AGEs compared with NGNL littermates (A). RAGE mRNA expression in the retina was increased in the setting of hyperglycemia and AGE accumulation. RAGE transcripts were highest in the retinas of HGHL mice, with a nearly twofold elevation compared with basal levels in NGNL littermates as well as a significant increase compared with NGHL mice (B). Results are expressed as the mean ± SEM. * $P < 0.05$; ** $P < 0.01$.

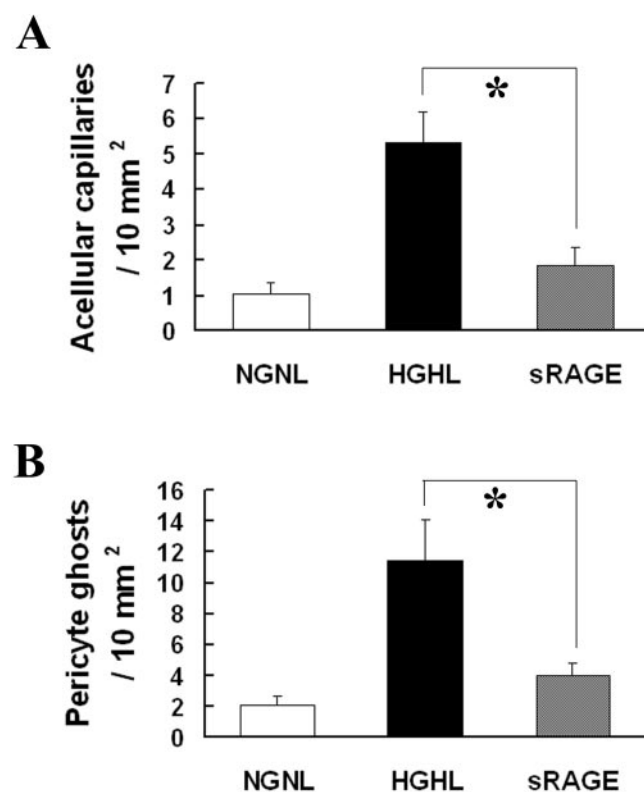


FIGURE 6. Effect of RAGE antagonism on vascular changes in HGHL mice. sRAGE-treated mice had significantly less acellular capillaries (A) and pericyte ghosts (B) in the retina compared with untreated HGHL mice. Treatment of these mice also reduced the latency delays observed in the OPs, with a significant reduction in the implicit times of OP2, OP3 and ΣOPs (the summation of OPs). * $P < 0.05$.

TABLE 6. sRAGE Effect on ERG Latencies at Age 6 Months

	NGNL (n = 18)	HGHL (n = 14)	sRAGE (n = 10)
b-Wave	32.0 ± 2.0	34.5 ± 2.9	33.3 ± 2.5
OP1	23.4 ± 1.4	24.6 ± 1.7	23.6 ± 1.4
OP2	32.0 ± 2.0	33.8 ± 2.2	32.2 ± 2.0*
OP3	42.6 ± 2.9	44.9 ± 3.1	42.0 ± 3.0*
ΣOPs	98.0 ± 6.2	103.3 ± 6.8	97.7 ± 6.1*

Data are expressed as the mean latency ± SD (ms).

* Significant ($P < 0.05$) compared to HGHL group.

mia was the most important contributor to the development of these AGEs, as HGHL mice also exhibited increased AGE accumulation in the ECM in these studies (though this increase was shown to be significant in this group only by ELISA). Consistent with a role for RAGE ligands such as AGEs in the development of retinopathy, we detected significant upregulation of RAGE transcripts in the retinas of HGHL mice that had the highest AGE accumulation and retinal disease. The amplification of RAGE in the setting of its ligands is consistent with the known biology of RAGE in other organ systems, and this property magnifies the effect of the RAGE axis in local cellular responses.^{26,40}

Importantly, in this study, antagonism of the RAGE axis ameliorated both neuronal dysfunction and vascular disease. The electrophysiologic benefit we observed suggests that RAGE contributes to neuronal dysfunction in the diabetic retina. The mechanisms of OP generation in the normal retina, the associated alterations observed in these neuronal responses in diabetic eyes, and the extent to which altered Müller cell glutamate metabolism, signaling, and gene expression contribute to perturbation of these signals remains to be determined. Antagonism of RAGE also reduced the progression of vascular lesions of diabetic retinopathy in HGHL mice. This vascular effect may relate to an a priori neuronal benefit to RAGE-expressing Müller cells, but the ample data on AGE toxicity and perturbation in retinal vascular endothelial cells also suggests that antagonism of circulating serum AGEs with sRAGE may reduce these perturbations and the resultant anatomic disease. The precise neurovascular mechanisms altered with ligand interaction with RAGE in the retina remain the subject of current and future investigations, but the amelioration of neurovascular features of diabetic retinopathy observed in this study identifies the RAGE axis as an important therapeutic target in the prevention and treatment of diabetic complications in the retina.

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