Endothelin Receptor-A Antagonist Attenuates Retinal Vascular and Neuroretinal Pathology in Diabetic Mice

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PURPOSE. We sought to determine the effects of atrasentan, a selective endothelin-A receptor antagonist, on the retinal vascular and structural integrity in a db/db mouse, an animal model of type 2 diabetes and diabetic retinopathy.

METHODS. Diabetic mice, 23 weeks old, were given either atrasentan or vehicle treatment in drinking water for 8 weeks. At the end of the treatment period, eyes underwent trypsin digest to assess the retinal vascular pathology focusing on capillary degeneration, endothelial cell, and pericyte loss. Paraffin-embedded retinal cross sections were used to evaluate retinal sublayer thickness both near the optic nerve and in the retinal periphery. Immunohistochemistry and TUNEL assay were done to evaluate retinal cellular and vascular apoptosis.

RESULTS. Compared with untreated db/db mice, atrasentan treatment was able to ameliorate the retinal vascular pathology by reducing pericyte loss ($29.2\% \pm 0.4\%$ vs. $44.4\% \pm 2.0\%$, respectively, $P < 0.05$) and capillary degeneration as determined by the percentage of acellular capillaries ($8.6\% \pm 0.3\%$ vs. $3.3\% \pm 0.41\%$, respectively, $P < 0.05$). A reduction in inner retinal thinning both at the optic nerve and at the periphery in treated diabetic mice was also observed in db/db mice treated with atrasentan as compared with untreated db/db mice ($P < 0.05$). TUNEL assay suggested that atrasentan may decrease enhanced apoptosis in neuroretinal layers and vascular pericytes in the db/db mice.

CONCLUSIONS. Endothelin-A receptor blockade using atrasentan significantly reduces the vascular and neuroretinal complications in diabetic mice. Endothelin-A receptor blockade is a promising therapeutic target in diabetic retinopathy.

Keywords: endothelins, pericyte, diabetic retinopathy, diabetic mice, retinal vessels

Diabetic retinopathy (DR) is a long-term manifestation of microvascular disease secondary to diabetes and affects 80% of diabetic individuals after 10 years.¹ It is the leading cause of blindness in among working-aged Americans and fifth leading cause worldwide.^{2,3}

Pericyte loss is the first cellular sign in DR, highlighting the important role played by these cells in its pathogenesis.⁴ Pericytes are mural cells encased within the vascular basement membrane that they share with the vascular endothelial cells. They are crucial in maintaining the vasculature via mediating blood vessel repair,⁵ while establishing and maintaining the inner blood-retinal barrier in concert with endothelial cells.⁶ The retina has one of the highest metabolic demands in the body, and pericytes function as hypoxia sensors to support the retinal metabolic function.⁷ To maintain these important functions, the retina, similar to the brain, has one of the highest ratios of pericytes to endothelial cells in the body.⁵ Despite its importance, the pathophysiology of pericyte loss in early DR remains poorly defined.⁸ Understanding the mechanism of pericyte loss is critical to advancing our understanding of the pathogenesis of early changes in DR.

Another early finding in DR is elevated endothelin-1^{9,10} levels and a positive correlation between plasma endothelin levels and the extent of microangiopathy in patients with type 2 diabetes has been reported.^{9,10} Endothelin-1 is one of the most potent vasoconstrictors in the body¹¹; its actions are mediated

via two receptors, ET_A and ET_B, both found in retinal pericytes.¹² Although ET_A has selective affinity for endothelin-1, ET_B has equal affinity for all isoforms of endothelin and is found on pericytes and endothelial cells, as well as retinal neural and glial cells.¹³ ET_A receptor has been shown to play an important role in mediating decreased retinal blood flow that is seen in early DR,¹⁴ a reflection of ET_A receptor role in vasoconstriction, fibrosis, and increased extracellular matrix via protein kinase C (PKC) and increased intracellular calcium.¹⁵

The important role played by endothelin-1 in diabetes has stimulated a recent interest in exploring the efficacy of ET_A receptor antagonist therapy in various diabetic complications, especially nephropathy.^{16–18} Endothelin-1 has been shown to play a role in podocyte disruption in diabetic nephropathy, primarily via ET_A receptor, ultimately leading to proteinuria.^{19,20} Glomerular podocytes have features that resemble pericytes in the eye, and because endothelin-1 is involved in various pericyte functions, including contraction,²¹ in this study we sought to explore the hypothesis that pericyte loss could be prevented by specific ET_A receptor blockade. We therefore studied the effect of atrasentan, a selective blocker of ET_A receptor, on the retinal vasculature and neuroretinal pathology in db/db mice, an animal model of type 2 diabetes. This study shows, for the first time, a significant benefit of an ET_A blocker on pericyte loss in a rodent model of DR. Capillary

FIGURE 1. Trypsin digest ($\times 600$) and E/P ratio. (a) Trypsin digest of control db/m mouse retina stained with H&E. The endothelial cell (white arrow) and pericyte (black arrow) are highlighted. (b) Compared with db/m, db/db had statistically increased E/P ratio ($*P < 0.001$). Atrasentan-treated db/db was able to significantly normalize the E/P ratio seen in untreated db/db ($\dagger P < 0.001$).

vascular obliteration and neuroretinal thinning were also improved. These findings expand our understanding of the role played by endothelin-1 in the pathogenesis of DR and identify the ET_A receptor as a potential therapeutic target.

METHODS

Animals

All experiments followed the guidelines of ARVO and were approved by the Institutional Animal Care and Use Committee of Northwestern University. The db/db female mice and db/m controls were fed a standard chow diet ad libitum, kept in a 14:10-hour light/dark cycle and monitored from 8 to 23 weeks of age. At 23 weeks of age, db/db mice were randomly assigned to one of two groups: group 1 ($n = 8$) received atrasentan (8 mg/kg/d) provided in the drinking water. A second group of db/db mice ($n = 9$) and age-matched db/m controls ($n = 8$) received diluted vehicle in their drinking water. After 8 weeks, animals were euthanized using 0.2 mL pentobarbital. Eyes were enucleated and fixed in 10% neutral buffered formalin at room temperature for 24 hours. Subsequently, they were transferred to 70% ethanol and stored at 4°C.

Trypsin Digest

The cornea and lens were removed and the retina gently dissected from the sclera and choroid. The isolated retinas were subjected to trypsin digest, as described by Kuwabara and Cogan,²² with modifications as described previously.²³ Retinas were washed in filtered water to separate the neural layers from the vasculature and then left overnight at room temperature. The following day, retinas were placed in 3% trypsin (1:250; Amresco, Solon, OH, USA) and 0.1 M Tris Buffer (pH 7.8) and incubated at 37°C with gentle shaking for 1.0 to 1.5 hours. The retinal vasculature was separated from the nonvascular components via a series of water washes and manipulation under a microscope. The vascular digest was mounted on a slide, allowed to dry, and stained with hematoxylin and eosin (H&E) and examined using a Nikon 80i Eclipse upright microscope (Nikon, Tokyo, Japan).

Masking of the Independent Readers

For trypsin digest, each slide was coded using a random series of letters (e.g., a, b, c), before it was examined by the reader.

For retinal thickness analysis, the images taken of the predefined retinal regions were coded by a series of letters that were stored separately. For both approaches, the readers were masked to the coding during their grading of the images/slides and were unmasked only during the data analysis phase.

Quantification of Endothelial Cell/Pericyte Ratio, Capillary Cellular Density, and Acellular Capillaries

Endothelial cells and pericytes were identified based on characteristic morphologic features. Endothelial cell nuclei are elongated and lie within the vessel wall along the axis of the capillary, whereas pericyte nuclei are spherical, stain densely, and generally have a protuberant position on the capillary wall (Fig. 1a).²⁴

The vascular cells were counted under the microscope at $\times 60$ magnification. A square reticle was placed in one ocular of the microscope to facilitate counting. Endothelial cell/pericyte (E/P) ratio was calculated as described by Midena et al.²⁵ For each vascular digest, a total of 200 cells were counted and the number of endothelial cells and pericytes was noted. To evaluate the cellular density in the capillaries, we recorded the number of reticle squares needed to count 50 endothelial cells and pericytes at $\times 60$ around the midretina.²⁵ To allow comparisons between groups, results were normalized to the number of cells in db/m controls.

Acellular capillaries were defined as those with no nuclei along their entire length (Fig. 2a), as described previously.²⁶ Briefly, capillary diameter had to be at least 20% of the adjacent capillary diameter to be considered acellular, whereas narrower structures were considered strands or artifacts of the digest and thus excluded. Five fields in each eye were randomly selected around the midretina (1.25 mm² retinal area per field at $\times 40$).

Two independent observers masked to the treatment assignment measured the E/P ratio, vascular cellular density, and acellular capillaries per five high-power fields. For statistical comparisons, we used the average of the measurements obtained by the two observers.

Paraffin-Embedded Retinal Sections

Paraffin-embedded retinal sections (5 μ m) were deparaffinized in Xylene, and rehydrated through a series of ethanol

FIGURE 2. Trypsin digest and acellular capillaries (per five retinal fields, $\times 400$). (a) Trypsin digest of db/db retina showing acellular capillaries (red arrow; $\times 400$). (b) Untreated db/db had statistically significant increase in acellular capillaries compared with both db/m ($*P < 0.001$) and atrasentan-treated db/db mice ($\dagger P < 0.001$).

concentrations. Retinal thickness analysis, TUNEL assay, and immunohistochemistry were performed as described below.

Retinal Thickness Analysis

Deparaffinized sections were stained with H&E. Images were taken using a Nikon 80i Eclipse upright microscope with a Roper Scientific Photometrics CoolSNAP CF camera (Photometrics, Tucson, AZ, USA) at $\times 120$. Sections that traversed the optic nerve were chosen for this analysis. Retinal thickness was measured 150 to 200 pixels (81–108 μm) away from either side of the optic nerve as well as in the retinal periphery. The thickness of the following retinal layers was measured: retinal nerve fiber layer plus ganglion cell layer, inner plexiform layer, inner nuclear layer, outer plexiform layer, outer nuclear layer, and the photoreceptor layer. The individual sublayer thickness was calculated using the *distance_between_polylines.java* plug-in²⁷ and using ImageJ software (National Institutes of Health, Bethesda, MD, USA). This tool allows multiple segments of each sublayer to be automatically calculated and averaged, avoiding user-induced bias or error. This was done in a masked fashion by two independent observers with good inter-rater reliability (intraclass correlation coefficient =

0.9966). The average of measurements obtained by the two observers is reported.

TUNEL Assay and Immunohistochemistry

To explore whether pericyte loss occurred via apoptosis, we performed TUNEL assay for apoptosis on retinal cross sections immunostained with α -smooth muscle actin (α -SMA), a pericyte marker.²⁸ Deparaffinized sections underwent high-temperature antigen retrieval by incubation in sodium citrate buffer (10 mM sodium citrate, 0.05% Tween-20, pH 6.0) at 100°C for 20 minutes. TUNEL assay was conducted following the manufacturer's instruction (APO-BrdU TUNEL Assay Kit; Molecular Probes, Eugene, OR, USA). Following TUNEL assay, sections were placed in blocking solution (10% donkey serum with 1% BSA in Tris-buffered saline) for 1 hour and then incubated with anti- α -SMA (1:200, ab5694; Abcam, Cambridge, MA, USA) overnight at 4°C . The following day, sections were washed and incubated in donkey anti-rabbit IgG secondary antibody (1:200, DyLight 594, ab96921; Abcam) for 1 hour at room temperature.

Sections were counterstained with 4',6-diamidino-2-phenylindole (DAPI; R37606; Molecular Probes, Eugene, OR, USA) and washed in 0.1% Sudan black in 70% ethanol for 25 minutes

FIGURE 3. Capillary cellular density ($\times 600$). Graphs were normalized to the number of reticles required to count 50 endothelial cells and 50 pericytes in db/m for better representation. (a) No difference was seen in endothelial cell density among the groups. (b) Untreated db/db had statistically decreased pericytes compared with both db/m ($*P < 0.001$) and atrasentan-treated db/db mice ($\dagger P < 0.001$).

TABLE 1. Summary of Metabolic Status and Vascular Changes

Group	Body Weight, g	Blood Glucose, mg/dL	E/P Ratio	Endothelial Cell Density*	Pericyte Density*	Acellular Capillary†
db/m, <i>n</i> = 8	27 ± 0.7‡	164 ± 12‡	3.73 ± 0.28§	50.0 ± 1.3	50.0 ± 1.3§	2.7 ± 0.3§
db/db, <i>n</i> = 9	57.7 ± 2.0	398 ± 64	6.63 ± 0.41	55.4 ± 2.2	29.2 ± 0.4	8.6 ± 0.3
db/db, atrasentan, <i>n</i> = 8	56.1 ± 1.9	272 ± 52	4.35 ± 0.19	52.9 ± 1.8	44.4 ± 2.0	3.3 ± 0.4

* Capillary density: Values are normalized to control db/m mice and are based on the number of reticle squares needed to count 50 endothelial cells (87) and pericytes (207) in db/m.

† Acellular capillaries (per five retinal fields).

‡ Reflects a statistically significant difference ($P < 0.05$) comparing db/m to untreated db/db group. There was no statistically significant difference in blood glucose between db/m and atrasentan-treated db/db mice (see text). No significant differences were found between the two db/db groups regarding body weight or blood glucose.

§ Reflects that db/m mice are significantly different from db/db mice.

|| Reflects that atrasentan-treated db/db mice are significantly different from the untreated db/db group, but not different from db/m mice.

to remove autofluorescence. Sections were washed in PBS, mounted, and sealed.

Statistical Analysis

Results are expressed as mean ± SEM. Initially, ANOVA was performed to determine presence of significant differences among the groups. This was followed by two-tailed Student's *t*-test between groups. For blood glucose analysis, Mann-Whitney-Wilcoxon test was used. Differences were considered statistically significant at a *P* value less than 0.05.

RESULTS

Metabolic Status

The blood glucose and body weight of the three groups are shown in Table 1. At the end of the study, the db/m mice had significantly lower mean blood glucose (164 ± 12 mg/dL) than untreated db/db (164 ± 12 vs. 398 ± 64 mg/dL, $P = 0.03$). The atrasentan-treated db/db mice also had higher levels of glucose than the db/m mice but the difference did not reach statistical significance (272 ± 52 vs. 164 ± 12 mg/dL, $P = 0.11$). There were no significant differences in random blood glucose between db/db treated with atrasentan or untreated db/db (272 ± 52 and 398 ± 64 mg/dL, $P = 0.10$). To take into account a potential effect of atrasentan on blood glucose and eye findings, a subset analysis was performed (see below). No significant differences were seen in weight between the two db/db groups and both db/db groups had markedly increased body weight as compared with db/m mice (Table 1).

Vascular Changes: Atrasentan Treatment Significantly Improved E/P Ratio, Pericyte Density, and Acellular Capillaries in db/db Mice

The measurements obtained by two graders performing the E/P ratio, pericyte density and acellular capillaries showed good interrater reliability; the average intraclass correlation coefficients for these three measurements were 0.9307, 0.9798, and 0.9526, respectively.

Capillary Cellular Density and E/P Ratio

E/P ratio was significantly higher in db/db mice than in db/m controls (6.63 ± 0.41 vs. 3.73 ± 0.28, respectively, $P < 0.001$; Fig. 1b). In contrast, the E/P ratio for atrasentan-treated db/db mice was not significantly different from the db/m control group (4.35 ± 0.19 vs. 3.73 ± 0.28, respectively, $P = 0.09$). These findings could theoretically be explained by either loss of pericytes and/or increased endothelial cells in db/db as

compared with db/m mice. Accordingly, we also evaluated capillary cellular density to determine whether the normalization of E/P ratio in atrasentan-treated db/db eyes was due to improved pericyte survival or increased endothelial cell loss (Fig. 3). Endothelial cell counts did not show significant differences among the four groups. We normalized the pericyte cellular density to db/m controls and found that db/db mice had statistically reduced pericyte density as compared with db/m controls (29.2 ± 0.4 vs. 50.0 ± 1.3, respectively, $P < 0.001$; Table 1). In contrast, pericyte density was significantly increased in atrasentan-treated compared with untreated db/db mice (44.4 ± 2.0 vs. 29.2 ± 0.4, respectively, $P < 0.001$). Overall, pericyte density in the atrasentan-treated db/db was not significantly different from the db/m group (44.4 ± 2.0 vs. 50.0 ± 1.3, respectively, $P = 0.06$). No significant differences in these parameters were found between atrasentan-treated db/db mice and db/m controls (see Table 1).

Acellular Capillaries

The average number of acellular capillaries visualized in five fields for each of the groups is shown (Fig. 2b). Consistent with retinopathy, db/db mice had significantly more acellular capillaries compared with control db/m (8.6 ± 0.3 vs. 2.7 ± 0.3, respectively, $P < 0.001$). Atrasentan-treated db/db mice had significantly decreased number of acellular capillaries compared with untreated db/db mice (3.3 ± 0.41 vs. 8.6 ± 0.3, respectively, $P < 0.001$).

Subgroup Analysis

To examine a potential effect of atrasentan on blood glucose levels, we performed an analysis of vascular findings in a subgroup of animals based on blood glucose criteria of more than 200 mg/dL to define diabetes. For this subanalysis, one db/m mice with glucose of 200 mg/dL or higher and five db/db mice (treated or untreated) with glucose lower than 200 mg/dL were excluded. After exclusion, the mean glucose of the three subgroups were as follows: db/m: *n* = 7, 151.0 ± 3.1; db/db: *n* = 7, 471.0 ± 51.5; db/db plus atrasentan: *n* = 5, 362.0 ± 38.0 mg/dL. In this subgroup, both db/db groups had blood glucose levels significantly higher than db/m ($P < 0.05$) and not significantly different from each other. Within this subgroup, we found that average vascular densities and statistical significance remained essentially unchanged as compared with the entire dataset (Table 1). Specifically, pericyte density remained significantly lower in untreated db/db (28.7 ± 0.4) compared with db/m controls (50.0 ± 1.4, $P < 0.001$), or compared with atrasentan-treated db/db (42.8 ± 2.1, $P < 0.001$). E/P ratio was significantly higher in untreated db/db mice compared with db/m controls (6.50 ± 0.37 vs. 3.63 ± 0.28, respectively, $P < 0.001$), or to the atrasentan-treated

FIGURE 4. Colocalization of TUNEL (apoptosis, *green*) and α -SMA (pericytes, *red*) in the ocular vasculature is seen only in untreated db/db (**d–f**) (*arrows*). No colocalization is seen in db/m (**a–c**) or atrasentan-treated db/db mice (**g–i**). Sections are counterstained with DAPI (nuclei, *blue*; $n = 3$ per group).

db/db group (6.50 ± 0.37 vs. 4.26 ± 0.17 , $P < 0.001$). Acellular capillaries remained significantly higher in untreated db/db mice compared with db/m mice (8.6 ± 0.26 vs. 2.6 ± 0.26 , $P < 0.001$) or compared with atrasentan-treated db/db mice (8.6 ± 0.26 vs. 3.9 ± 0.39 , $P < 0.001$). Therefore, the effect of atrasentan on these vascular alterations remained highly significant as compared with db/m mice, even though the blood glucose is significantly different between the two subgroups (151.0 ± 3.1 vs. 362.0 ± 38.0 mg/dL, $P < 0.05$). Of note, the atrasentan-treated db/db group continued to have blood glucose levels that were approximately 100 mg lower than the untreated db/db mice, which did not achieve statistical significance ($P = 0.10$).

TUNEL and α -SMA Colocalize in db/db Eyes

To further confirm that increased E/P ratio in db/db mice was due to pericyte loss, we performed TUNEL assay and immunostaining with α -SMA on three separate retinal sections per group. TUNEL assay and α -SMA colocalized in retinal vessels in db/db eyes (Figs. 4d–f), a finding that was not seen in either db/m control or atrasentan-treated db/db mice (Figs. 4a–c, g–i, respectively).

Retinal Thickness: Atrasentan Treatment Improved Retinal Thickness in db/db Mice

The average thickness of retinal sublayers is shown (Table 2). Near the optic nerve, the inner retinal sublayers were reduced in the untreated db/db compared with db/m eyes, although no comparison reached statistical significance (Figs. 5a, 5c). Atrasentan-treated db/db eyes showed a trend for improved inner retinal thickness compared with untreated db/db (Fig.

5c). In the periphery, all retinal sublayers were reduced in the untreated db/db group compared with db/m, with the inner retinal sublayers reaching statistical significance (Figs. 5b, 5d). Atrasentan-treated db/db had improved inner retinal thickness compared with untreated db/db; differences reached statistical significance for the inner nuclear layer (46.1 ± 2.8 vs. 37.8 ± 3.6 , $P < 0.05$).

Inner Retinal Apoptosis Is Seen Centrally and Peripherally in Untreated db/db but Only Peripherally in Atrasentan-Treated db/db

To evaluate whether decreased retinal thickness in db/db mice was related to increased apoptosis, we performed TUNEL assay on three retinal sections per group. Near the optic nerve, untreated db/db eyes showed inner retinal apoptosis (Figs. 6c, 6d); this was not seen in atrasentan-treated db/db (Figs. 6a, 6b, 6e, 6f). In the periphery, we saw inner retinal apoptosis in both untreated and atrasentan-treated db/db groups, suggesting that retinal thinning was secondary to cellular apoptosis (Figs. 6i–l). In addition, untreated db/db also showed mild apoptosis in the outer nuclear layer. No apoptosis was seen in control db/m mice (Figs. 6g, 6h). These data, combined with retinal thickness analysis, suggests that atrasentan was able to improve retinal thickness, but not completely eliminate cellular apoptosis in the peripheral retina of db/db mice.

DISCUSSION

This study used obese db/db mice, a well-established animal model of type 2 diabetes, to examine the effect of ET_A receptor blockade on vascular and neuroretinal aspects of DR. Similar to

TABLE 2. Retinal Thickness (in Pixels)

	NFL/GCL	IPL	INL	OPL	ONL	PR
Near optic nerve						
db/m, <i>n</i> = 3	78.1 ± 2.5	121.6 ± 13.5	71.7 ± 2.7	31.6 ± 5.5	81.6 ± 14.5	60.5 ± 6.2
db/db, <i>n</i> = 3	61.4 ± 12.4	99.4 ± 19.3	61.3 ± 5.5	24.9 ± 0.53	77.6 ± 8.58	63.4 ± 9.2
db/db, atrasentan, <i>n</i> = 3	73.6 ± 1.9	114.4 ± 3.0	70.4 ± 5.6	35.2 ± 2.1	94.0 ± 13.6	82.4 ± 18.4
Near periphery						
db/m, <i>n</i> = 3	36.6 ± 4.0*	86.1 ± 8.6*	65.6 ± 5.4*	35.8 ± 2.2*	78.2 ± 10.5	74.7 ± 8.3
db/db, <i>n</i> = 35	21.9 ± 2.0	51.1 ± 5.2	37.8 ± 3.6†	27.4 ± 1.7	64.8 ± 4.8	56.2 ± 4.3
db/db, atrasentan, <i>n</i> = 3	24.8 ± 2.6	69.1 ± 10.2	46.1 ± 2.8‡	29.8 ± 1.8	65.4 ± 4.3	58.7 ± 1.8

One pixel is equivalent to 0.54 μm. INL, inner nuclear layer; IPL, inner plexiform layer; NFL/GCL, nerve fiber layer/ganglion cell layer; ONL, outer nuclear layer; OPL, outer plexiform layer; PR, photoreceptor layer.

* Significant difference seen was between db/m and untreated db/db mice in NFL/GCL (*P* < 0.05), IPL (*P* < 0.03), INL (*P* < 0.03), OPL (*P* < 0.04).

† Significant difference seen was between atrasentan-treated db/db and untreated db/db mice in INL (*P* < 0.05).

‡ Significant difference seen was between atrasentan-treated db/db and db/m mice in INL (*P* < 0.05).

a previous study, we found there is an increase in E/P ratio and acellular capillaries in db/db as compared with db/m mice.²⁵ We found that selective blockade of the ET_A receptor using atrasentan for 8 weeks caused normalization in the E/P ratio and improved vascular network integrity, along with decreased acellular capillaries in db/db mice. Furthermore, atrasentan-treated db/db eyes showed significantly improved peripheral retinal thickness, as well as improved central and peripheral neuroretinal apoptosis. Overall, these results suggest a protective role for specific ET_A receptor antagonist in this animal model of DR.

The E/P ratio is generally used as a convenient way to reflect alterations in pericytes relative to endothelial cells, although both cell types could potentially be affected by endothelin-1 excess, and/or blockade of the ET_A receptor. We wanted to explore whether atrasentan treatment caused normalization of E/P ratio through improved pericyte survival or loss of endothelial cells. Our assessment of capillary cellular density showed variations in pericyte and not endothelial cell density among the groups (Fig. 3). Immunohistochemistry showed colocalization of TUNEL, an apoptosis marker, and α-SMA, a marker of pericytes, in the vasculature of untreated db/db eyes, reflecting pericyte apoptosis, a finding that was not seen in control db/m or atrasentan-treated db/db eyes (Fig. 4). The improvement in the E/P ratio, pericyte cellular density, and acellular capillaries combined, suggest that atrasentan plays a protective role favoring pericyte survival in the retina. Similar effects have been seen in the kidney, where ET_A receptor antagonists have been shown to improve renal function, prevent disruption of the podocyte cytoskeleton, and significantly reduce proteinuria, both in rodent models¹⁹ and in humans.²⁹

Although endothelin-1 has not been directly implicated in pericyte loss in DR, several lines of evidence support this possibility. Endothelin-1 is mitogenic to pericytes in a normoglycemic environment.³⁰ Nuclear factor (NF)-κβ, similar to endothelin-1, supports pericyte survival in normoglycemia, but when activated in a hyperglycemic environment, NF-κβ becomes pro-apoptotic.³¹ Thus, one can extrapolate the potential that endothelin-1 in a hyperglycemic environment may have a similar reversal in function. In fact, it has been shown that pericyte mitogenic activity is reduced in diabetic patients.³² This suggests that under hyperglycemic pathological conditions, endothelin-1-induced activation of the ET_A receptor may have a reduced mitogenic effect or perhaps an alteration in function that favors pericyte loss.

Another potential link between elevated endothelin-1 levels and pericyte loss is the protein kinase, PKC-δ, which is upregulated in diabetes and has been shown to play an

important role in pericyte apoptosis in diabetic mice.³³ PKC-δ and its downstream target, p38αMAPK, activate two independent pathways involved in pericyte apoptosis in hyperglycemic environment: increased reactive oxygen species leading to activation of NF-κβ, and activation of SHP-1 resulting in PDGFR-β inactivation.³³ Studies have further shown in the setting of hyperglycemia, PKC-δ and PKC-β are the main mediators of increased endothelin-1 in the retinal vascular cells.³⁴ Furthermore, endothelial cell culture experiments have shown that endothelin-1 can directly induce PKC-δ translocation resulting in further increased endothelin-1 activity, likely via ET_A receptor, potentially creating a vicious cycle.^{35,36} It is thus reasonable to argue that these pathways could be activated in DR, whereby an increase in endothelin-1 stimulates PKC-δ activity and subsequent retinal pericyte death.

Endothelin-1 plays an important role in maintaining vasculature in a hemodynamic environment through paracrine signaling between endothelial cells and pericytes.³⁷ In cell culture models, a high-flow environment has been shown to have pro-survival effects on endothelial cells, while increasing pericyte apoptosis. The use of a dual endothelin receptor antagonist reduced the pro-survival effect on endothelial cells and reduced pericyte apoptosis.³⁷ The fact that ET_A receptor is primarily found on pericytes, along with its involvement in regulating PKC-δ activity, suggest that excessive ET_A receptor activation in the setting of a hyperglycemic environment may be associated with pericyte apoptosis. Similarly, inhibiting these molecular pathways through selective ET_A receptor blockade can explain the improved pericyte survival, as demonstrated in our study as an improved E/P ratio and a decrease in acellular capillaries. We further confirmed this protective effect on the pericytes by immunohistochemical dual staining with TUNEL, which showed colocalization of TUNEL and α-SMA in db/db vasculature, a finding that was abolished by atrasentan (Fig. 4).

Our studies show that the peripheral retina in diabetic db/db mice may be more susceptible to pathologic changes of DR. We observed that untreated db/db mice had more prominent apoptosis and thinning in the peripheral retinal sublayers compared with the central retina, suggesting that the retinal periphery maybe more susceptible early on. Furthermore, atrasentan treatment was able to reduce apoptosis and retinal thinning in both regions of the retina, but the protective effect on retinal thickness reached statistical significance only in the peripheral retina, perhaps because of the relatively higher severity of apoptosis. The increased susceptibility of peripheral retina also has been shown in the Ins2^{Akita} mouse, where the inner plexiform layer is reduced by 16.7% centrally versus 27%

FIGURE 5. Histological cross-section of retina near optic nerve (a) and periphery (b): nerve fiber layer/ganglion cell layer (NFL/GCL), inner plexiform layer (IPL), inner nuclear layer (INL), outer plexiform layer (OPL), outer nuclear layer (ONL), photoreceptor layer (PR). (c) Thickness of retinal sublayers (*central*): no differences were seen among the three groups ($n = 3$ per group). (d) Thickness of retinal sublayers (*peripheral*): ($n = 3$ db/m; $n = 3$ atrasentan-treated db/db; $n = 5$ untreated db/db). *Significant difference was seen between db/m and untreated db/db mice for NFL/GCL ($P < 0.05$), IPL ($P < 0.03$), INL ($P < 0.03$), and OPL thickness ($P < 0.04$). †Significant difference seen between atrasentan-treated db/db and untreated db/db mice for INL thickness ($P < 0.02$). ‡Significant difference seen between atrasentan-treated db/db and db/m mice for INL thickness ($P < 0.05$).

FIGURE 6. TUNEL assay (DAPI counterstain). Retinal sections taken at $\times 200$. Central retina (a–f). No TUNEL-positive areas in db/m (a, b) or atrasentan-treated db/db mice (e, f; insert 2). TUNEL positive in the inner retina, primarily GCL in untreated db/db mice (c, d; insert 1 and arrows). Peripheral retina (g–l). TUNEL positive in all nuclear layers (GCL, INL, ONL) in untreated db/db mice, but primarily in GCL/INL and GCL/INL in atrasentan-treated db/db mice (see inserts 2 and 3 and arrows; [i–l]; *artifact). No TUNEL-positive areas present in db/m mice (g, h); $n = 3$ per group).

peripherally after 22 weeks of diabetes.³⁸ We also found mild apoptosis in the outer nuclear layer in the periphery of untreated db/db, suggesting that the outer retina may be at risk for retinal thinning over time. This has also been observed in longitudinal studies of streptozotocin-induced mice and rats.^{39,40}

Inner retinal thinning has been reported in other animal models of diabetes, such as streptozotocin-induced rats, where it has been noted at 7.5 months postinduction, and in Ins2^{Akita} mice at 22 weeks of age.^{38,41} Similarly, retinal thinning has been noted in type 2 diabetic patients, who develop inner retinal thinning over time.⁴² In our study, we confirmed that retinal thinning was due to apoptosis via TUNEL assay (Fig. 6). Whereas prior studies have focused on retinal thickness near the optic nerve, our study shows that the timeline of neuroretinal changes may be distinct, when comparing the central and peripheral retina. Future studies investigating retinal changes in DR may help elucidate the course of

neuroretinal compromise, by comparing central and peripheral retinal thickness in various animal models.

The exact pathophysiology of neuroretinal thinning in DR remains unknown, although there are several proposed mechanisms. Decreased retinal blood flow secondary to capillary closure, pericyte loss, and endothelin-1-induced vasoconstriction can lead to ischemia and relative inner retinal compromise. Although decreased blood flow likely contributes to neuroretinal thinning via ischemia, it is likely that nonvascular metabolic pathways also may be involved. Studies have shown that neuroretinal function is compromised before the onset of vascular lesions and that neuroretinal defects may have an equally important role in DR as vascular defects.^{43,44} Endothelin-1 has been suggested to have a detrimental effect on neural survival, with increased endothelin-1 immunoreactivity in photoreceptors, inner nuclear layer, and ganglion cells in diabetic BB/W rats.⁴⁵ Endothelin-1 also has been strongly

implicated in astrogliosis, a process that causes the release of toxic mediators and ultimately neuronal death.^{46,47} Prior studies have shown that endothelin-1 enhances the glutamate-induced death of amacrine cells, which are also able to release endothelin-1, potentially affecting neighboring horizontal and bipolar cells.⁴⁸ In cultured rat retinal neurons, it has been shown that an ET_A receptor antagonist can suppress the retinal toxicity caused by endothelin-1.⁴⁸ These studies lend support to our finding that atrasentan-treated db/db mice had significantly improved retinal thickness, perhaps via its neuroprotective effects.

In this study, we initiated atrasentan treatment in db/db mice at 23 weeks of age, a time point when pathologic changes of DR, including capillary closure, are already present in db/db mice.²⁵ The fact that ET_A receptor blockade after onset of retinopathy was able to effect significant improvements in peripheral retinal thickness and pericyte survival in db/db mice suggests that earlier treatment, before the onset of vascular pathology, could potentially preserve the health of the peripheral retina. Longer follow-up, as well as studies of earlier treatment, may allow us to further explore these important questions.

It should be noted that the atrasentan-treated diabetic group had a blood glucose level intermediate between the control db/m and untreated db/db mice (Table 1). Although not statistically significant, the blood glucose of atrasentan-treated db/db mice was approximately 100 mg/dL lower than that of untreated db/db mice. To address the glycemic control issue, we did a subset analysis based on stricter glucose levels that essentially confirmed the differences in E/P ratio and acellular capillaries reported in Table 1. However, it is possible the improvement in the retinal vascular parameters was potentially enhanced by the improved metabolic control by atrasentan. Endothelin-1 has been shown to have an important detrimental role on glucose metabolism, although the exact mechanisms have not been fully elucidated.⁴⁹ Animal studies have shown ET_A receptor blockade improved glucose tolerance, hyperinsulinemia, and insulin sensitivity in Zucker fatty rats, an animal model of insulin resistance.⁵⁰ Other studies have shown that ET_A receptor blockade for 8 weeks delayed onset of hyperglycemia in nonobese mice, an animal model of type 1 diabetes.⁵¹ The effects of ET_A receptor blockade could augment the metabolically favorable effect on the pericytes, and may potentially explain the nonstatistically significant difference in mean blood glucose of 100 mg/dL seen in our treated db/db mice. Our study is not of sufficient power to study this aspect of ET_A receptor blockade, but this could be the focus of future research.

The use of endothelin receptor antagonists in diabetes has been explored in various studies with promising results. ET_A receptor antagonists have been shown to improve podocyte survival and protect against renal damage in diabetic nephropathy in streptozotocin-induced and Zucker diabetic rats.^{19,52,53} In healthy humans, as well as streptozotocin-induced mice, ET_A receptor antagonists can prevent decreased retinal blood flow secondary to endothelin-1 administration.^{14,54} Macitentan, a dual endothelin receptor antagonist, was shown to decrease abnormally elevated molecular biomarkers (e.g., endothelin-1, TGF- β , VEGF, fibronectin, collagen) in retina of db/db mice.⁵⁵ Our current study shows a novel role for endothelin-1 antagonists in preserving retinal vasculature as well as neuroretinal structure in db/db mice.

In summary, our study shows that specific ET_A receptor blockade in db/db mice can significantly improve pericyte survival, decrease their apoptosis, reduce acellular capillaries, and reduce peripheral retinal thinning, all of which are important pathologic findings in DR. Our results suggest a potential role for selective ET_A receptor blockade in the management of DR because pericyte loss and retinal thinning are important pathologic features of DR, further research is

needed to explore the exact mechanisms underlying the protective effects of ET_A receptor blockade. Furthermore, additional research is needed to explore whether endothelin receptor antagonists could play a role in prevention as well as therapy of diabetic microvascular complications.

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