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Retinylamine Benefits Early Diabetic Retinopathy in Mice*

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Background: The development of diabetic retinopathy (DR) is incompletely understood. Administered retinylamine is stored in the retinal pigmented epithelium (RPE) where it affects the ocular visual cycle.
Results: Retinylamine inhibited vascular and neural lesions of early DR.
Conclusion: Both the RPE and visual cycle are novel targets for the inhibition of DR.
Significance: Vision-related processes can contribute to DR.

Recent evidence suggests an important role for outer retinal cells in the pathogenesis of diabetic retinopathy (DR). Here we investigated the effect of the visual cycle inhibitor retinylamine (Ret-NH_2) on the development of early DR lesions. Wild-type (WT) C57BL/6J mice (male, 2 months old when diabetes was induced) were made diabetic with streptozotocin, and some were given Ret-NH_2 once per week. Lecithin-retinol acyltransferase (LRAT)-deficient mice and P23H mutant mice were similarly studied. Mice were euthanized after 2 (WT and *Lrat*$^{-/-}$) and 8 months (WT) of study to assess vascular histopathology, accumulation of albumin, visual function, and biochemical and physiological abnormalities in the retina. Non-retinal effects of Ret-NH_2 were examined in leukocytes treated *in vivo*. Superoxide generation and expression of inflammatory proteins were significantly increased in retinas of mice diabetic for 2 or 8 months, and the number of degenerate retinal capillaries and accumulation of albumin in neural retina were significantly increased in mice diabetic for 8 months compared with nondiabetic controls. Administration of Ret-NH_2 once per week inhibited capillary degeneration and accumulation of albumin in the neural retina, significantly reducing diabetes-induced retinal superoxide and expression of inflammatory proteins. Superoxide generation also was suppressed in *Lrat*$^{-/-}$ diabetic mice. Leukocytes isolated from diabetic mice treated with Ret-NH_2 caused significantly less cytotoxicity to retinal endothelial cells *ex vivo* than did leukocytes from control diabetics. Administration of Ret-NH_2 once per week significantly inhibited the pathogenesis of lesions characteristic of early DR in diabetic mice. The visual cycle constitutes a novel target for inhibition of DR.

Retinopathy is a common complication of diabetes and is the principal cause of blindness in adults in industrialized nations. DR[4] comprises a spectrum of histologic and functional abnormalities that are relatively unique for diabetes, including nonperfused and degenerate capillaries, pericyte "ghosts" (empty pockets in the basement membrane surrounding capillaries where pericytes had been located), microaneurysms, and increased vascular permeability (1). Retinal neurons also become dysfunctional as indicated by alterations in visual acuity, contrast sensitivity, color perception, and electrical activity (2–5) and begin to degenerate (6–8). The relationship between the vascular and neural lesions of DR is under investigation, but therapies that inhibit retinal vascular pathology in diabetes have not always reduced the defects in visual function (9, 10).

Multiple investigators have provided evidence suggesting that oxidative stress contributes to diabetes-induced vascular pathology in animals (11–13) and can induce proinflammatory proteins implicated in the pathogenesis of DR (14). Recently, specialized retinal neuronal cells (photoreceptors) have been identified as major contributors to the vascular damage in DR (15, 16), potentially via the generation of superoxide (17). Stitt and co-workers (15) reported that diabetes did not cause the expected decrease in density of the retinal microvasculature in mice lacking rhodopsin (which secondarily leads to photoreceptor degeneration). The authors concluded that loss of the outer retina reduced the severity of DR in that model.

In the retina, a photon of light is converted into an electrical signal by the phototransduction machinery of photoreceptors. When struck by light, opsin-bound 11-*cis*-retinal is photoi-

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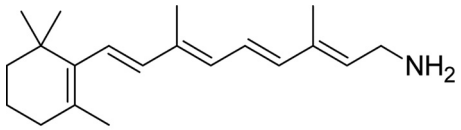
[4] The abbreviations used are: DR, diabetic retinopathy; ICAM-1, intercellular adhesion molecule-1; LRAT, lecithin-retinol acyltransferase; Ret-NH_2, retinylamine; RPE, retinal pigmented epithelium; RPE65, retinal pigmented epithelium-specific 65-kDa protein; ONL, outer nuclear layer.



Retinylamine

FIGURE 1. **Structure of Ret-NH_2.**

somerized to its all-*trans* stereoisomer. All-*trans*-retinal then is released from the opsin binding pocket, reduced to all-*trans*-retinol, and transported to the retinal pigmented epithelium (RPE) where it is esterified by lecithin-retinol acyltransferase (LRAT) and converted to 11-*cis*-retinol by the retinal pigmented epithelium-specific 65-kDa protein (RPE65). Finally, it is oxidized to 11-*cis*-retinal before traveling back to the photoreceptor outer segment to regenerate the visual pigments and photosensitivity of the photoreceptors. This regeneration pathway of chromophore is known as the visual (retinoid) cycle.

All-*trans*-retinylamine (Ret-NH_2) is structurally similar to vitamin A (Fig. 1) but acts as a visual cycle inhibitor by selectively inhibiting RPE65 in the RPE. RPE65 catalyzes the conversion of all-*trans*-retinyl esters to 11-*cis*-retinal during the visual cycle, and thus its inhibition results in slowing of the visual cycle in rod photoreceptor cells. Ret-NH_2 has also been shown to sequester reactive aldehydes (particularly all-*trans*-retinal) (18). Mice treated with all-*trans*-Ret-NH_2 displayed profound delayed dark adaptation following light exposure (19) but also evidenced slower progression of retinal degeneration (20).

The biological effect of administered Ret-NH_2 is prolonged because it is reversibly acylated by LRAT to form inactive *N*-retinylamides (21). This modified Ret-NH_2 is stored in the RPE (21, 22) and other tissues containing LRAT, including intestine and liver (23), testes (24), lung (25), stem cells (26), and mammary and renal epithelium (27). Thus, LRAT is critical for retention of retinoid in the eye and its removal from the circulation. *N*-Retinylamides can be mobilized back to Ret-NH_2 by hydrolysis, thus acting as a slow release depot of Ret-NH_2 in cells expressing LRAT. *N*-Retinylamides are the dominant form of the drug that accumulates in RPE. Their levels build up within 2 h after intraperitoneal injection of Ret-NH_2 to reach ~100 pmol/eye after which their levels slowly decline over the next 7–10 days, reaching a relatively low but stable level of 2–4 pmol/eye (19, 21, 28, 29). *Lrat*$^{-/-}$ mice are chromophore-deficient, and consequently their rod and cone visual functions are severely attenuated at an early age (30).

In the present report, we demonstrate that administration of Ret-NH_2 to diabetic animals inhibits both the vascular (capillary degeneration and abnormal leakage) and neural (visual function) defects that characterize early stages of DR. In addition, the unique actions of Ret-NH_2 provide unique insight into the pathogenesis of DR. Our findings suggest that RPE cells and possibly also the visual cycle contribute to the development of DR. Neither the RPE nor enzymes of the visual cycle have previously been identified as potential contributors to the pathogenesis of DR or as targets for therapeutic inhibition of that retinopathy.

Experimental Procedures

Experimental Animals—Male C57Bl/6J mice, *Lrat*$^{-/-}$ mice (30), and mice in which the P23H mutation of rhodopsin was knocked in (31) were randomly assigned to become diabetic or remain nondiabetic. Diabetes was induced by five sequential daily intraperitoneal injections of a freshly prepared solution of streptozotocin in citrate buffer (pH 4.5) at 60 mg/kg of body weight. After hyperglycemia was verified at least three times during the 2nd week after streptozotocin, diabetic mice were randomly assigned to remain as untreated diabetic controls or to be administered therapy. Insulin was given as needed to prevent weight loss without preventing hyperglycemia and glucosuria (0–0.2 unit of neutral protamine Hagedorn insulin subcutaneously, 0–3 times/week). Blood glucose and HbA1c were measured as reported previously (10, 32). Because blood sugar and insulin treatment are known to influence the development and severity of DR, considerable effort was made to keep glycemia similar in diabetics treated with and without Ret-NH_2. Treatment of animals conformed to the Association for Research in Vision and Ophthalmology Resolution on Treatment of Animals in Research as well as to institutional guidelines. Animals were studied for 2- or 8-month duration of diabetes to determine the effects of potential therapies on molecular and histopathologic changes of the retina, respectively. All animals were euthanized 4–6 days after the last injection of Ret-NH_2.

Ret-NH_2 was synthesized as described previously (19). Initially, a single dose of the amine was injected intraperitoneally into diabetic mice at doses of 0.05–1.0 mg/mouse, and retinal generation of superoxide and visual function were measured 3, 7, and 14 days later. Several diabetic mice died after administration of the 1 mg/mouse dose, so that dose was not studied further. Then the selected lower dose was administered once per week to diabetic mice for 2 months while assessing effects of therapy on retinal superoxide, expression of inflammatory proteins, and visual function. After selection of a safe and effective dose and frequency of its administration (0.2 mg/animal, once per week), a long term (8-month) study was performed to assess the effects of this treatment on diabetes-induced degeneration and dysfunction of retinal capillaries and visual function. In the 2- and 8-month studies, most animals were euthanized 4 days after the previous injection of retinylamine. Retinylamine was not given to nondiabetic animals.

Superoxide Generation—Freshly isolated retinas were incubated in 200 μl of Krebs-Hepes buffer (pH 7.2) with 5 or 30 mM glucose for 5 min at 37 °C in 5% CO_2. Luminescence indicating the presence of superoxide was measured 5 min after addition of 0.54 mM (final concentration) lucigenin as published previously (33–37). Luminescence intensity is reported in arbitrary units/mg of protein.

Leakage of Albumin into Neural Retina—Accumulation of the blood protein albumin in the neural retina has been viewed as a marker of increased vascular permeability (9, 38). At 8 months of diabetes, sterile FITC-BSA (50 μg/μl) in phosphate-buffered saline (0.138 M NaCl, 0.0027 M KCl (pH 7.4)) was injected into the tail veins of mice at 100 μg/g. After 20 min, mice were euthanized, and their eyes were fixed in

ice-cold 4% paraformaldehyde and then frozen in O.C.T. (optimal cutting temperature compound) in isopentane on dry ice after infusion with sucrose. Retinal cryosections were cut and viewed by fluorescence microscopy. Leakage of albumin was estimated from measurements of FITC-BSA in the inner plexiform layer of the neural retina using computer-assisted microscopy. Vascular permeability in diabetes is expressed as the ratio of FITC-dextran concentration in neural retina relative to that in plasma and compared with that of nondiabetic animals.

Diabetes-induced Retinal Histopathology—DR is a slowly developing disease, and rodent models develop only the early stages of this retinopathy during their lives. After 8 months of diabetes, mouse eyes were fixed in formalin, and one retina from each animal was isolated, washed in running water overnight, and digested for 2 h in elastase as we reported previously (39–41). When totally cleaned of neural cells, the isolated vasculature was laid out on a glass microscope slide, dried overnight, stained with hematoxylin and periodic acid-Schiff, dehydrated, and coverslipped. Degenerate (acellular) capillaries were quantitated in six to seven field areas corresponding to the mid-retina (200× magnification) in a masked manner. Acellular capillaries reported per square millimeter of retinal area were identified as capillary-sized vessel tubes having no nuclei along their length.

We estimated whether or not photoreceptors had degenerated in diabetic mice by counting the number of layers in the outer nuclear layer (ONL) in histologic cross-sections of retina. Using photomicrographs generated for the permeability measurements, we counted the number of cells of the ONL in two areas on either side of the optic nerve (~300 μm from the optic nerve), and the resulting values were averaged together to compute a single estimate for each animal. WT diabetic and nondiabetic mice from the 8-month study served as controls for another experiment run simultaneously (9); data from these control experiments are provided for the reader's convenience.

Immunoblotting—Retinas were isolated, sonicated, and centrifuged, and the supernatants were used for immunoblotting. Samples (50 μg) were fractionated by SDS-PAGE and electroblotted onto nitrocellulose membranes, and membranes were blocked in Tris-buffered saline containing 0.02% Tween 20 and 5% nonfat milk. Antibodies for ICAM-1 (1:2,000; Proteintech, Chicago, IL) and inducible isoform of nitric-oxide synthase (1:1,000 dilution; Santa Cruz Biotechnology, Santa Cruz, CA), and phospho-IκB and IκB (1:200 and 1:1,000 dilutions, respectively; both from Santa Cruz Biotechnology, Santa Cruz, CA) were applied followed by secondary antibody for 1 h. After washing, slides were visualized for enhanced chemiluminescence.

Measurement of Visual Cycle Intermediates—Eyes from WT control and mice diabetic for 2 months were collected after (*a*) 24-h dark adaptation and (*b*) bleaching followed by dark adaption (which shows that the mice were able to regenerate 11-*cis*-retinaldehyde after bleaching). For retinoid extraction and HPLC analyses, all procedures were carried out under dim red light. Retinoids were extracted from eyecups, including the retina and RPE. The tissue was transferred into 200 μl of 2 M NH_2OH (pH 6.8) and 200 μl of methanol and homogenized by sonification (five bursts for 5 s, 20% of maximum power). Retinoids were extracted from the homogenate as described previously (21). HPLC analysis was performed on a normal phase ZORBAX SIL column (Agilent Technologies) in 10% ethyl acetate, hexane at an isocratic flow rate of 1.4 ml/min. Individual retinoids were determined by their retention times and spectral characteristics as compared with those of authentic standards. For quantification of molar amounts, peak integrals were scaled with defined amounts of reference retinoids. The reference retinoids all-*trans*-retinyl palmitate, 11-*cis*-retinal, and all-*trans*-retinal were purchased from Toronto Research Chemicals (Toronto, Canada). Corresponding retinal oximes were obtained by their reaction with NH_2OH. The amount of a retinal isomer was determined by the total peak areas of both its *syn*- and *anti*-retinal oxime. Resulting amounts were summed to provide the total amount of 11-*cis*-retinal and all-*trans*-retinal in pmol/eye.

LRAT Activity Assay—Homogenates were made of both leukocytes from WT and $Lrat^{-/-}$ mice and from immortalized mouse retinal endothelial cells (42). All-*trans*-retinol esterification was carried out essentially as described (21). Mouse leukocytes or retinal endothelial cell lysates (~50 mg of protein) were incubated in 10 mM Tris/HCl buffer (pH 7.5), 1% bovine serum albumin with all-*trans*-retinol delivered in 1 μl of *N*,*N*-dimethylformamide to a final concentration of 10 μM. The total volume of the reaction mixture was fixed at 200 μl. Reactions were incubated at 30 °C for 1 h and then stopped by adding 300 μl of methanol followed by the same volume of hexane. Retinoids were extracted and analyzed by the chromatographic method described above in a stepped gradient of ethyl acetate in hexane (1% from 0 to 10 min and 10% up to 30 min at a flow rate of 1.4 ml/min).

Optokinetic Assessment of Photopic Visual Function—The spatial frequency threshold, a marker of visual acuity, and contrast sensitivity threshold were measured with the Virtual Optokinetic system (10, 43, 44). The maximum spatial frequency capable of driving head tracking was determined as the spatial frequency threshold. The contrast sensitivity at 8 months of study was measured at six spatial frequencies to detect functional defects in spatially sensitive retinal cells or in higher visual pathways. This was determined as the inverse of Michelson contrast without correction for luminance of the monitors. The experimenter was masked as to the identity of the experimental group.

Retina Explants—Eyes were enucleated from adult C57Bl/6J mice and immediately immersed in ice-cold DMEM containing 10% FBS, 100 units/ml penicillin, and 100 μg/ml streptomycin. The posterior pole (including the retina) was incubated for 3 days in DMEM in a humidified incubator with 5% CO_2 at 37 °C with the retina kept in contact with the RPE. The culture medium was changed every other day. At the end of this incubation, the retina was separated from the RPE prior to the assay for superoxide described above.

Endothelial Co-culture with Leukocytes—The retinal endothelial cells were grown in control medium (DMEM with 5 mM glucose) containing 10% serum. The serum concentration was reduced to 2% just before cells were placed in either 5 mM glucose or high glucose (30 mM). Medium was changed every other

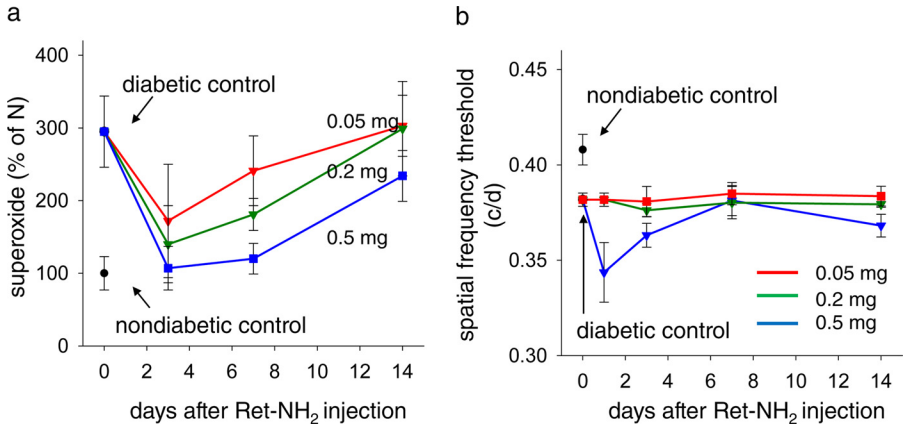


FIGURE 2. **Effects of a single dose of Ret-NH_2 on retinal superoxide production and visual function in 4-month-old mice diabetic for 2 months.** Retinal superoxide generation (*a*) and spatial frequency threshold (*b*) were measured 1, 3, 7, and 14 days after a single intraperitoneal injection of Ret-NH_2. Superoxide was measured by the lucigenin method, and spatial frequency threshold was measured via the optokinetic method. $n = 5–9$ in all groups for superoxide, and $n = 4–6$ for spatial frequency threshold. *Error bars* represent S.D. *N*, nondiabetic; *c/d*, cycle/degree.

day for 3 days. When cells reached 80% confluence (~300,000 cells), freshly isolated leukocytes from blood (100,000 cells) were added and incubated for an additional 6 h after which cells and media were collected and washed with PBS. Cells were stained with an antibody against CD144 to identify endothelial cells, and the viability of the endothelial cells was identified by flow cytometry based on 7-aminoactinomycin D staining. Cell death was expressed as the percentage of endothelial cells that stained with dyes. Approximately 10,000 cells were counted in each sample. Experiments were repeated two times with similar results each time.

Statistical Analyses—Data are expressed as means ± S.D. All statistical analyses were performed with analysis of variance followed by Fisher's test (StatView for Windows (SAS Institute Inc.) and Prism (GraphPad)) except for the full contrast sensitivity curve, which was analyzed by repeated measures analysis of variance to account for testing each animal at multiple spatial frequencies and *t* test in retinoid analyses. Values of $p < 0.05$ were considered statistically significant.

Results

Glycemia was elevated in all diabetic animals, and administration of Ret-NH_2 once per week for the duration of this study did not alter this observation. Average glycated hemoglobin over the entire duration of the 8-month experiment was 3.4 ± 0.2, 11.1 ± 0.6, and 10.8 ± 0.7% for the nondiabetic, diabetic control, and diabetic treated with Ret-NH_2 groups, respectively, and average nonfasted blood glucose values for these groups over the 8-month study were 148 ± 22, 528 ± 52, and 482 ± 66 mg/dl. Final body weights in these groups were 46 ± 3, 29 ± 2, and 29 ± 3 g, respectively. Data from the 2-month experiment were similar. Chronic administration of Ret-NH_2 had no detectable effect on glycemia or health of the animals. Because a fraction of glucose exists as an aldehyde *in vivo* and Ret-NH_2 sequesters aldehydes, the possibility that Ret-NH_2 might lower blood glucose was considered. However, our data indicate that the observed effects of Ret-NH_2 were not mediated by lowering blood glucose.

Dose-ranging Study in Diabetic Mice—Our initial studies sought to determine the dose and frequency of Ret-NH_2 administration that would be efficacious in diabetes using the diabetes-induced increase in retinal generation of superoxide as the end point. After a single injection of Ret-NH_2, all doses of the compound tested reduced the retinal production of superoxide in a dose-dependent manner (Fig. 2*a*) with the 0.5 mg/mouse dose totally normalizing superoxide generation for 1 week and other doses having a lesser effect. The highest dose, however, transiently impaired visual function (spatial frequency threshold; Fig. 2*b*), whereas the lower doses neither improved nor further impaired visual function. This dose-ranging study led to the selection of a once weekly administration of 0.2 mg/mouse Ret-NH_2 for subsequent studies.

Inhibition of Diabetes-induced Retinal Histopathology and Permeability by Ret-NH_2—Long term (8 months) diabetes resulted in a significant ($p < 0.0005$) increase in the number of degenerate (acellular) capillaries in the retina of control animals (Fig. 3, *a* and *b*). Diabetes of 8 months also resulted in a significant ($p < 0.01$) increase in the levels of albumin extravasation into the nonvascular retina (*i.e.* in the neural retina between vessels) in the inner plexiform layer during the preceding 20 min (Fig. 3*c*). Weekly injection of Ret-NH_2 significantly reduced the diabetes-induced degeneration of retinal capillaries and albumin accumulation in the retina ($p < 0.001$ and $p < 0.05$, respectively).

Visual Function—We also measured the spatial frequency threshold and contrast sensitivity in diabetes, both of which are psychophysical measures that assess the function of retinal and central visual pathways. Diabetes of 2- and 8-month durations significantly ($p < 0.01$) lowered the spatial frequency threshold, but Ret-NH_2 had no significant benefit on this defect (0.399 ± 0.004, 0.357 ± 0.007, and 0.366 ± 0.007 cycle/degree for nondiabetic controls, diabetic controls, and diabetics treated with RetNH_2 for 2 months, respectively, and 0.396 ± 0.009, 0.359 ± 0.007, and 0.364 ± 0.009 cycle/degree for 8 months of study). Contrast sensitivity was measured at six spatial frequencies at 8 months of diabetes, and repeated measures analysis indicated that diabetes significantly inhibited the contrast sensitivity curve ($p < 0.0001$), and Ret-NH_2 had a small (but significant; $p < 0.05$) benefit on this defect.

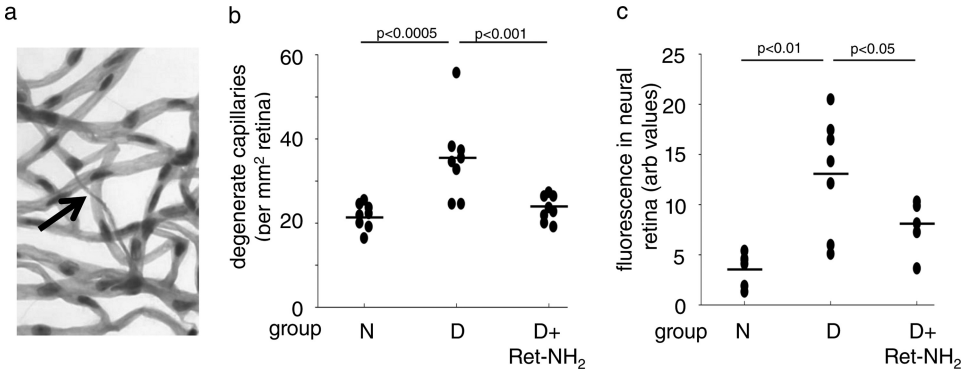


FIGURE 3. **Long term administration of Ret-NH_2 significantly inhibited lesions characteristic of DR.** A representative degenerate retinal capillary (*arrow*) is shown in *a*. Diabetes of 8-month duration in control mice significantly increased degeneration of retinal capillaries (*b*) and accumulation of FITC-albumin in the neural retina (*c*). Ret-NH_2 (0.2 mg/mouse) administered once per week (from the onset of diabetes) for this duration significantly reduced both of these lesions. Vascular histopathology was quantitated microscopically following isolation of the vasculature by the elastase digestion method. Fluorescence in the neural retina was assessed histologically in areas of the inner plexiform layer where there was no obvious vasculature following intravenous injection of FITC-BSA. *Horizontal lines* indicate group means. (*b*, $n = 8$ in all groups; *c*, $n = 5–7$ in all groups). *N*, nondiabetic; *D*, diabetic; *arb*, arbitrary.

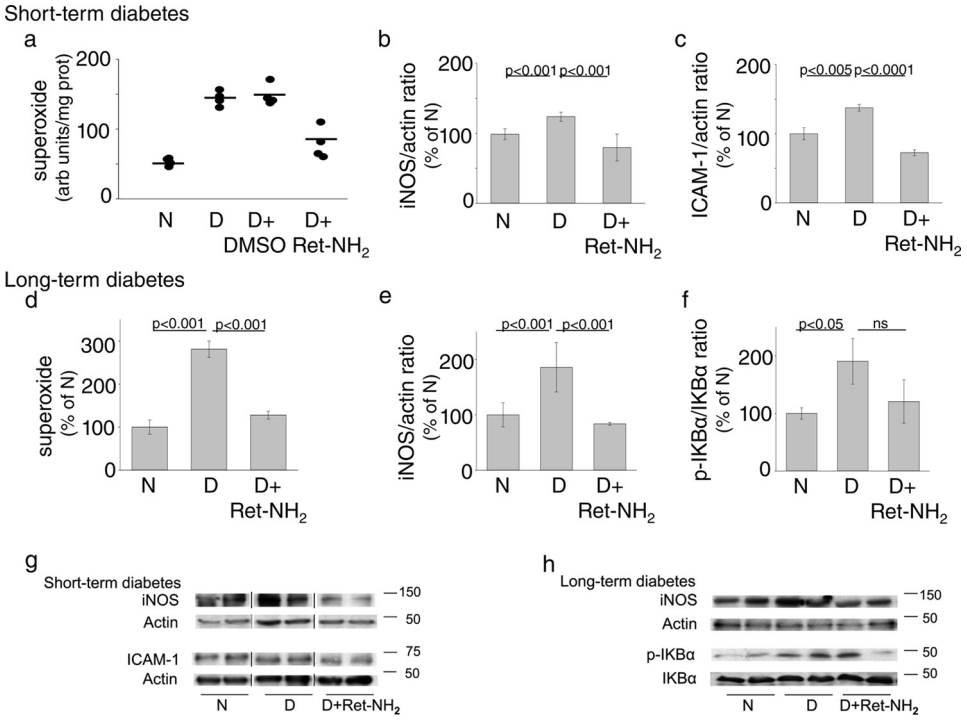


FIGURE 4. The effect of Ret-NH_2 on diabetes-induced increases in retinal superoxide and expression of proinflammatory proteins at 2 (*a–c*) and 8 (*d–f*) months of diabetes is shown. *a* includes a dimethyl sulfoxide (*DMSO*) control to demonstrate that this solvent had no effect on the measured parameters. *Horizontal lines* in a indicate group means. Diabetes was induced at 2 months of age. Superoxide was measured by the lucigenin method, and inflammatory proteins were measured by immunoblotting of retinal homogenates. Representative immunoblots from the 2- (*g*) and 8 (*h*)-month studies are shown (appropriate molecular weight standards are provided to the *right*; and areas where the membranes were cut to remove omitted lanes are indicated by *vertical lines*). $n = 4–7$ in all groups. *Error bars* represent S.D. *iNOS*, inducible isoform of nitric-oxide synthase; *D*, diabetic; *N*, nondiabetic; *p-IκB*, phospho-IκB; *ns*, not significant.

Effect of Ret-NH_2 on Retinal Oxidative Stress and Inflammation—To investigate the mechanism by which Ret-NH_2 inhibited dysfunction and degeneration of retinal cells and capillaries in diabetes, we measured several parameters found in other studies to be causally related to diabetes-induced retinopathy. Thus, we focused initially on oxidative stress and inflammation.

Diabetes significantly increased superoxide and inflammation (inducible isoform of nitric-oxide synthase, ICAM-1, and phospho-IκB as assessed by immunoblots) in the retina at both 2- (Fig. 4, *a–c* and *g*) and 8 (Fig. 4, *d–f* and *h*)-month durations of diabetes. Weekly administration of Ret-NH_2 for the entire duration of these studies markedly reduced the diabetes-induced increase in retinal superoxide and the inducible isoform of nitric-oxide synthase and ICAM-1 expression. The ratio of phospho-IκB to total IκB tended to be normalized by Ret-NH_2 therapy, but the results did not achieve statistical significance in our sample.

Because Ret-NH_2 is known to inhibit photoreceptor degeneration caused by accumulation of all-*trans*-retinal in retinal degeneration models (20), we considered the possibility that diabetes might cause photoreceptor loss and that might be preventable by Ret-NH_2 therapy. In contrast to photoreceptor loss in retinal degeneration (and to the observed degeneration of

TABLE 1

Effect of 2 months of diabetes on visual cycle intermediates in eyes from C57Bl/6J mice (pmol/eye)

N, nondiabetic; D, diabetic.

Group	11-*cis*-Retinal	All-*trans*-retinal	Retinyl esters
Unbleached			
N	353 ± 24[a]	54 ± 7	71 ± 5
D	395 ± 32	47 ± 5	225 ± 57[b]
Bleached			
N	415 ± 40	53 ± 7	128 ± 15
D	416 ± 11	40 ± 1[c]	182 ± 52

[a] Values are mean ± S.D.
[b] Different from nondiabetic at $p < 0.001$.
[c] Different from nondiabetic at $p < 0.05$.

retinal capillaries in the current study of diabetic mice), diabetes of 8-month duration did not produce a significant loss of photoreceptor cells compared with nondiabetic controls (12.6 ± 0.8 layers of nuclei in ONL *versus* 11.2 ± 0.7, respectively), although local losses in the retina might have been missed by our methods. Weekly treatment of diabetic mice with Ret-NH_2 had no significant effect on photoreceptor numbers (12.4 ± 1.7; not significant compared with diabetic or nondiabetic WT controls). Moreover, diabetes of 2-month duration did not alter levels of 11-*cis*-retinal and did not increase all-*trans*-retinal in dark-adapted retinas (Table 1). Nondiabetic and diabetic mice that were (*a*) dark-adapted and (*b*) bleached with subsequent dark adaptation showed similar retinoid composition between groups, indicating that the visual cycle is functional in the diabetics (mice were able to regenerate 11-*cis*-*retinaldehyde* after bleaching). However, diabetes did significantly increase levels of retinyl esters that contribute to 11-*cis*-retinal formation. Because diabetes did not cause photoreceptor degeneration or alterations in levels of all-*trans*-retinal in our study, we conclude that the observed beneficial effects of Ret-NH_2 were not mediated by prevention of these defects.

LRAT catalyzes the formation of retinyl esters, which are storage forms of vitamin A (30, 45). Ret-NH_2 is a derivative of vitamin A, and thus LRAT also amidates Ret-NH_2, thereby allowing it to be stored in cells that contain LRAT (21). LRAT also participates in the visual cycle to regenerate 11-*cis*-retinal for continuing vision. To investigate whether LRAT is required for the beneficial effects of Ret-NH_2, we studied *Lrat*-deficient diabetic mice. In retinas from WT controls diabetic for 2-month duration, the expected increase in retinal superoxide generation compared with controls was observed, and Ret-NH_2 administration (once per week) inhibited this increase (Fig. 5). Surprisingly, retinal superoxide generation was also greatly inhibited in diabetic mice deficient in LRAT compared with wild-type nondiabetic controls. Thus, even in the absence of Ret-NH_2, LRAT deficiency prevented the diabetes-induced increase in superoxide generation by the retina.

To confirm the *in vivo* findings, posterior eyes from nondiabetic WT and $Lrat^{-/-}$ mice were organ cultured for 3 days in levels of glucose comparable with those seen in diabetic (30 mM) and nondiabetic (5 mM) patients, and then retinal superoxide was measured. Retinal explants from WT mice in 30 mM glucose showed a significant increase in retinal superoxide generation compared with those incubated in 5 mM glucose, whereas retinas from $Lrat^{-/-}$ mice incubated in elevated glucose did not show an increase in the generation of superoxide (Fig. 6).

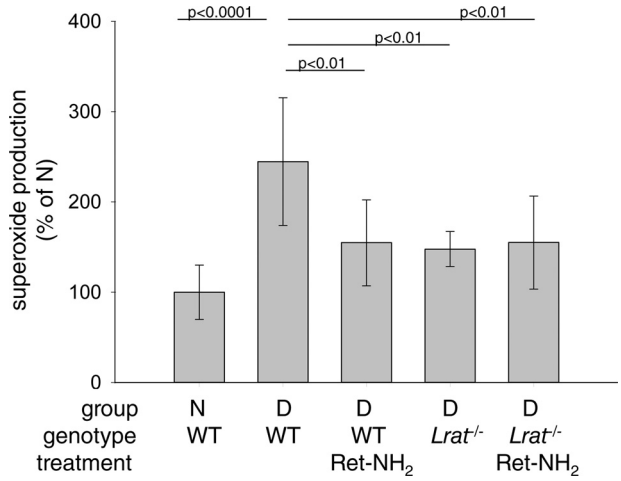


FIGURE 5. **Diabetes-induced increase in superoxide generation by mouse retina is inhibited by weekly treatment with Ret-NH_2 or by LRAT deficiency compared with wild-type nondiabetic controls.** Nondiabetic *Lrat*-deficient mice were not studied. Ret-NH_2 given to $Lrat^{-/-}$ mice did not further inhibit retinal superoxide production. The duration of diabetes was 2 months, and all mice were 4 months of age at the time of the superoxide assay. Ret-NH_2 was administered weekly from the onset of diabetes. Superoxide was measured by the lucigenin method. $n = 5$ in all groups. *Error bars* represent S.D. *N*, nondiabetic; *D*, diabetic.

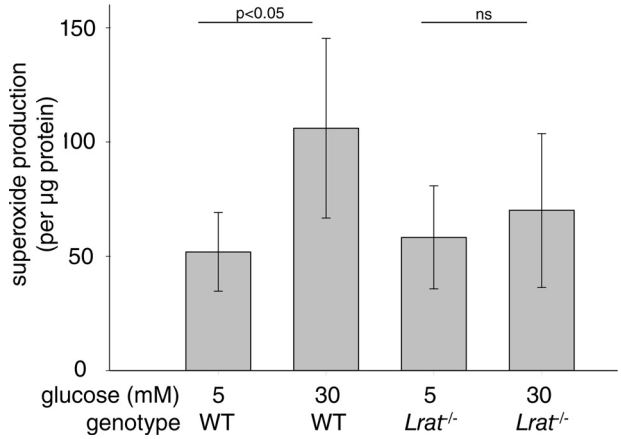


FIGURE 6. **Retinal explants involving posterior eyecups from WT or $Lrat^{-/-}$ mice reveal that superoxide generation in elevated glucose is derived from the retina and is reduced by the absence of LRAT (in the RPE).** Eyecups were obtained from nondiabetic C57Bl/6J (WT) or $Lrat^{-/-}$ mice at age 2–3 months. The duration of incubation was 3 days, and medium was changed after 2 days. Superoxide was measured by the lucigenin method. $n = 5$–6 in all groups. *Error bars* represent S.D. *ns*, not significant.

Because *Lrat* deficiency has been reported to cause slow degeneration of photoreceptors (30), we evaluated the number of nuclear layers in the ONL of 4-month-old WT and $Lrat^{-/-}$ mice to learn whether photoreceptor degeneration could contribute to the absence of retinal oxidative stress in $Lrat^{-/-}$ mice. Our LRAT-deficient mice had 18% fewer nuclear layers in the ONL than did age-matched WT controls (8.9 ± 0.3 and 10.8 ± 2.8 nuclear layers adjacent to the optic nerve in $Lrat^{-/-}$ mice and WT mice, respectively). However, it seems unlikely that the total inhibition of retinal superoxide generation in our $Lrat^{-/-}$ diabetic mice (Fig. 6) was due solely to this modest loss of photoreceptors compared with that in WT mice.

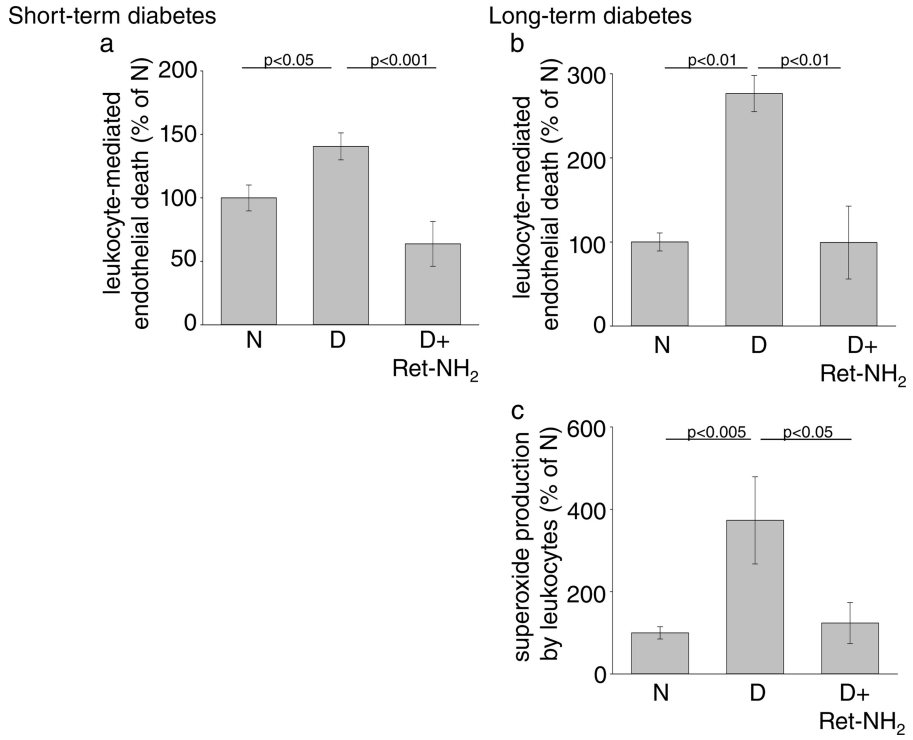


FIGURE 7. **Leukocytes isolated from mice diabetic (*D*) for 2 (*a*) or 8 (*b*) months caused more cytotoxicity to retinal endothelial cells and produced more superoxide (*c*; diabetic for 8 months) than did leukocytes from age-matched nondiabetic mice (*N*).** *In vivo* treatment of some diabetic mice with Ret-NH_2 (*D* + *Ret-NH_2*) from the onset of diabetes suppressed all of these abnormalities. $n = 3–5$ in all groups. *Error bars* represent S.D.

We have implicated leukocytes as contributing to the development of DR (39–41, 46), and killing of retinal endothelial cells has been demonstrated with leukocytes from both diabetic patients (46) and animals (41). We isolated the leukocyte fraction from the blood of animals in the various experimental groups and assessed the effects of *in vivo* Ret-NH_2 treatment on both basal superoxide generation by these leukocytes and leukocyte-mediated killing of retinal endothelial cells. Weekly administration of Ret-NH_2 to diabetic animals significantly inhibited both of these diabetes-induced defects (Fig. 7).

To determine whether the inhibition of leukocyte-mediated cytotoxicity against endothelial cells by Ret-NH_2 was dependent on LRAT, we tested whether LRAT activity is present in leukocytes and endothelial cells and whether LRAT deficiency in mice diabetic for 2 months altered leukocyte-mediated killing of retinal endothelial cells compared with that in WT diabetic animals. Measurement of LRAT enzymatic activity in freshly isolated leukocytes or the retinal endothelial cell line revealed a lack of LRAT activity in both cell types (Fig. 8). In studies to determine whether LRAT deficiency altered the diabetes-induced leukocyte-mediated killing of retinal endothelial cells, we showed that leukocytes from $Lrat^{-/-}$ animals diabetic for 2 months showed no inhibition or exacerbation of leukocyte-mediated endothelial death compared with wild-type nondiabetic controls (Fig. 9); *Lrat*-deficient nondiabetics were not studied. Thus, inhibition of diabetes-induced abnormalities in leukocytes by Ret-NH_2 *in vivo* apparently is not due to a direct effect on the leukocytes or endothelial cells *per se* and appears more likely to be mediated indirectly where Ret-NH_2 is stored under the influence of LRAT. Although deficiency of LRAT sufficed to inhibit diabetes-induced oxidative stress in the retina, it lacked a similar effect on leukocyte-mediated cytotoxicity to endothelial cells.

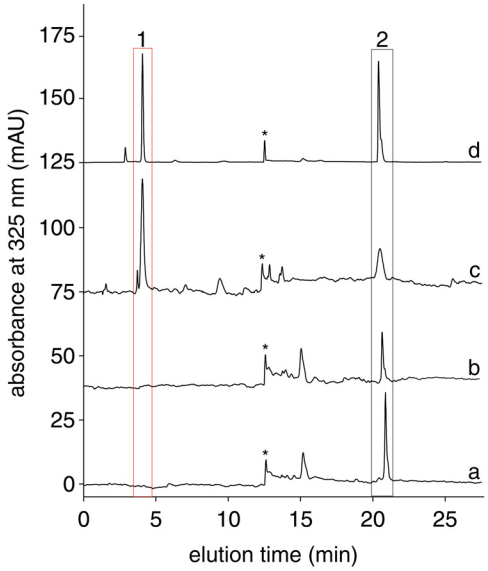


FIGURE 8. **Lack of LRAT enzymatic activity in leukocytes or retinal endothelial cells as evidenced by the absence of all-*trans* ester formation.** Leukocytes were isolated from nondiabetic mice. Analysis of the retinoid composition extracted after incubation of all-*trans*-retinol with leukocyte or retinal endothelial cell extracts (chromatograms "*a*" and "*b*", respectively) did not reveal the presence of all-*trans*-retinyl esters, which were readily detectable in a sample extracted from bovine RPE microsomes used as a positive control (chromatogram "*c*"). Chromatogram "*d*" represents chromatographic separation of synthetic standards of all-*trans*-retinyl palmitate (*peak 1*) and all-*trans*-retinol (*peak 2*). The *asterisks* mark the location of a step increase of ethyl acetate concentration in the mobile phase. *mAU*, milli-absorbance units.

We postulated that disrupted cellular metabolism or damage to the outer retina/RPE might activate circulating leukocytes,

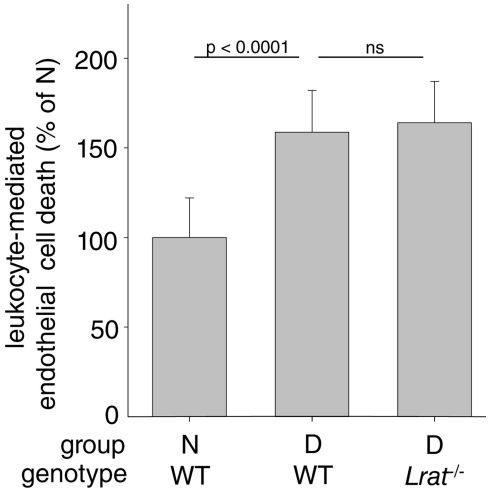


FIGURE 9. **Blood leukocytes from diabetic (*D*) *Lrat*$^{-/-}$ mice cause cytotoxicity to retinal endothelial cells comparable with that seen with leukocytes from WT diabetic mice.** Nondiabetic *Lrat*-deficient mice were not studied. The duration of diabetes was 2 months (age, 4 months). The effect of leukocytes from nondiabetic (*N*) mice on retinal endothelial cells is included for comparison. $n = 5$ in all groups. *Error bars* represent S.D. *ns*, not significant.

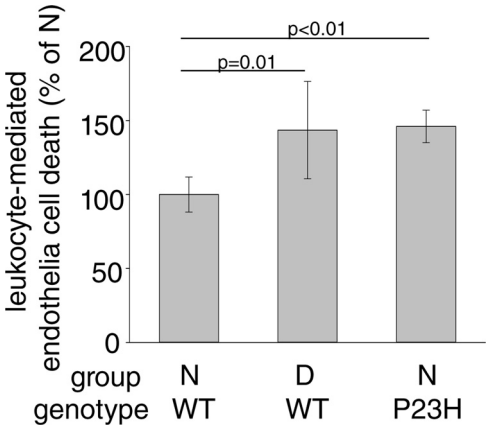


FIGURE 10. **Experimental damage to retinal photoreceptors by the P23H mutation of rhodopsin in nondiabetic mice (*N*) causes circulating leukocytes to become cytotoxic toward retinal endothelial cells.** All animals were studied at 4 months of age. $n = 3–4$ in all groups. *Error bars* represent S.D.

thus contributing to their increased cytotoxicity, and that such cytotoxicity could be inhibited by Ret-NH_2 administration. The first part of this postulate was tested by measuring leukocyte-mediated killing of retinal endothelial cells in nondiabetic animals having different kinds of localized retinal injury (slow photoreceptor degeneration due to mutant photoreceptor rhodopsin (P23H mutation) (31) or LRAT deficiency from RPE). As shown in Figs. 9 and 10, deficiencies of LRAT in the RPE or a P23H mutation of rhodopsin in photoreceptor cells led to increased killing of retinal endothelial cells by circulating leukocytes. Thus, even dysfunction or damage to the outer retina that might not be clinically detectable could suffice to increase cytotoxicity of leukocytes against retinal endothelial cells under a variety of conditions. Mechanisms by which this localized injury to the outer retina causes activation of circulating leukocytes are not known.

Discussion

Ret-NH_2 is an amine-containing compound that was shown previously to inhibit retinal degeneration after acute light-induced phototoxicity (28) in *Rdh8*$^{-/-}$*Abca4*$^{-/-}$ mice (47). Evidence suggested that this beneficial effect was mediated largely by slowing the visual cycle involving the conversion of all-*trans*-retinal into its 11-*cis* configuration (19, 28, 48) at RPE65 within the RPE (28, 49) and by sequestering excessive levels of the reactive aldehyde all-*trans*-retinal (50, 51).

We now report that Ret-NH_2 also inhibits early stages of DR, including increased permeability and degeneration of retinal capillaries. The retinoid had little or no beneficial effect on visual function parameters tested, similar to some other therapies (9, 10) that inhibit diabetes-induced retinal vascular histopathology but not visual dysfunction. Perhaps these sites differ with respect to their sensitivity to inhibition by retinylamine, or their pathogenesis differs in some ways. The pathology of DR differs from that of models of retinal degeneration in several ways, including the relative sparing of photoreceptors in most diabetic patients and animals and the lack of accumulation of toxic retinoids such as all-*trans*-retinal and its metabolites. Thus, the observed beneficial effects of Ret-NH_2 in diabetes are unlikely to result from these mechanisms.

Both oxidative stress and inflammatory systems have been implicated in the capillary degeneration characteristic of early DR, and inhibition of these abnormalities preserves the retinal vasculature despite diabetes (11–14, 32, 37, 40, 41, 46, 52–55). The mechanism by which superoxide generation (which occurs via both mitochondria and NADPH oxidase (17)) causes degeneration of retinal capillaries in diabetes remains unclear, but the phenomenon has been well validated (11–13). In the present study, we found that Ret-NH_2 reduced diabetes-induced defects related to both oxidative stress and some markers of inflammation in the retina, thus offering some insight into the mechanism of the beneficial effect of this retinoid therapy in diabetes. Ret-NH_2 tended to inhibit the diabetes-induced increase in retinal phospho-IκB, although it did not achieve statistical significance in this sample, raising the possibility that some aspects of the inflammatory cascade might be more sensitive to treatment by Ret-NH_2 than others. Ret-NH_2 is unlikely to act as a direct antioxidant or free radical scavenger as administered here because of its short half-life in blood (28) and once per week administration.

Ret-NH_2 is known to inhibit RPE65, an enzyme of the visual cycle that converts all-*trans*-retinol esters back to 11-*cis*-retinal (56, 57). Deletion of another enzyme of the visual cycle, LRAT, also inhibited diabetes-induced generation of superoxide by the retina, but we acknowledge that comparison of *Lrat*-deficient diabetics with *Lrat*-deficient nondiabetics might require modification of this conclusion. The visual cycle itself could contribute to the diabetes-induced generation of superoxide by the retina, but additional work will be needed to test this possibility. Retinyl esters participate in 11-*cis*-retinal formation (49) and are shown herein to accumulate to supranormal levels in diabetic retina, suggesting an impairment of retinoid dynamics in diabetic mice. However, other investigators (58) have not found evidence of abnormal visual cycle activity in diabetes. Maintenance of the visual cycle requires support from numerous mitochondria in photoreceptor cells, and our previous evidence (17, 37) suggests that much of the superoxide generated by the retina in diabetes is generated by such mitochondria. Administra-

tion of Ret-NH_2 was reported by us to cause prolonged suppression of visual cycle activity (18, 19). Because regeneration of 11-*cis*-retinal by the visual cycle activity is greatest in daylight, it seems likely that Ret-NH_2 would exert its effects on RPE65 during this period even though Ret-NH_2 can also affect ion channels in the dark (59). Energy consumption by the retina is lesser in the light than in the dark due to the activity of light-gated ion channels (60), but considerable mitochondrial activity remains even in the light, and diabetes increases the generation of superoxide in both light and dark environments (17).

LRAT has multiple functions that pertain to normal retinal function and damage in a disease like diabetes. LRAT is critical for the retention of retinoids in the retina, circulation, and certain peripheral tissues. Moreover, LRAT also amidates Ret-NH_2, thereby allowing this retinoid to be stored in tissues containing LRAT. Of particular relevance to the retina, LRAT participates in the visual cycle to regenerate 11-*cis*-retinal for vision. *Lrat*$^{-/-}$ mice do undergo slow photoreceptor degeneration (Ref. 61 and the present study), but as diabetes did not cause significant photoreceptor loss, the beneficial effect of Ret-NH_2 in diabetes is unlikely due to stopping degeneration of these cells.

Leukocytes also play an important role in the pathogenesis of DR (14, 32, 39–41, 46). A contribution of white blood cells to the pathogenesis of DR was suggested initially by evidence that diabetes-induced degeneration of retinal capillaries was significantly reduced in mice deficient in CD18 or ICAM-1 (52). The idea was further supported in mice wherein the interaction of leukocytes with endothelial adhesion molecules was blocked by neutrophil inhibitory factor (39) or mice wherein several proteins involved in inflammation or oxidative stress were deleted selectively from myeloid-derived cells (40, 41). Such leukocyte-mediated damage to the vascular endothelial cells in diabetes has been replicated *ex vivo* in a co-culture system where leukocytes from diabetic or nondiabetic mice or patients were incubated with retinal endothelial cells (39, 41, 46). Leukocytes can damage retinal endothelial cells and vasculature by releasing small lipids such as leukotrienes (35, 40, 62). Because leukocytes activated by lesions in one tissue can contribute to pathology at more distant sites (63, 64), we considered the possibility that metabolic dysfunction or damage to the photoreceptors or RPE in diabetes could also activate leukocytes, which might then damage distant retinal vasculature. We tested this idea first in a model of photoreceptor degeneration (P23H mutation) and found that this genetic mutation that initially directly affects only rods resulted in systemic activation of leukocytes. This was evidenced by increased killing of endothelial cells by leukocytes collected from the peripheral circulation. In diabetes, the retinal vasculature seems unusually susceptible to such leukocyte-mediated damage because hyperglycemia up-regulates ICAM-1 on the endothelial surface of retinal endothelial cells more than in virtually all other vascular beds, most probably due to the high metabolic activity of unique retinal cells like photoreceptors.

Ret-NH_2 exerted a beneficial effect on circulating leukocytes by inhibiting diabetes-induced damage to retinal endothelial cells, thus offering a mechanism by which this amide might inhibit vascular disease. We postulate that Ret-NH_2 inhibits diabetes-induced dysfunction/damage to the RPE or photoreceptors, thus reducing the activation of leukocytes that damage retinal vasculature. A direct effect of the drug on leukocytes seems unlikely because (*a*) we administered Ret-NH_2 at low concentrations only once per week, (*b*) neither leukocytes nor endothelial cells showed evidence of LRAT activity (and thus no storage of Ret-NH_2), and (*c*) acute (1-h) incubation of leukocytes *ex vivo* with Ret-NH_2 did not significantly inhibit diabetes-induced cytotoxicity of leukocytes toward endothelial cells.

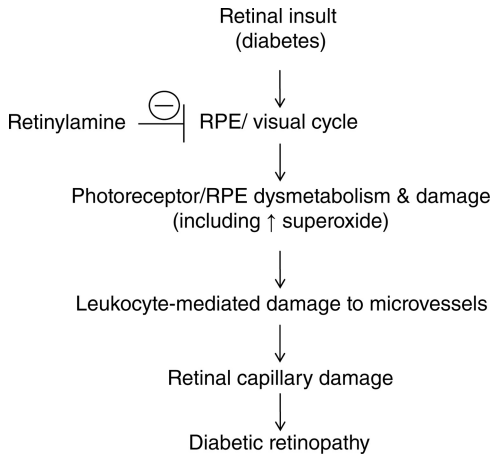


FIGURE 11. **Postulated mechanism by which weekly injections of Ret-NH_2 protect against the vascular lesions of DR.** Diabetes-induced damage to the RPE or alterations subsequent to visual cycle activity could activate circulating leukocytes by unknown mechanisms, and such leukocytes could preferentially damage the retinal vasculature. Because Ret-NH_2 is known to be stored in RPE under the influence of LRAT (neither leukocytes nor endothelial cells have LRAT), its initial beneficial effects likely occur in the RPE with consequent secondary positive effects then observed in leukocytes and retinal vasculature in diabetes.

DR was previously shown by multiple investigators to be inhibited by another amine-containing compound, namely aminoguanidine (65–71). A major action of aminoguanidine was attributed to the sequestration of carbonyls and dicarbonyls (72–74). Ret-NH_2 also can react with reactive aldehydes such as all-*trans*-retinal, but the low dose and infrequent administration of Ret-NH_2 in our experiments suggest that the beneficial effects of this amine in diabetes are unlikely to be mediated solely by its action as a carbonyl trap. Clinical development of aminoguanidine as a therapy for diabetic complications was discontinued due to safety concerns (75).

The unique effects of Ret-NH_2 as reported herein offer novel mechanistic insights into the pathogenesis of the vascular lesions of DR. We postulate that slowing the visual cycle at RPE65 by Ret-NH_2 moderates subsequent phototransduction and the demand on mitochondria, which then reduces superoxide generation and slows the rate of capillary degeneration. One potential mechanism for capillary degeneration involves leukocytes (Fig. 11). Another not mutually exclusive possibility is that alterations within the photoreceptor/RPE induce secondary changes in Müller cells or retinal microglia, which are known to develop oxidative stress and a proinflammatory state in diabetes (76–81) and which could interact with photoreceptor cells and other retinal neurons and the retinal vasculature.

To date, Ret-NH_2 has been found to be safe, and it has the considerable advantage of only needing to be administered

once per week. Current efforts are underway to extend this interval even longer. The metabolism of Ret-NH_2 leads to natural vitamin A products, so there are no known contraindications as long as the selected dose does not impair visual function. Because Ret-NH_2 is stored in RPE, we postulate that the benefits of this compound in DR are derived from local effects on RPE65 in the RPE, thus implicating the RPE in the pathogenesis of this retinopathy. Because RPE65 also is part of the visual cycle that regenerates retinoids, these studies also suggest a role for the visual cycle in the pathogenesis of DR. Obviously, vision depends on a functioning visual cycle, so inhibition of the visual chromophore regeneration by Ret-NH_2 or any similar agent must be titrated so that abnormalities such as superoxide generation are prevented while vision is unaffected. The present results suggest that this objective can be achieved. Both the RPE and the visual cycle constitute novel targets for the amelioration of DR.

Author Contributions—H. L., Y. D., and A. A. V. performed molecular and physiologic analyses and analyzed data. J. T. evaluated histopathology. C. A. L. performed measurements of visual function and maintained animal colony. M. G prepared retinylamine and measured enzyme activity. A. M. and D. A. A. assessed permeability. J. A. and J. v. L. measured retinoids. T. S. K. designed the experiments and analyzed data. K. P. and T. S. K. wrote the manuscript.

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