Retinylamine benefits early diabetic retinopathy in mice*

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Abbreviations: DR, diabetic retinopathy; ICAM-1, intercellular adhesion molecule-1; iNOS, inducible isoform of nitric oxide synthase; LRAT, lecithin retinol acyltransferase; Ret-NH2, retinylamine; RPE, retinal pigmented epithelium; RPE65, retinal pigmented epithelium-specific 65 kDa protein; WT, wildtype

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.

ABSTRACT
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-NH2) 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-NH2 once per week. Lecithin-retinol acyltransferase (LRAT)-deficient mice and P23H mutant mice were similarly studied. Mice were euthanized after 2 months (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-NH2 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 to nondiabetic controls. Administration of Ret-NH2 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-NH2 caused significantly less cytotoxicity to retinal endothelial cells ex vivo than did leukocytes from control diabetics. Administration of Ret-NH2 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 is comprised of 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 pro-inflammatory 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 collaborators (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 photoisomerized 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-NH2) is structurally similar to vitamin A (Figure 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-NH2 has also been shown to sequester reactive aldehydes (particularly all-trans-retinal) (18). Mice treated with all-trans-Ret-NH2 displayed profound delayed dark adaptation following light exposure (19), but also evidenced slower progression of retinal degeneration (20).

The biological effect of administered Ret-NH2 is prolonged because it is reversibly acylated by LRAT to form inactive N-retinylamides (21). This modified Ret-NH2 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-NH2 by hydrolysis, thus acting as a slow release depot of Ret-NH2 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 IP injection of Ret-NH2 to reach ~100 pmols per eye, after which, their levels slowly decline over the next 7 to 10 days, reaching a relatively low but stable level of 2-4 pmols per 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-NH2 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-NH2 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 5 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 3 times during the second 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 units of NPH insulin subcutaneously, 0-3 times per 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-NH₂. Treatment of animals conformed to the ARVO Resolution on Treatment of Animals in Research, as well as to institutional guidelines. Animals were studied for 2 or 8 months durations 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-NH₂.

Ret-NH₂ was synthesized as previously described (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 months) 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% CO₂. 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 per mg 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 (NaCl, 0.138 M; KCl, 0.0027 M; 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 (IPL) 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 to 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 previously reported (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 6-7 field areas corresponding to the mid-retina (200 x 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 2 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 readers’ convenience.

Immuno blot s. The retina was isolated, sonicated, and centrifuged, and the supernatants were used for immunoblots. Samples (50 µg) were fractionated by SDS-PAGE, electrophoresed 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 iNOS (1:1,000 dilution; Santa Cruz Biotechnology, Santa Cruz, CA), p-IκB and κB (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-cisRAL after bleach). For retinoid extraction and HPLC analyses, all procedures were carried out under dim red light. Retinoids were extracted from eye cups including the retina and RPE. The tissue was transferred into 200 µl of 2 M NH₂OH (pH 6.8) and 200 µl of methanol and homogenized by sonification (5 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 NH₂OH. 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 per eye.

LRAT activity assay. Homogenates were made of both leukocytes from WT and Lrat<sup>−/−</sup> mice, and from immortalized mouse retinal endothelial cells (42). all-trans-Retinol esterification was carried out essentially as described in (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, 10% up to 30 min, at the 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 6 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, penicillin (100 U/ml) and streptomycin (100 µg/ml). The posterior pole (including the retina) was incubated for 3 days in DMEM in a humidified incubator with 5% CO<sub>2</sub> 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). Media was changed every other 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 6 additional 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-AAD 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 ± SDs. All statistical analyses were performed with ANOVA followed by Fischer's test (StatView for Windows (SAS Institute Inc) and Prism (graphpad)), except for the full contrast sensitivity curve which was analyzed by repeated measures ANOVA 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-NH₂ 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-NH₂ groups, respectively, and average nonfasted blood glucose values for these groups over the 8 month study were 148 mg/dl ± 22, 528 ± 52, and 482 ± 66. Final body weights in these groups were 46±3 g, 29±2, and 29±3, respectively. Data from the 2-month experiment were similar. Chronic administration of Ret-NH₂ had no detectable effect on glycemia or health of the animals. Because a fraction of glucose exists as an aldehyde in vivo and Ret-NH₂ sequesters aldehydes, the possibility that Ret-NH₂ might lower blood glucose was considered. However, our data indicate that the observed effects of Ret-NH₂ 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-NH₂ administration that would be efficacious in diabetes, using the diabetes-induced increase in retinal production of superoxide as the endpoint. After a single injection of Ret-NH₂, all doses of the compound tested reduced the retinal production of superoxide in a dose-dependent manner (Figure 2a), with the 0.5 mg/mouse dose totally normalizing superoxide generation for one week and other doses having a lesser effect. The highest dose, however, transiently impaired visual function (spatial frequency threshold; Figure 2b), 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 of Ret-NH₂ for subsequent studies.

**Inhibition of diabetes-induced retinal histopathology and permeability by Ret-NH₂.** 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 (Figure 3a,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 (IPL) during the preceding 20 min (Figure 3c). Weekly injection of Ret-NH₂ 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 being psychophysical measures that assess the function of retinal and central visual pathways. Diabetes of 2 and 8 months duration significantly (p<0.01) lowered the spatial frequency threshold, but Ret-NH₂ had no significant benefit on this defect (0.399 c/d ±0.004, 0.357 ± 0.007, and 0.366 ± 0.007 for nondiabetic controls, diabetic controls and diabetics treated with RetNH₂ for 2 months, respectively, and 0.396 ± 0.009, 0.359 ± 0.007, and 0.364 ± 0.009 for 8 months of study). Contrast sensitivity was measured at 6 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-NH₂ had a small (but significant; p<0.05) benefit on this defect.

**Effect of Ret-NH₂ on retinal oxidative stress and inflammation.** To investigate the mechanism by which Ret-NH₂ 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 (iNOS, ICAM-1, p-ιKB as assessed by immunoblots) in the retina at both 2 (Figure 4a-c, g) and 8 (Figure 4d-f, h) months duration of diabetes. Weekly administration of Ret-NH₂ for the entire duration of these studies markedly reduced the diabetes-induced increase in retinal superoxide and iNOS and ICAM-1 expression. The ratio of p-IKB to
total IκB tended to be normalized by Ret-NH₂ therapy, but the results did not achieve statistical significance in our sample.

Because Ret-NH₂ 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-NH₂ therapy. In contrast to photoreceptor loss in retinal degeneration (and to the observed degeneration of retinal capillaries in the current study of diabetic mice), diabetes of 8 months duration did not produce a significant loss of photoreceptor cells compared to 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-NH₂ had no significant effect on photoreceptor numbers (12.4 ± 1.7; not significant compared to diabetic or nondiabetic WT controls). Moreover, diabetes of 2 months 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-cisRAL after bleach). Diabetes did however 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-NH₂ 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-NH₂ is a derivative of vitamin A, and thus LRAT also amidates Ret-NH₂, 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 if LRAT is required for the beneficial effects of Ret-NH₂, we studied Lrat-deficient diabetic mice. In retinas from WT controls diabetic for 2 months duration, the expected increase in retinal superoxide generation compared to controls was observed, and Ret-NH₂ administration (once per week) inhibited this increase (Figure 5). Surprisingly, retinal superoxide generation was also greatly inhibited in diabetic mice deficient in LRAT compared to wildtype nondiabetic controls. Thus, even in absence of Ret-NH₂, 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 to those seen in diabetic (30 mM) and nondiabetic (5 mM) patients, and then retinal superoxide measured. Retinal explants from WT mice in 30 mM glucose showed a significant increase in retinal superoxide generation compared to those incubated in 5 mM glucose, whereas retinas from Lrat⁻/⁻ mice incubated in elevated glucose did not increase the generation of superoxide (Figure 6).

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 if 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 (Figure 6) was due solely to this modest loss of photoreceptors compared to that in WT mice.

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 effects of in vivo Ret-NH₂ treatment on both basal superoxide generation by these leukocytes and leukocyte-mediated killing of retinal endothelial cells. Weekly administration of Ret-NH₂ to diabetic animals significantly inhibited both of these diabetes-induced defects (Figure 7).

To determine if the inhibition of leukocyte-mediated cytotoxicity against endothelial cells by Ret-NH₂ was dependent on LRAT, we tested if LRAT activity is present in leukocytes and endothelial cells, and if LRAT deficiency in mice diabetic for 2 months altered leukocyte-mediated killing of retinal endothelial cells compared to 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 (Figure 8). In studies to determine if 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 to wildtype nondiabetic controls (Figure 9); Lrat-deficient nondiabetes were not studied. Thus, inhibition of diabetes-induced abnormalities in leukocytes by Ret-NH2 in vivo apparently is not due to a direct effect on the leukocytes or endothelial cells per se, and appears more likely mediated indirectly where Ret-NH2 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.

We postulated that disrupted cellular metabolism or damage to the outer retina/RPE might activate circulating leukocytes thus contributing to their increased cytotoxicity and that such cytotoxicity could be inhibited by Ret-NH2 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 in photoreceptor cells led to increased killing of RPE). As shown in Figures 9 and 10, deficiencies of LRAT sufficed to inhibit diabetes-induced oxidative stress in the retina, it lacked a similar effect on leukocyte-mediated cytotoxicity to endothelial cells.

DISCUSSION

Ret-NH2 is an amine-containing compound that previously was shown 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-NH2 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-NH2 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-NH2 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-NH2 tended to inhibit the diabetes-induced increase in retinal p-iKB, although it did not achieve statistical significance in this sample, raising a possibility that some aspects of the inflammatory cascade might be more sensitive to treatment by Ret-NH2 than others. Ret-NH2 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-NH2 is known to inhibit the RPE-specific protein 65 kDa (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 to 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 supra-normal 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. Administration of Ret-NH2 was reported by us to cause prolonged suppression of visual cycle activity (18,19). Because regeneration of 11-cis-retinal by the visual cycle is greatest in daylight, it seems likely that Ret-NH2 would exert its effects on RPE65 during this period even though Ret-NH2 can also affect ion-channels in the dark (59). Energy consumption by the retina is less 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-NH2, 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 (61 and present study), but as diabetes did not cause significant photoreceptor loss, the beneficial effect of Ret-NH2 in diabetes is unlikely due to stopping these cells degenerate.

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-NH2 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-NH2 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-NH2 at low concentrations only once per week, (b) neither leukocytes nor endothelial cells showed evidence of LRAT activity (and thus no storage of Ret-NH2), and (c) acute (1 hour) incubation of leukocytes ex vivo with Ret-NH2 did not significantly inhibit diabetes-induced cytotoxicity of leukocytes towards endothelial cells.

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-NH2 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 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 (Figure 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 pro-inflammatory 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.

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CONFLICTS OF INTEREST
K.P. and M.G. are inventors of U.S. Patent No. 8722669, “Compounds and Methods of Treating Ocular Disorders,” and U.S. Patent No. 20080275134, “Methods for Treatment of Retinal Degenerative Disease,” issued to Case Western Reserve University (CWRU), whose values may be affected by this publication. CWRU may license this technology for commercial development. K.P. is a member of the scientific board of Vision Medicine, Inc., involved in developing visual cycle modulators, and their values may be affected by this publication. The other authors declare that they have no conflicts of interest with the contents of this article.

AUTHOR CONTRIBUTIONS
HL, YD and AV performed molecular and physiologic analyses and analyzed data, JT evaluated histopathology, CAL performed measurements of visual function and maintained animal colony, MG prepared retinylamine and measured enzyme activity, AM and DAA assessed permeability, JA and JvL measured retinoids, TSK designed the experiments and analyzed data, KP and TSK wrote the manuscript.
REFERENCES


LEGENDS

Figure 1. Structure of Ret-NH₂.

Figure 2. Effects of a single dose of Ret-NH₂ on retinal superoxide production and visual function in 4-month-old mice diabetic for 2 months. Retinal superoxide generation and spatial frequency threshold were measured 1, 3, 7 and 14 days after a single intraperitoneal injection of Ret-NH₂. 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.

Figure 3. Long-term administration of Ret-NH₂ significantly inhibited lesions characteristic of DR. A representative degenerate retinal capillary is shown in (a). Diabetes of 8 months duration in control mice significantly increased (b) degeneration of retinal capillaries and (c), accumulation of FITC-albumin in the neural retina. Ret-NH₂ (0.2 mg per 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 (IPL) where there was no obvious vasculature following intravenous injection of FITC-BSA. Horizontal lines indicate group means. (panel b, n=8 in all groups; panel c, n=5-7 in all groups).

Figure 4. Effect of Ret-NH₂ on diabetes-induced increases in retinal superoxide and expression of pro-inflammatory proteins at 2 (a-c) and 8 (d-f) months of diabetes. Panel a includes a 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 by immunoblots 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.

Figure 5. Diabetes-induced increase in superoxide generation by mouse retina is inhibited by weekly treatment with Ret-NH₂ or by LRAT deficiency compared to wildtype nondiabetic controls. Nondiabetic Lrat⁻⁻ mice were not studied. Ret-NH₂ 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-NH₂ was administered weekly from the onset of diabetes. Superoxide was measured by the lucigenin method. n=5 in all groups.

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 media was changed after 2 days. Superoxide was measured by the lucigenin method. n=5-6 in all groups.

Figure 7. Leukocytes isolated from mice diabetic (D) for (a) 2 months or (b) 8 months caused more cytotoxicity to retinal endothelial cells, and produced more superoxide (c; 8 months diabetes) than did leukocytes from age-matched nondiabetic mice (N). In vivo treatment of some D mice with Ret-NH₂ (D+Ret-NH₂) from the onset of diabetes suppressed all of these abnormalities. n=3-5 in all groups.

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, that were readily detectable in a sample extracted from bovine RPE microsomes, a positive control (chromatogram “c”).

Figure 9. Blood leukocytes from diabetic (D) Lrat⁻/⁻ mice cause cytotoxicity to retinal endothelial cells comparable to that seen with leukocytes from WT diabetic mice. Nondiabetic Lrat-deficient mice were not studied. 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.

Figure 10. Experimental damage to retinal photoreceptors by the P23H mutation of rhodopsin in nondiabetic mice (N) causes circulating leukocytes to become cytotoxic towards retinal endothelial cells. All animals were studied at 4 months of age. n=3-4 in all groups.

Figure 11. Postulated mechanism by which weekly injections of Ret-NH₂ 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-NH₂ 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.
Table 1. Effect of 2 months diabetes on visual cycle intermediates in eyes from C57Bl/6J mice (pmol/eye).

<table>
<thead>
<tr>
<th>group</th>
<th>11-cis-retinal</th>
<th>all-trans-retinal</th>
<th>retinyl esters</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unbleached</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N</td>
<td>353 ±24</td>
<td>54 ± 7</td>
<td>71 ± 5</td>
</tr>
<tr>
<td>D</td>
<td>395 ±32</td>
<td>47 ± 5</td>
<td>225 ±57†</td>
</tr>
<tr>
<td>Bleached</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N</td>
<td>415 ±40</td>
<td>53 ± 7</td>
<td>128 ±15</td>
</tr>
<tr>
<td>D</td>
<td>416 ±11</td>
<td>40 ± 1*</td>
<td>182 ±52</td>
</tr>
</tbody>
</table>

Different from N at *p<0.05 or †p<0.001

1 Mean ± SD
Retinylamine
Fig. 2

(a) Superoxide (% of N) vs. days after Ret-NH$_2$ injection.

(b) Spatial frequency threshold (c/d) vs. days after Ret-NH$_2$ injection.

Diabetic control:
- 0.05 mg
- 0.2 mg
- 0.5 mg

Nondiabetic control:
- 0.05 mg
- 0.2 mg
- 0.5 mg

Legend:
- Red line: 0.05 mg
- Green line: 0.2 mg
- Blue line: 0.5 mg
Fig. 3

(a) Degenerate capillaries

(b) Fluorescence in neural retina (arb values)

(c) Group comparison (p-values indicated)

ND  D  +  Ret-NH₂

p<0.0005  p<0.001

p<0.01  p<0.05

p<0.01  p<0.05
Fig. 5

A bar graph showing superoxide production (% of N) for different groups and genotypes. The x-axis represents the group, genotype, and treatment, while the y-axis represents superoxide production. The graph includes error bars indicating variability. Significant differences are indicated by p-values: p<0.0001, p<0.01.
Fig. 6

Superoxide production (per µg protein) vs glucose concentration and genotype

- **p<0.05** for glucose 30mM WT vs Lrat-/-. 
- **ns** for other comparisons.

- **Glucose concentrations:** 5mM and 30mM
- **Genotypes:** WT and Lrat^-/-

Data points:
- **5mM:**
  - WT: 
  - Lrat^-/-: 
- **30mM:**
  - WT: 
  - Lrat^-/-: 

Error bars indicate variability.
Fig. 7

Short-term diabetes

Leukocyte-mediated endothelial death (% of N)

Long-term diabetes

Leukocyte-mediated endothelial death (% of N)

Superoxide production by leukocytes (% of N)

Ret-NH2

N  D  D+

N  D  D+

N  D  D+

p<0.05  p<0.001

p<0.01  p<0.01

p<0.005  p<0.05
Fig. 9

Leukocyte-mediated endothelial cell death (% of N)

<table>
<thead>
<tr>
<th>Group</th>
<th>Genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>WT</td>
</tr>
<tr>
<td>D</td>
<td>Lrat⁻/⁻</td>
</tr>
</tbody>
</table>

p < 0.0001 ns ns
Fig. 10

Leukocyte-mediated endothelial cell death (% of N)

group

WT

P23H

N

N

p<0.01

0 50 100 150 200

0 100 150 200
Retinal insult (diabetes)

Retinylamine \rightarrow RPE/visual cycle

Photoreceptor/RPE dysmetabolism & damage (including ↑ superoxide)

Leukocyte-mediated damage to microvessels

Retinal capillary damage

Diabetic retinopathy