Published in final edited form as:

Neurochem Res. 2018 August ; 43(8): 1500–1510. doi:10.1007/s11064-018-2563-2.

Mechanisms underlying early-stage changes in visual performance and retina function after experimental induction of sustained dyslipidemia

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Abstract

Visual and retinal function was measured in a mouse model of chemically induced, sustained dyslipidemia to determine the contribution of dyslipidemia to the pathogenesis of retinopathy in the context of metabolic syndrome. Fifteen male C57BL/6Cr1 mice were divided into three groups. Poloxamer 407 (P-407), 14.5% w/w was delivered at a rate of 6 μ l/day by implanted osmotic mini-pumps either subcutaneously (P-407 SQ) or intraperitoneally (P-407 IP) to P-407-treated mice, whereas saline was administered at the same rate to control mice using only the subcutaneous route of administration. Total cholesterol (TC) and true triglyceride (TG) levels were quantified from plasma. Optomotor responses to stimuli of varying spatial frequency or contrast were used to measure visual acuity and contrast sensitivity. Retinal function was determined using Ganzfeld flash electroretinography (ERG). At 32 days, TC for the P-407 IP group was significantly elevated compared to saline controls (169.4 ± 16.5 mg/dl, $0.001 < P < 0.01$). TG levels for both the P-407 SQ (59.3 ± 22.4 mg/dl, $0.01 < P < 0.05$) and P-407 IP groups (67.7 ± 18.0 mg/dl, $0.001 < P < 0.01$) were significantly elevated relative to controls. Electroretinography demonstrated a very significant decline in the b/a ratio (1.80 ± 0.11 , $P < 0.01$) for the P-407 IP group. The b/a ratio exhibited a moderate, significant correlation with TC levels ($r = -0.4425$, $P = 0.0392$) and a strong, very significant correlation with TG levels ($r = -0.6190$, $P = 0.0021$). Delivery of P-407 via osmotic mini-pump resulted in the sustained, significant elevation of plasma TC and TG levels. This elevation in plasma lipid levels was correlated with a decline in inner retinal function.

Keywords

C57BL/6; cholesterol; dyslipidemia; hypercholesteremia; hyperlipidemia; metabolic syndrome; mouse; plasma lipid; Poloxamer 407; retina; triglyceride; visual impairment

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Introduction

Diabetic retinopathy (DR), as one of the complications subsequent to the metabolic syndrome, which is characterized by hyperglycemia, hypertension and dyslipidemia, is a serious threat to vision that is poised to become even more problematic with the increasing worldwide incidence of diabetes¹. In fact, DR is the leading cause of blindness among working people (ages 20–74) in developed countries². The estimated treatment cost of patients with DR and its complications is \$500 million annually in the United States^{3,4}. Based on the current prevalence rates of the metabolic syndrome, it is projected that by the year 2050, up to one third of the total adult U.S. population could potentially have diabetes, which would result in an enormous increase in the number of cases of DR⁵. Thus, and in order to develop more effective DR therapies, the contribution of different aspects of metabolic disorder to DR development need to be identified.

DR is classified as either non-proliferative or proliferative based on the stage of disease progression. Non-proliferative DR (NPDR) is characterized by increased permeability of retinal vessels, microaneurysms, exudate deposits, basement membrane thickening, and microhemorrhages^{6,7}. Advanced proliferative DR (PDR) is characterized by neovascularization, preretinal or vitreous hemorrhages, and fibrovascular proliferation^{8,9}. The development of glaucoma, retinal detachment, and vision loss may also happen at this stage of PDR. As epidemiological studies have unequivocally established that DR is a disease with both neurological and microvascular pathologies, with the neurological component preceding the microvascular complications⁶, different aspects of the metabolic syndrome potentially contribute differentially to one or both of these two complications of DR. This potentially also contributes to the issue that DR is not easily diagnosed at early stages, but is more readily noticed at advanced stages or when edema occurs.

Approximately 60% of individuals with Type 2 diabetes mellitus (T2DM) and nearly all individuals with Type 1 diabetes mellitus (T1DM) show at least some signs of DR^{10,11}. Because symptoms that comprise the metabolic syndrome associated with T2DM include hypertension, hyperglycemia, and dyslipidemia, which, in experimental animal models, can be replicated collectively, but not individually for each condition their respective contributions to DR remain poorly understood. Therefore, the purpose of the present study was to delineate the mechanisms involved with the pathogenesis of retinal dysfunction resulting from dyslipidemia in the absence of other symptoms of the metabolic syndrome. This was accomplished in a well-established mouse model of dyslipidemia^{12,13,14,15}, in which plasma insulin and blood glucose remain unperturbed¹⁶, hypertension is not observed and atherogenesis only occurs significantly after disease induction^{12,13,14,15}, a period of time that was not pursued in the present study. Combining these experimental conditions allowed a unique mechanistic focus and the present work to be undertaken in an attempt to isolate the effects of dyslipidemia alone on the development of retinal dysfunction, as a factor potentially contributing to DR.

The mouse model of dyslipidemia we employed is capable of elevating plasma lipids to any desired level without the confounding variable of altered blood glucose and insulin. Additionally, this mouse model is able to specifically elevate the two groups of lipids

[triglycerides (TG) and very-low-density lipoprotein (VLDL)-cholesterol] that are potentially the most problematic, independent risk factors for the development of DR¹⁷, while simultaneously producing low levels of high-density lipoprotein (HDL)-cholesterol. Mice are made dyslipidemic with a triblock copolymer called poloxamer 407 (P-407)¹⁸. The P-407-induced mouse model of dyslipidemia can elevate plasma TG to any desired concentration, whereas, in contrast, classic diet-induced, non-genetically-modified mouse models of hyperlipidemia cannot. An additional benefit of major significance is the fact that the P-407 mouse model of dyslipidemia produces an environment of cellular oxidative stress due to lipids spontaneously and continuously undergoing lipid peroxidation. This is important, since lipid peroxidation is significantly higher in individuals with metabolic syndrome¹⁹, but especially also in DR patients²⁰. Lipid peroxidation produces a chronic inflammatory state and, therefore, the effects of the toxic byproducts of lipid peroxidation on retinal neurodegeneration may be evaluated in the context of dyslipidemia alone, without, for example, the confounding influence of advanced glycation end products (AGEs) that result from hyperglycemia²¹.

High triglycerides and low HDL-cholesterol (HDL-C) levels are prominent lipid abnormalities in the metabolic syndrome as a result of insulin resistance. Recently, the third report of the National Cholesterol Education Program Adult Treatment Panel III (NCEP ATP III) guidelines stipulate that a patient with the metabolic syndrome must meet several criteria, two of which include triglycerides ≥ 150 mg/dl (irrespective of gender) and HDL-C less than 50 mg/dl (women) or less than 40 mg/dl (men).²² In addition to the triglyceride and HDL-C abnormalities mentioned above, those patients with the metabolic syndrome exhibit a high prevalence of small, dense LDL particles, which are extremely atherogenic. Most patients with hyperlipidemia typically have an elevation in plasma total cholesterol and LDL-cholesterol (LDL-C), with or without an elevation in serum triglycerides.²³ The elevation in plasma LDL-C often gives rise to oxidized LDL (oxLDL), which is a known atherogenic stimulus. Additionally, most humans with dyslipidemias exhibit a reduced level of HDL-C. The mouse model used in the present study is able to recapitulate several of the phenotypic signature symptoms associated with lipid abnormalities observed in humans with the metabolic syndrome, which include low HDL-C, elevated triglycerides, and an increase in very-low-density lipoprotein associated with cholesterol (VLDL-C). However, because P-407 functions as a non-specific lipase inhibitor *in vivo*, the rise in plasma triglycerides is much greater than the rise in plasma cholesterol following P-407 administration to mice.

To more easily maintain a sustained atherogenic plasma lipid profile without the increased stress and risk of animal loss associated with repeated intraperitoneal injections, we employed implantable osmotic pumps to continuously deliver P-407 at a defined rate to mice for one month. This strategy was based on the rationale that an effective delivery of P-407 utilizing osmotic pumps would simplify an examination of the effects of an elevated and sustained atherogenic plasma lipid profile on visual and retinal function in otherwise intact, yet dyslipidemic mice.

Materials and Methods

Materials

Poloxamer 407 (Pluronic F-127, N.F.) was obtained from Sigma (St. Louis, MO), shipped directly to Charles River Laboratories, and used as received. The Alzet® osmotic pumps were purchased from DURECT, Inc. (Cupertino, CA) by Charles River Laboratories (Raleigh, NC). The osmotic pumps (model number 2004) had a total internal reservoir volume of 186 microliters and a delivery rate of 0.25 microliters/hr and, thus, could deliver poloxamer 407 for a total of 4 weeks following subcutaneous implantation. Commercial enzyme-based kits to measure the concentration of total cholesterol and triglycerides were purchased from Wako Diagnostics (Richmond, VA).

Animal Protocols

Three groups of male C57BL6 mice having a body mass of approximately 20 g each were obtained from Charles River Laboratories 4 days after subcutaneous implantation of osmotic pumps. The control group (saline) was comprised of 5 mice that were implanted subcutaneously on their dorsal side midway between the ears with an osmotic mini-pump filled with normal saline. The second group (P-407 SQ) contained 5 mice similar to the first with the exception that the osmotic pump contained P-407 dissolved in normal saline at a concentration of 14.5% w/w. Both experimental groups received a continuous infusion of 27 mg of P-407 over the course of 28 days, which is equivalent to 1.35 g/kg of body weight. One P-407 SQ animal died before any data was collected, and measurements from another were excluded after day 20 due to pump failure. The concentration of P-407 was not arbitrarily selected, but rather, was chosen after *in vitro* experiments had demonstrated that the slightly-viscous P-407 solution would remain in a liquid state and easily flow from the pump orifice, since P-407 is a triblock polymeric compound that undergoes reverse thermal gelation. The third group of 5 (P-407 IP) consisted of mice that had been implanted subcutaneously at the same location as mice in the first two groups, but additionally had a narrow-bore cannula that connected the orifice of the osmotic pump, which contained 14.5% w/w P-407 in normal saline, with the peritoneal cavity, so that P-407 was delivered directly into the peritoneal space. Note that the size of the model 2004 osmotic pump precludes peritoneal implantation in a 20 g mouse per the manufacturer's specifications.

Mice were housed individually in plastic shoebox cages at 25 °C with *ad libitum* access to water and maintained on a 12 hr light/dark cycle. Teklad Global 18% Protein Rodent Diet (Envigo, New Jersey; catalog #2918.15) was fed *ad libitum* except during the 5 hour fasting period immediately prior to each blood draw. Saline controls gained $2.6 \text{ g} \pm 0.7$, the P-407 SQ group gained $3.7 \text{ g} \pm 1.3$, and the P-407 IP group gained $3.0 \text{ g} \pm 1.1$ in weight over the course of the study. There were no significant differences in weight between any of the groups (Supplementary Figure S1). All experimental animal procedures were approved by the institutional animal care and use committee and performed in accordance with institutional and federal guidelines and the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Quantification of Plasma Lipid Concentrations

The concentrations of total cholesterol and triglycerides were determined in plasma using the commercial enzyme-based kits/reagents described above. Mice were deprived of food, but had free access to water, 5 h before tail vein blood sampling. A minimum of 100 microliters of blood was collected in heparinized plastic capillary tubes from each mouse at the time points depicted in Figure 1. Collected blood samples were kept on ice, centrifuged at 10,000 x g, the plasma harvested, and immediately frozen at -80°C until the time of analysis.

At the time of lipid determination, plasma samples were thawed on ice, mixed, and the samples combined with the appropriate reagents to determine either total cholesterol or true triglycerides. Absorbance at 600 nm was determined on a Flex Station 3 microplate reader (Molecular Devices, Sunnyvale, CA) at 37°C . A multi-lipid calibrator (Wako Diagnostics, Richmond, VA) at a concentration of 98 mg/dl was used as the standard for the determination of both plasma total cholesterol and true triglycerides

Methods for TG determination usually involve hydrolysis of triglycerides to glycerol and free fatty acids followed by chemical or enzymatic measurement of the glycerol released. Many triglyceride assays do not differentiate between endogenous glycerol and that released from triglycerides, and thus report “total triglycerides”, which consequently overestimates the concentration of triglycerides. When the endogenous glycerol content is decomposed to a non-reactive state or measured separately from the glycerol released from triglycerides, as in the assay used here (Wako Diagnostics, Richmond, VA), “true triglycerides” are reported.

Determination of Visual Function

Due to the length of time required for measurements of visual and retinal function and the number of animals in the study, visual acuity (VA), contrast sensitivity (CS), and electroretinography (ERG) measurements were staggered as shown in Figure 1. Quantification of visual function using measures previously established in a mouse model of ocular disease²⁴ was performed using an Optomotry™ system (Cerebral Mechanics, Lethbridge, AB). Visual acuity is reported as the maximum spatial frequency which elicits an optomotor response. Visual acuity testing was performed at 100% contrast beginning at a low spatial frequency (0.042 cycles/degree [c/d]) with a grating rotating at $12^{\circ}/\text{s}$. Contrast sensitivity is reported as the reciprocal of the lowest contrast which elicits an optomotor response. Contrast sensitivity testing was performed by decreasing the contrast while maintaining the spatial frequency at 0.042 c/d. Both tests utilized a random staircase method with the observer blind to the treatment group as well as the spatial frequency or contrast level.

Electroretinography

Electroretinography (ERG) responses were recorded using a Ganzfeld-electroretinography and adjunct anesthesia system (HMsERG Rodent System, Ocuscience LLC, Henderson, NV), following standard procedures adapted for mice. ERG testing was staggered as shown in Figure 1. Animals were dark adapted for a minimum of 12 hours and all subsequent procedures were performed under dim red light. One drop of 0.5% tropicamide was instilled

in each eye 13 – 20 minutes before the first flash. Anesthesia was induced with 1.75 % isoflurane at an oxygen flow rate of 1 L/min for three minutes and thereafter maintained at 1.5% at the same flow rate. Core body temperature was sustained at 37°C with a warming pad controlled by a rectal thermometer. Ground and reference electrodes were placed near the base of the tail and just above or below the ears, respectively. Goniovisc ophthalmologic solution (HUB Pharmaceuticals, LLC. Rancho Cucamonga, CA) was applied to each eye prior to application of silver-embedded thread electrodes. Optically clear Aclar 3.5 mm contact lenses (Ocuscience LLC, Henderson, NV) filled with Goniovisc were applied over the thread electrodes.

Background activity was recorded for 20 ms prior to each flash and the response was recorded for at least 180 ms afterward. All traces were processed post-acquisition with a 60 Hz noise filter and 150 Hz low pass filter. Rod function was evaluated using 10 flashes with an intensity of 10 mcd-s/m² at 0.5 Hz. Mixed rod and cone function was measured using a single flash with an intensity of 10 cd-s/m². Oscillatory potentials were derived from mixed rod and cone traces prior to the application of noise and low pass filters using a 65–300 Hz bandpass filter with amplitudes calculated from baseline to peak. Cone function was assessed using 32 flashes with an intensity of 10 cd-s/m² at 2 Hz on a 30 cd-s/m² background after 10 minutes of background adaptation. Since ERGs are prone to artifacts caused by electrode malfunction as well as electrical, mechanical, and physiological noise, numbers that fell 1.5 times the interquartile range outside the upper or lower interquartile range were excluded from the mean.

Statistics

Data are expressed as mean ± standard deviation of the mean. One-way ANOVA with Dunnett's or Turkey's post-test was used to determine statistical significance. P values > 0.05 were not considered statistically significant, 0.05 to 0.01 were considered significant (*), 0.01 to 0.001 were considered very significant (**), and < 0.001 were considered extremely significant (***). The Pearson product-moment correlation coefficient (r) was calculated to evaluate relationship strengths. Correlations were identified as strong for r values > 0.6, moderate for r values of 0.4 to 0.6, weak for r values of 0.2 to 0.4, and no correlation for r values < 0.2. Statistical analyses were performed using GraphPad Prism 5.0 software (GraphPad Software Inc., La Jolla, CA).

RESULTS

Delivery of P-407 by osmotic pump induces significant dyslipidemia

To determine the effectiveness of P-407 administration by osmotic pump, plasma total cholesterol (TC) and true triglyceride concentrations (TG) were measured once a week for the duration of the study. Total plasma cholesterol ranged from 91.4 to 137.9 mg/dl with an average of 118.5 mg/dl over the course of 32 days for saline controls. When P-407 was administered intraperitoneally (P-407 IP), TC concentrations ranged between 93.2 mg/dl to 169.4 mg/dl with an average value of 143.6 mg/dl. For subcutaneous delivery of P-407 (P-407 SQ), TC concentrations ranged between 86.9 to 157.8 mg/dl with an average value of 131.6 mg/dl. At all but the first time point throughout the 32-day study, TC was significantly

($P < 0.05$) greater for the P-407 IP group when compared to corresponding mean TC for saline controls (Figure 2A; Supplementary Table 1). In general, the P-407 SQ group exhibited modestly elevated TC concentrations compared with the corresponding values observed for saline controls with a significant ($0.01 < P < 0.05$) increase seen only at day 27. Total cholesterol concentrations for P-407 SQ mice fell approximately midway between those determined for saline controls and P-407 IP. When mean TC concentrations for mice in the P-407 IP group were compared to corresponding mean values for mice in the P-407 SQ group a statistical difference was found only at day 13 ($0.001 < P < 0.01$).

Plasma true triglyceride concentrations resulting from the controlled release of P-407 from implanted osmotic pumps for 32 days followed a similar trend as plasma total cholesterol; specifically, $TG_{P-407\ IP} > TG_{P-407\ SQ} > TG_{saline}$. For saline controls, TG ranged from 14.6 to 26.7 mg/dl, averaging 18.5 mg/dl over the course of 32 days. Intraperitoneal administration of P-407 resulted in TG concentrations ranging from 43.2 mg/dl to 67.7 mg/dl with an average value of 65.1 mg/dl (3.5 fold higher than controls). When P-407 was delivered subcutaneously, TG concentrations ranged from 20.1 mg/dl to 59.3 mg/dl with an average of 38.6 mg/dl (a 2.1 fold increase over controls). At each time point throughout the study, TG concentrations were significantly ($P < 0.05$) elevated for the P-407 IP group (Figure 2B; Supplementary Table 2) when compared to the corresponding mean TG for saline controls. For the P-407 SQ group, TG concentrations were significantly ($P < 0.05$) elevated on day 20 and day 37 when compared to saline controls. When mean TG concentrations for mice in the P-407 IP group were compared to corresponding mean values for mice in the P-407 SQ group, a statistical difference was found at day 13 ($P < 0.001$), day 20 ($0.01 < P < 0.05$), and day 27 ($P < 0.001$).

Visual function declines with retinal function

Visual acuity and contrast sensitivity were quantified in each cohort as measures of visual function. Visual function was assessed at the beginning of the study to verify that no animals had pre-existing visual deficits (Supplementary Figure S2). At the conclusion of the study, visual acuity (VA) measured 0.463 ± 0.022 c/d for the saline controls ($n = 10$ eyes), 0.456 ± 0.032 c/d for the P-407 SQ group ($n = 6$ eyes), and 0.463 ± 0.031 c/d for the P-407 IP group ($n = 10$ eyes). When compared to saline controls, there was no significant difference in VA with either the P-407 SQ group or the P-407 IP group (Figure 3A). Final contrast sensitivity (CS) values were 2.47 ± 0.18 for the saline controls ($n = 10$ eyes), 2.47 ± 0.20 for the P407 SQ group ($n = 6$ eyes), and 2.57 ± 0.35 . Contrast sensitivity was not significantly different for either the P-407 SQ or the P-407 IP group when compared to saline controls (Figure 3B). Visual acuity correlated strongly ($r = 0.6192$) and very significantly ($P = 0.0007$) with contrast sensitivity (Figure 3C). Although measures of visual function were not significantly different when compared as grouped means, both VA and CS correlate moderately ($r = 0.4759$ and 0.4808 , respectively) and significantly ($P = 0.0252$ and 0.0235 , respectively) with declining b/a-wave ratios, a measure of inner retinal function.

Inner retinal function is diminished in dyslipidemic animals

For measures of rod function, there were no significant differences in amplitude (Figure 4A) or implicit time (Figure 4B) for either P-407 treated group when compared to saline

controls. A very significant ($0.001 < P < 0.05$) increase in a-wave amplitude (Figure 4D) was observed in tests of mixed rod and cone function when comparing the P-407 IP group to saline controls. The P-407 IP group also demonstrated a very significant ($0.001 < P < 0.05$) decrease in b/a wave ratio (Figure 4G). Delays in both a- and b-wave cone implicit times (Figure 4I) were also very significant ($0.001 < P < 0.05$) for the P-407 IP group. There were no statistically significant differences observed in retinal function of P-407 SQ animals compared to controls (Figure 4A–J). However, values for both the b/a-wave ratio and the cone a- and b-wave implicit times for the P-407 SQ group fall midway between the control group and the P-407 IP group, as their TC and TG values do. Although not statistically significant, the second and third oscillatory potential (OP) amplitudes as well as the sum of OP amplitudes were lower for the P-407 IP group (Supplementary Figure S3) than the saline controls.

Increased lipid levels correlate with decreased visual and inner retinal function

Pearson product-moment correlation coefficient calculations were performed to examine relationship strengths between TC or TG levels and parameters of visual and retinal function. End-point TC levels demonstrated a positive, moderate ($r = 0.5175$), significant ($P = 0.0163$) correlation with mixed rod and cone a-wave amplitude (Figure 5A) as well as a negative, moderate ($r = -0.4425$) and significant ($P = 0.0392$) correlation with the b/a-wave ratio (Figure 5B). True triglyceride levels measured at the final time-point correlated weakly ($r = -0.3961$) but significantly ($P = 0.0451$) with visual acuity (Figure 5C). In measures of rod function, the b-wave amplitude exhibited a moderate (-0.4263) and significant ($P = 0.0378$) association with end-point TG levels (Figure 5D). Final TG concentrations also showed a positive, moderate ($r = 0.4688$), significant ($P = 0.032$) correlation with mixed rod and cone a-wave amplitude (Figure 5E) as well as a negative, strong ($r = -0.6190$), very significant ($P = 0.0021$) correlation with the b/a wave ratio (Figure 5F).

Discussion

Implantable osmotic pumps have not been used previously in the P-407 hyperlipidemia model because the reverse thermal gelation properties of P-407 limits the maximum deliverable dose for a 20 g mouse to about 43.5 mg/kg/day compared to the typical dose of 300–500 mg/kg every three days. Since P-407 elevates plasma lipids in a predictable, dose-dependent manner²⁵, the maximum attainable TG and TC levels would be limited to about 375 mg/dl and 175 mg/dl respectively, using the osmotic pumps with a zero-order (constant) rate of P-407 administration. The present study demonstrates the effectiveness of an implantable osmotic pump in sustaining an atherogenic plasma lipid profile without the risks of repeated intraperitoneal injections. Although these levels are lower than those typically induced to study, for example, the effects of lipid-lowering drugs, they more closely approximate the more common situation of moderate hyperlipidemia. This moderate, yet significant, elevation of both TC and TG make this a useful pre-clinical or early-stage animal model with which to evaluate the effects of elevated serum lipids on early neurological changes in the retina as part of mechanistically isolated aspect of metabolic syndrome.

Although there are several case reports characterizing visual and/or retinal function in humans with severe hyperlipidemia resulting in a condition called lipemia retinalis^{26–28}, there are few that examine retinal function in humans with moderate hyperlipidemia prior to the onset of clinical visual dysfunction²⁹. In the rare human cases where severe hyperlipidemia (>1000 mg/dl) causes visual deficits, function is generally recovered with prompt reduction of lipid levels^{26–28}. In the present study, we did not observe a significant difference in visual function (visual acuity or contrast sensitivity) in either hyperlipidemic group (*i.e.*, P-407 SQ and P-407 IP) compared to the saline control group. However, we did find that the b/a-wave ratio was significantly reduced in the P-407 IP group and that for the same group this hallmark of retinal function was moderately and significantly correlated with both visual acuity and contrast sensitivity. This, along with the observation that higher TG levels also correlated significantly with lower visual acuity suggests that dyslipidemia results in a presymptomatic decline in visual function that is not apparent when simply comparing visual function measurements among the experimental groups. Reversible changes in retinal function, such as a severely reduced scotopic a-wave and a normal, but smaller than average, scotopic b-wave, and photopic cone responses have also been observed in severe hyperlipidemia.²⁶ Zhang and colleagues measured ERGs in humans with moderate hyperlipidemia (TC = 232.4 ± 40.5 mg/dl, TG = 231.9 ± 108.0) in the absence of other complicating systemic or ocular disorders.²⁹ They observed significant a- and b-wave amplitude declines and implicit time delays in rods, mixed rods and cones, and isolated cones as well as oscillatory potential sums and flicker responses²⁹. Although the lipid levels we measured are comparable to the ones of human subjects in the study by Zhang *et al.*, the duration of hyperlipidemia in our study is much shorter and equivalent to approximately 3.4 years duration after adjusting for the mouse life span³⁰ compared to the 17.4 ± 6.9 years in the study performed by Zhang *et al.*²⁹ indicating that prolonged hyperlipidemia is likely to increase the early presymptomatic decline in retinal and visual function we observed.

Hyperlipidemia as a component of the metabolic syndrome predisposing individuals to type 2 diabetes mellitus, leads to DR in more than half of affected individuals.^{17, 31} The P-407 mouse model of hyperlipidemia specifically elevates triglycerides and very-low-density lipoprotein, both of which are independent risk factors for DR in humans. The model also produces cellular oxidative stress, analogous to higher levels of lipid peroxidation seen in patients with DR, without inducing hyperglycemia or hypertension. In combination, these properties of the P-407 mouse model of hyperlipidemia render it a potentially valuable tool for evaluating the effects of dyslipidemia alone on the development of DR, thereby allowing one to mechanistically dissect the contributions of hyperlipidemia, hyperglycemia, and hypertension to DR in combination with other mouse models. Significant delays in oscillatory potential implicit time and amplitude are the most commonly observed ERG alterations in humans with diabetes^{32–34} as well as in rodent models^{33, 35–37}. These changes are reported to occur prior to ophthalmoscopic and angiographic changes^{32, 34} or deterioration of the blood-retinal barrier³⁸. We did not observe any significant changes in oscillatory potential amplitudes or implicit times (Supplementary Figure S3), suggesting that this effect is specific to, or more heavily influenced by, the effects of elevated blood glucose rather than hyperlipidemia and/or a result of longer exposure to hyperlipidemia.²⁹ Our finding of a significantly reduced b/a-wave amplitude is similar to that reported in Alloxan

induced diabetic mice³⁹ and streptozotocin induced diabetic rats^{33, 36} at early time points, prior to the development of diabetic retinopathy. While severely reduced b/a-wave ratios are typically associated with retinal vascular obstruction, the dosage and duration of P-407 administered in the present study was not sufficient to cause atherosclerotic lesions¹³. Instead, it is more likely that the increase in oxidatively-modified LDL (ox-LDL) and the decrease in protective apoA1 containing HDL conferred by this model^{13, 40, 41} result in microvascular injury or disruption of neurovascular coupling. Indeed, ox-LDL is toxic to cultured retinal capillary endothelial cells and pericytes⁴² and has been observed in inner retinal tissue prior to the onset of clinically diagnosed DR in humans⁴³ as well as in Akita mice⁴⁴.

The intimate association of Müller cells with the inner retinal vasculature⁴⁵ as well as their many roles in supporting neuronal function and viability⁴⁶ make them a possible target of oxidative stress associated with hyperlipidemia⁴⁷. The significantly decreased b/a-wave ratio and increased a-wave amplitude we observed could be explained by Müller cell dysfunction. Since the ERG P_{II} (b-wave) component, which is contributed by Müller and bipolar cells, destructively interferes with the P_{III} (a-wave) component⁴⁸, which is contributed by photoreceptors, the a-wave amplitude would be expected to increase as Müller cell function is altered due to reduced interference.

Conclusions

In summary, we identified previously unknown changes in visual and retinal function in a mouse model of hyperlipidemia in the absence of hyperglycemia. Specifically, significant changes in parameters of retinal function, which correlate with increasing plasma TC and TG, suggest that retinal function may be negatively impacted by hyperlipidemia and/or the accompanying increase in oxidative stress and risk of lipid oxidation. Although these changes may not be severe enough to cause a clinically symptomatic phenotype of changes in visual function in humans, hyperlipidemia alone may initiate conditions that are conducive to further damage potentially resulting in more severe forms of DR.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The research presented in the present publication was supported in part by NIH grants AG027956 from NIH/NIA, RR027093 from NIH/NCRR, EY022774 and EY027005 from NIH/NEI (PK). The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health. Additional support by the Felix and Carmen Sabates Missouri Endowed Chair in Vision Research, the Vision Research Foundation of Kansas City (PK) and a departmental challenge grant by Research to Prevent Blindness (PK, TPJ) is gratefully acknowledged. The authors thank Margaret, Richard and Sara Koulen for generous support and encouragement.

List of Abbreviations

AGE advanced glycation end product

ANOVA	analysis of variance
CS	contrast sensitivity
DR	diabetic retinopathy
ERG	electroretinography
HDL	high-density lipoprotein
IP	intraperitoneally
LDL	low-density lipoprotein
NPDR	non-proliferative diabetic retinopathy
ox-LDL	oxidatively-modified low-density lipoprotein
P-407	Poloxamer 407
PDR	proliferative diabetic retinopathy
SD	standard deviation
SQ	subcutaneously
T1DM	type 1 diabetes mellitus
T2DM	type 2 diabetes mellitus
TC	total cholesterol
TG	true triglyceride
VA	visual acuity
VLDL	very-low-density lipoprotein

References

1. Bolinger MT, Antonetti DA. Moving Past Anti-VEGF: Novel Therapies for Treating Diabetic Retinopathy. *Int J Mol Sci*. 2016;17.
2. Cai X, McGinnis JF. Diabetic Retinopathy: Animal Models, Therapies, and Perspectives. *J Diabetes Res*. 2016; 2016:3789217. [PubMed: 26881246]
3. Centers for Disease C, Prevention. Blindness caused by diabetes--Massachusetts, 1987–1994. *MMWR Morb Mortal Wkly Rep*. 1996; 45:937–941. [PubMed: 8927019]
4. Javitt JC, Aiello LP, Chiang Y, Ferris FL 3rd, Canner JK, Greenfield S. Preventive eye care in people with diabetes is cost-saving to the federal government. Implications for health-care reform. *Diabetes Care*. 1994; 17:909–917. [PubMed: 7956643]
5. Boyle JP, Thompson TJ, Gregg EW, Barker LE, Williamson DF. Projection of the year 2050 burden of diabetes in the US adult population: dynamic modeling of incidence, mortality, and prediabetes prevalence. *Popul Health Metr*. 2010; 8:29. [PubMed: 20969750]
6. Antonetti DA, Klein R, Gardner TW. Diabetic retinopathy. *N Engl J Med*. 2012; 366:1227–1239. [PubMed: 22455417]

7. Williams R, Airey M, Baxter H, Forrester J, Kennedy-Martin T, Girach A. Epidemiology of diabetic retinopathy and macular oedema: a systematic review. *Eye (Lond)*. 2004; 18:963–983. [PubMed: 15232600]
8. Lai CM, Dunlop SA, May LA, et al. Generation of transgenic mice with mild and severe retinal neovascularisation. *Br J Ophthalmol*. 2005; 89:911–916. [PubMed: 15965177]
9. Cheung N, Mitchell P, Wong TY. Diabetic retinopathy. *Lancet*. 2010; 376:124–136. [PubMed: 20580421]
10. Fong DS, Aiello L, Gardner TW, et al. Retinopathy in diabetes. *Diabetes Care*. 2004; 27(Suppl 1):S84–87. [PubMed: 14693935]
11. Aiello LP, Gardner TW, King GL, et al. Diabetic retinopathy. *Diabetes Care*. 1998; 21:143–156. [PubMed: 9538986]
12. Johnston TP, Baker JC, Hall D, Jamal S, Palmer WK, Emeson EE. Regression of poloxamer 407-induced atherosclerotic lesions in C57BL/6 mice using atorvastatin. *Atherosclerosis*. 2000; 149:303–313. [PubMed: 10729380]
13. Johnston TP, Korolenko TA, Sahebkar A. The P-407-Induced Mouse Model of Dose-Controlled Hyperlipidemia and Atherosclerosis: Twenty-five Years Later. *J Cardiovasc Pharmacol*. 2017
14. Palmer WK, Emeson EE, Johnston TP. Poloxamer 407-induced atherogenesis in the C57BL/6 mouse. *Atherosclerosis*. 1998; 136:115–123. [PubMed: 9544738]
15. Johnston TP, Coker JW, Paigen BJ, Tawfik O. Sex does not seem to influence the formation of aortic lesions in the P-407-induced mouse model of hyperlipidemia and atherosclerosis. *J Cardiovasc Pharmacol*. 2002; 39:404–411. [PubMed: 11862120]
16. Johnston TP, Waxman DJ. Circulating free fatty acids are increased independently of PPARgamma activity after administration of poloxamer 407 to mice. *Can J Physiol Pharmacol*. 2008; 86:643–649. [PubMed: 18758514]
17. Verges B. Pathophysiology of diabetic dyslipidaemia: where are we? *Diabetologia*. 2015; 58:886–899. [PubMed: 25725623]
18. Gilinsky MA, Johnston TP, Zhukova NA, et al. Methylated arginine analogues: their potential role in atherosclerosis and cognition using the poloxamer-407-induced mouse model of dyslipidemia. *Can J Physiol Pharmacol*. 2016:1–10.
19. de Souza Bastos A, Graves DT, de Melo Loureiro AP, et al. Diabetes and increased lipid peroxidation are associated with systemic inflammation even in well-controlled patients. *J Diabetes Complications*. 2016; 30:1593–1599. [PubMed: 27497685]
20. Mancino R, Di Pierro D, Varesi C, et al. Lipid peroxidation and total antioxidant capacity in vitreous, aqueous humor, and blood samples from patients with diabetic retinopathy. *Mol Vis*. 2011; 17:1298–1304. [PubMed: 21633716]
21. Singh VP, Bali A, Singh N, Jaggi AS. Advanced glycation end products and diabetic complications. *Korean J Physiol Pharmacol*. 2014; 18:1–14. [PubMed: 24634591]
22. National Cholesterol Education Program Expert Panel on Detection E, Treatment of High Blood Cholesterol in A. Third Report of the National Cholesterol Education Program (NCEP) Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults (Adult Treatment Panel III) final report. *Circulation*. 2002; 106:3143–3421. [PubMed: 12485966]
23. Kaur J. A comprehensive review on metabolic syndrome. *Cardiol Res Pract*. 2014; 2014:943162. [PubMed: 24711954]
24. Burroughs SL, Kaja S, Koulen P. Quantification of deficits in spatial visual function of mouse models for glaucoma. *Invest Ophthalmol Vis Sci*. 2011; 52:3654–3659. [PubMed: 21330670]
25. Wout ZG, Pec EA, Maggiore JA, Williams RH, Palicharla P, Johnston TP. Poloxamer 407-mediated changes in plasma cholesterol and triglycerides following intraperitoneal injection to rats. *J Parenter Sci Technol*. 1992; 46:192–200. [PubMed: 1474430]
26. Fu V, Scanga HL, Medsinge A, Nischal KK. Improved visual function with dietary intervention in a child with lipemia retinalis. *J AAPOS*. 2014; 18:488–490. [PubMed: 25439305]
27. Nagra PK, Ho AC, Dugan JD Jr. Lipemia retinalis associated with branch retinal vein occlusion. *Am J Ophthalmol*. 2003; 135:539–542. [PubMed: 12654375]

28. Rymarz E, Matysik-Wozniak A, Baltaziak L, Prystupa A, Sak J, Grzybowski A. Lipemia retinalis - an unusual cause of visual acuity deterioration. *Med Sci Monit.* 2012; 18:CS72–75. [PubMed: 22847206]
29. Zhang B, Qiu Q, Yin L, Yao Y, Wang C, Wu X. Measurement of retinal function with flash-electroretinography in Chinese patients with hyperlipidemia. *Graefes Arch Clin Exp Ophthalmol.* 2014; 252:1385–1392. [PubMed: 25030239]
30. Flurkey KM, Curren J, Harrison DE. Chapter 20 - Mouse Models in Aging Research A2 - Fox, James G. In: Davisson MT, Quimby FW, Barthold SW, Newcomer CE, Smith AL, editors *The Mouse in Biomedical Research* (Second Edition) Burlington: Academic Press; 2007 637672
31. Korolenko TA, Johnston TP, Tuzikov FV, et al. Early-stage atherosclerosis in poloxamer 407-induced hyperlipidemic mice: pathological features and changes in the lipid composition of serum lipoprotein fractions and subfractions. *Lipids Health Dis.* 2016; 15:16. [PubMed: 26801626]
32. Jackson GR, Barber AJ. Visual dysfunction associated with diabetic retinopathy. *Curr Diab Rep.* 2010; 10:380–384. [PubMed: 20632133]
33. Li Q, Zemel E, Miller B, Perlman I. Early retinal damage in experimental diabetes: electroretinographical and morphological observations. *Exp Eye Res.* 2002; 74:615–625. [PubMed: 12076083]
34. Tzekov R, Arden GB. The electroretinogram in diabetic retinopathy. *Surv Ophthalmol.* 1999; 44:53–60. [PubMed: 10466588]
35. Hancock HA, Kraft TW. Oscillatory potential analysis and ERGs of normal and diabetic rats. *Invest Ophthalmol Vis Sci.* 2004; 45:1002–1008. [PubMed: 14985323]
36. Kohzaki K, Vingrys AJ, Bui BV. Early inner retinal dysfunction in streptozotocin-induced diabetic rats. *Invest Ophthalmol Vis Sci.* 2008; 49:3595–3604. [PubMed: 18421077]
37. Shinoda K, Rejdak R, Schuettauf F, et al. Early electroretinographic features of streptozotocin-induced diabetic retinopathy. *Clin Exp Ophthalmol.* 2007; 35:847–854. [PubMed: 18173414]
38. Yoshida A, Kojima M, Ogasawara H, Ishiko S. Oscillatory potentials and permeability of the blood-retinal barrier in noninsulin-dependent diabetic patients without retinopathy. *Ophthalmology.* 1991; 98:1266–1271. [PubMed: 1923365]
39. Gaucher D, Chiappore JA, Paques M, et al. Microglial changes occur without neural cell death in diabetic retinopathy. *Vision Res.* 2007; 47:612–623. [PubMed: 17267004]
40. Yasuda T, Johnston TP, Shinohara M, Inoue M, Ishida T. The effect of poloxamer 407 on the functional properties of HDL in mice. *J Pharm Pharmacol.* 2012; 64:677–687. [PubMed: 22471363]
41. Johnston TP, Zhou X. Oxidation of low-density lipoprotein cholesterol following administration of poloxamer 407 to mice results from an indirect effect. *J Cardiovasc Pharmacol.* 2007; 49:246–252. [PubMed: 17438410]
42. Lyons TJ, Li W, Wells-Knecht MC, Jokl R. Toxicity of mildly modified low-density lipoproteins to cultured retinal capillary endothelial cells and pericytes. *Diabetes.* 1994; 43:1090–1095. [PubMed: 8070608]
43. Wu M, Chen Y, Wilson K, et al. Intraretinal leakage and oxidation of LDL in diabetic retinopathy. *Invest Ophthalmol Vis Sci.* 2008; 49:2679–2685. [PubMed: 18362112]
44. Zou MH, Li H, He C, Lin M, Lyons TJ, Xie Z. Tyrosine nitration of prostacyclin synthase is associated with enhanced retinal cell apoptosis in diabetes. *Am J Pathol.* 2011; 179:2835–2844. [PubMed: 22015457]
45. Carlson EC, Audette JL, Swinscoe JC. Ultrastructural evidence for morphological specificity in isolated bovine retinal capillary basement membranes. *J Ultrastruct Mol Struct Res.* 1988; 98:184–198. [PubMed: 3373070]
46. Newman E, Reichenbach A. The Muller cell: a functional element of the retina. *Trends Neurosci.* 1996; 19:307–312. [PubMed: 8843598]
47. Wu M, Yang S, Elliott MH, et al. Oxidative and endoplasmic reticulum stresses mediate apoptosis induced by modified LDL in human retinal Muller cells. *Invest Ophthalmol Vis Sci.* 2012; 53:4595–4604. [PubMed: 22678501]
48. Granit R. The components of the retinal action potential in mammals and their relation to the discharge in the optic nerve. *J Physiol.* 1933; 77:207–239. [PubMed: 16994385]

Figure 1. Experimental timeline

Due to the length of testing procedures, measurement of visual and retinal function was staggered over five days so that one animal from each cohort was tested each day.

Figure 2. Plasma total cholesterol and true triglyceride levels

Intraperitoneally (P-407 IP) administered poloxamer 407 (P-407) results in a sustained and significant elevation of plasma total cholesterol when compared to saline controls (**A**). A rapid-onset, sustained, and significant increase in plasma triglyceride levels is seen with IP delivery of P-407 when compared to saline controls (**B**). When P-407 is delivered subcutaneously (P-407 SQ), the increase in triglyceride levels is delayed but still sustained and significant. Saline controls are symbolized with squares, subcutaneous P-407 with triangles, and intraperitoneal P-407 with inverted triangles. Data are expressed as mean $\pm$ SD. One-way ANOVA with Turkey's post-test was used to determine statistical significance. When compared to saline controls, P values from 0.05 to 0.01 are represented as *, 0.01 to 0.001 as **, and < 0.001 as ***. When comparing P-407 IP to P-407 SQ, P values are represented as above but with a number sign (#).

Figure 3. Visual function

When compared with saline controls, visual acuity (A) and contrast sensitivity (B) are not significantly different in animals with moderately but significantly elevated TG (P-407 IP and P-407 SQ) and TC (P-407 IP). Contrast sensitivity correlates strongly and very significantly with visual acuity (C). Declining visual function correlates weakly but significantly with declining retinal function (D). Saline controls are symbolized with squares, subcutaneous P-407 with triangles, and intraperitoneal P-407 with inverted triangles. Data are expressed as mean $\pm$ SD. One-way ANOVA with Dunnett's post-test was used to determine statistical significance. P values from 0.05 to 0.01 are represented as *, 0.01 to 0.001 as **, and < 0.001 as ***.

Figure 4. Retinal function

Full-field flash electroretinography was used to evaluate rod (A–C), mixed rod and cone (D–F), and cone function (H–J). Representative traces for one eye from each group are provided in C, F, and J respectively. Neither rod amplitudes (A) nor implicit times (B) were significantly altered in dyslipidemic animals. A very significant increase in mixed rod and cone a-wave amplitude (D) was observed for the P-407 IP group when compared to saline controls. The P-407 IP group also exhibited a very significant decrease in b/a wave ratio (G) compared to controls. Cone implicit times (I) for both the a- and b-waves were very significantly delayed in the P-407 IP group. Saline controls are symbolized with squares, subcutaneous P-407 with triangles, and intraperitoneal P-407 with inverted triangles. Data are expressed as mean ± SD. One-way ANOVA with Dunnett's post-test was used to determine statistical significance. P values from 0.05 to 0.01 are represented as *, 0.01 to 0.001 as **, and < 0.001 as ***.

Figure 5. Relationships between plasma lipid levels and visual or retinal function

Total plasma cholesterol levels correlate moderately and significantly with mixed rod and cone a-wave amplitude (A) and b/a-wave ratio (B). True triglyceride levels (TG) have a weak but significant relationship with visual acuity (C). Rod b-wave (D) and mixed rod and cone a wave (E) amplitudes demonstrated moderate and significant correlations with TG levels. A strong and very significant relationship was observed between TG and the b/a wave ratio (F). Statistical values from correlations A–F (G). Saline controls are symbolized with squares, subcutaneous P-407 with triangles, and intraperitoneal P-407 with inverted triangles. Data are expressed as mean $\pm$ SD. One-way ANOVA with Dunnett's post-test was used to determine statistical significance. P values from 0.05 to 0.01 are represented as *, 0.01 to 0.001 as **, and < 0.001 as ***.