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Authors

Liu, Haitao
Tang, Jie
Du, Yunpeng
[et al.](#)

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Photoreceptor Cells Influence Retinal Vascular Degeneration in Mouse Models of Retinal Degeneration and Diabetes

Haitao Liu,¹ Jie Tang,¹ Yunpeng Du,¹ Aicha Saadane,² Deoye Tonade,³ Ivy Samuels,⁴ Alex Veenstra,⁴ Krzysztof Palczewski,³ and Timothy S. Kern¹⁻⁴

¹Department of Medicine, Case Western Reserve University, Cleveland, Ohio, United States

²Department of Ophthalmology and Visual Science, Case Western Reserve University, Cleveland, Ohio, United States

³Department of Pharmacology and Cleveland Center for Membrane and Structural Biology, Case Western Reserve University, Cleveland, Ohio, United States

⁴Veterans Administration Medical Center Research Service 151, Cleveland, Ohio, United States

Correspondence: Timothy S. Kern, W309 Wood Building Case Western Reserve University, 10900 Euclid Avenue, Cleveland, OH 44106, USA; tsk@case.edu.

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PURPOSE. Loss of photoreceptor cells is associated with retinal vascular degeneration in retinitis pigmentosa, whereas the presence of photoreceptor cells is implicated in vascular degeneration in diabetic retinopathy. To investigate how both the absence and presence of photoreceptors could damage the retinal vasculature, we compared two mouse models of photoreceptor degeneration (*opsin*^{-/-} and *Rbo*^{P23H/P23H}) and control C57Bl/5J mice, each with and without diabetes.

METHODS. Retinal thickness, superoxide, expression of inflammatory proteins, ERG and optokinetic responses, leukocyte cytotoxicity, and capillary degeneration were evaluated at 1 to 10 months of age using published methods.

RESULTS. Retinal photoreceptor cells degenerated completely in the opsin mutants by 2 to 4 months of age, and visual function subsided correspondingly. Retinal capillary degeneration was substantial while photoreceptors were still present, but slowed after the photoreceptors degenerated. Diabetes did not further exacerbate capillary degeneration in these models of photoreceptor degeneration, but did cause capillary degeneration in wild-type animals. Photoreceptor cells, however, did not degenerate in wild-type diabetic mice, presumably because the stress responses in these cells were less than in the opsin mutants. Retinal superoxide and leukocyte damage to retinal endothelium contributed to the degeneration of retinal capillaries in diabetes, and leukocyte-mediated damage was increased in both opsin mutants during photoreceptor cell degeneration.

CONCLUSIONS. Photoreceptor cells affect the integrity of the retinal microvasculature. Deterioration of retinal capillaries in opsin mutants was appreciable while photoreceptor cells were present and stressed, but was less after photoreceptors degenerated. This finding proves relevant to diabetes, where persistent stress in photoreceptors likewise contributes to capillary degeneration.

Keywords: retina, photoreceptors, microvasculature, opsin

Normally, the structure and vascularity of the retina are positively correlated to meet the metabolic demands of retinal neurons.^{1,2} The retinal vasculature provides nutrients for the metabolic activity of the inner retina, whereas the choroidal circulation nourishes the photoreceptor cells, the major source of the retina's metabolic demand.

Previous studies have indicated that photoreceptor cell loss in patients³ or in animal models of retinitis pigmentosa⁴⁻⁶ is associated with reduced blood flow or atrophy of the retinal capillary network.⁷ Likewise, photoreceptor cell degeneration in vitamin E deficiency,⁸ in polycystic kidney disease,⁹ and in Abyssinian cats¹⁰ has been associated with damage to the retinal vasculature, leading authors to conclude that the vascular changes were secondary to the photoreceptor cell degenerative disease process. Therapeutics such as safranal^{5,11} or RTL220¹² that inhibited degeneration of the outer layers of

the retina also partially prevented capillary degeneration in those models. This evidence suggests that loss of photoreceptor cells precedes and contributes to the degeneration of the retinal vasculature. Developmentally, the early loss of photoreceptor cells in animal models of retinal degeneration also has been shown to profoundly inhibit development of the retinal vasculature.¹³

In contrast, the loss of photoreceptor cells essentially eliminated the degeneration of retinal capillaries caused by diabetes. De Gooyer and collaborators¹⁴ induced diabetes in opsin-deficient mice, where photoreceptor cells degenerated secondary to the opsin deficiency, and these mice were protected from the expected diabetes-induced reduction in density of the retinal microvasculature compared to nondiabetic controls. These findings were interpreted as indicating that the photoreceptor cells contributed to the development

of diabetic retinopathy. Results of a survey sent to diabetic patients who also had retinitis pigmentosa (and thus photoreceptor cell degeneration)¹⁵ likewise suggested that diabetic retinopathy was less severe in patients who had photoreceptor cell degeneration. Moreover, Ramsey and Arden¹⁶ reported that suppression of the dark-induced opening of rod ion channels inhibited retinal edema in diabetic patients (presumably by decreasing vascular permeability).

Evidence that the absence of photoreceptor cells both contributes to the dysfunction/degeneration of the vasculature under normal conditions and inhibits the diabetes-induced degeneration of retinal capillaries is both unconfirmed and unexplained. In the present study, we compared diabetic and nondiabetic wild-type (WT) control mice with two mouse models of photoreceptor cell degeneration (*opsin*^{-/-} mice and P23H mutant opsin knock-in *Rho*^{P23H/P23H} mice¹⁷) with respect to retinal capillary degeneration, oxidative stress, and inflammatory changes affecting their retinas. The P23H opsin mutation is the most common cause of human autosomal dominant retinitis pigmentosa.^{18,19}

METHODS

Experimental Animals

Male C57Bl/6J mice, opsin-deficient mice, and mice in which the P23H mutation of rhodopsin was knocked in (*Rho*^{P23H/P23H})¹⁷ were randomly assigned to become diabetic or remain nondiabetic. Both mutant strains were on the C57Bl/6 background. The *opsin*^{-/-} mice were from the same line as that studied previously by de Gooyer et al.⁶ Diabetes was induced in 2-month-old mice by five sequential daily intraperitoneal injections of a freshly prepared solution of streptozotocin in citrate buffer (pH 4.5) at 60 mg/kg body weight (bw). Injected animals were regarded as diabetic only after hyperglycemia (>275 mg/dL) was verified at least three times during the second week after treatment with streptozotocin. Insulin (0–0.2 units of neutral protamine Hagedorn (NPH) insulin subcutaneously, zero to three times per week) was given as needed to prevent weight loss without preventing hyperglycemia and glucosuria. Blood glucose and HbA1c (hemoglobin A1c) were measured as reported previously.^{20–22} Streptozotocin-induced diabetes mimics type 1 diabetes, but the study conclusions likely are relevant also to type 2 diabetes, because there is no consistent evidence that photoreceptors or the pathogenesis of diabetic retinopathy differs between type 1 and 2 diabetes. Treatment of animals conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, as well as to institutional guidelines.

Vascular Histopathology

Eyes were removed from anesthetized animals at different ages and durations of diabetes and from age-matched nondiabetic controls and fixed in formalin, and one retina from each animal was isolated, washed in running water overnight, and digested in elastase as previously reported.²² When devoid of neuronal cells, the isolated vasculature was placed on a glass microscope slide, dried overnight, stained with hematoxylin and periodic acid Schiff, dehydrated, and coverslipped. Degenerated (acellular) capillaries were quantitated in up to six to seven field areas corresponding to the midretina (200× magnification) in a masked manner. Acellular capillaries (reported per square millimeter of retinal area) were identified as capillary-sized vessel tubes having no nuclei anywhere along their lengths.

Ultrahigh-Resolution Spectral-Domain Optical Coherence Tomography Imaging

Spectral-domain optical coherence tomography (SD-OCT; BiopTigen, Durham, NC, USA) was used for in vivo imaging of mouse retinas. Mice were anesthetized by intraperitoneal injection of ketamine/xylazine (10 mg/100 g bw + 1 mg/100 g bw). Pupils were dilated with 1% tropicamide. Five pictures acquired in the B-scan mode were used to construct each final averaged image. Thicknesses of the retina and outer nuclear layer (ONL) were measured at distances of 0.15, 0.30, and 0.45 mm from the optic nerve, and the average thickness at 0.45 mm from the disc is reported. For comparison, thicknesses were measured also in formalin-fixed, sucrose-infiltrated cryosections.

Superoxide Generation

Freshly isolated retinas were incubated in 200 μ L Krebs-Hepes buffer (20 mM HEPES in 0.12 mM CaCl₂ 0.08 mM MgSO₄), pH 7.2, with 5 or 25 mM glucose for 5 minutes at 37°C in 5% CO₂. Luminescence indicating the presence of superoxide was measured at 5 minutes after addition of 0.54 mM (final concentration) lucigenin, as published previously.^{22–27} Luminescence intensity is stated in relative units per milligram protein. Superoxide data from opsin-deficient mice at 2 months of diabetes were reported previously,²⁸ and are reanalyzed differently here to emphasize superoxide generation in the experimental groups relative to nondiabetic C57Bl/6J controls.

Immunoblots

Isolated retinas were lysed in protease and phosphatase inhibitors,²⁹ sonicated, and centrifuged, and the supernatants (50–80 μ g) were fractionated by SDS-PAGE and electroblotted onto nitrocellulose membranes; membranes were blocked overnight with Tris-buffered saline containing 0.05% Tween 20 and 5% nonfat milk (4°C). Rabbit antibodies for intercellular adhesion molecule 1 (ICAM-1) (1:2000; Proteintech, Chicago, IL, USA), inducible nitric oxide synthase (iNOS) (1:1000 dilution; Santa Cruz Biotechnology, Santa Cruz, CA, USA), and p-I κ B α and nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor α (I κ B α) (1:200 and 1:1000 dilutions, respectively; Santa Cruz Biotechnology) were applied, followed by rabbit secondary antibody (Sigma-Aldrich Corp., St. Louis, MO, USA) for 1 hour. After washing, immunoreactive bands were visualized by enhanced chemiluminescence. Protein levels were quantified relative to β -actin loading controls (1:5000; Abcam, Inc., Cambridge, MA, USA) in the same samples.

Optokinetic Assessment of Visual Function

The spatial frequency threshold (a marker of visual acuity in rodents) and contrast sensitivity were measured with the Virtual Optokinetic system.^{20,30,31} The maximum spatial frequency that resulted in head tracking was determined as the spatial frequency threshold. Only a single spatial frequency (0.064 cyc/deg) was measured to estimate contrast sensitivity. The experimenter was masked as to the identity of the experimental animals. Electroretinograms were measured as reported previously.³²

Leukocyte-Mediated Damage of Endothelial Cells

With respect to leukocyte-mediated cytotoxicity against retinal endothelial cells,^{21,33–37} transformed retinal endothelial cells³⁸ were grown in control medium Dulbecco's modified Eagle's

TABLE. Glycemia in Wild-Type, *opsin*^{-/-}, and P23H Knock-In Mutant Mice Over 8 Months of Study

Group	Genotype	n	Blood Glucose,		
			Final bw, g	Nonfasting, mg/dL	HbA1c, %
Nondiabetic	WT	13	43 ± 5	138 ± 14	3.1 ± 0.1
Nondiabetic	<i>opsin</i> ^{-/-}	5	47 ± 8	138 ± 14	3.0 ± 0.0
Nondiabetic	P23H	6	44 ± 3	139 ± 19	3.1 ± 0.1
Diabetic	WT	14	27 ± 2	490 ± 40	9.6 ± 0.3
Diabetic	<i>opsin</i> ^{-/-}	7	27 ± 3	484 ± 40	9.3 ± 0.6
Diabetic	P23H	6	27 ± 3	496 ± 66	10.0 ± 0.4

medium (DMEM with 5 mM glucose) containing 10% bovine serum. The serum concentration was reduced to 2% just before cells were placed either in 5 mM glucose or in high glucose (25 mM). Media were changed every other day for 3 days. When cells reached 80% confluence (~300,000 cells), freshly isolated leukocytes from blood (100,000 cells) of animals in different experimental groups were added and incubated for 6 additional hours, after which cells 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 determined by flow cytometry based on 7-AAD staining.³⁹ Endothelial cell death was expressed as the percentage of endothelial cells that stained positively with dye. Approximately 10,000 cells were counted in each sample. Experiments were repeated two times with similar results.

Statistical Analyses

Data are expressed as mean ± SD. All statistical analyses were performed with 2-way ANOVA followed by Fischer's post hoc test. Values of $P < 0.05$ were considered statistically significant.

RESULTS

There were no significant differences with respect to bw or glycemia between nondiabetic members of the mouse strains studied. Glycemia was elevated in all diabetic animals, and the severity of diabetes did not differ among the diabetic groups. Average HbA1c and blood glucose over the entire duration of the 8-month experiment were significantly greater than normal in all diabetic groups ($P < 0.01$) as summarized in the Table.

For comparison to the amount of capillary degeneration in the various models, we measured photoreceptor integrity and visual function. Assessments of these parameters of structure and function were made throughout the study, as summarized in Figure 1.

Photoreceptors Degenerate in Opsin Mutants but Not in Diabetic C57Bl/6 Controls

Optical coherence tomography analysis indicated that the thickness of the retina was reduced by more than half by 2 months of age in both the opsin-deficient nondiabetic mice and the P23H knock-in nondiabetic mice (Fig. 2). Most of this decrease was due to loss of photoreceptor cells, which were largely undetectable by OCT in either of the mutant strains by 4 months of age. Histologically, the P23H knock-in mutants had 1.4 ± 0.2 layers of nuclei in the ONL at 4 months of age compared to 11.7 ± 0.6 layers in age-matched C57Bl/6J controls ($P < 0.001$). (Similar results were obtained for opsin-deficient nondiabetic mice [Fig. 2]). Even at 3 weeks of age, the ONL was only half as thick in P23H knock-in mutant mice (34

Age (mo)	<1	2	3	4	5	6	7	8	9	10
Duration diabetes (mo)		STZ		2		4		6		8
OCT	X		X						X	
OKT				X					X	
ERG									X	
Histology	X									X
Microvessel analysis	X		X							X
Superoxide				X						X
Immunoblots				X						X
Co-culture				X						X

FIGURE 1. Summary of tests performed throughout the experiment.

± 1 μm) as in normal mice (59 ± 1 μm), indicating that photoreceptor cell degeneration began soon after birth.

Diabetes 8 months in duration had no effect on either retinal or ONL thickness in C57Bl/6J mice (Fig. 2), indicating that diabetes itself is not a major cause of photoreceptor cell degeneration.

Visual Function Is Lost in Opsin Mutants and Is Impaired in Diabetic C57Bl/6 Mice

Both spatial frequency threshold and contrast sensitivity are psychophysical measures that assess the function of retinal and central visual pathways. By 4 months of age, nondiabetic mice from the opsin knockout or P23H knock-in strains showed essentially no evidence of visual function, consistent with the observed retinal degeneration. Diabetes 2 months in duration (4 months age) in WT C57Bl/6J mice caused a significant decrease in both parameters of visual function compared to nondiabetic controls (Fig. 3; both $P < 0.05$), despite no photoreceptor degeneration. As a confirmatory test, ERGs were examined in WT and P23H mutant groups at 9 to 10 months of age (7–8 months of diabetes). The mutant nondiabetic and diabetic mice showed essentially no b-wave activity compared to WT controls, and scotopic and photopic b-wave were not significantly different between diabetic and nondiabetic WT controls, again having no photoreceptor degeneration (Fig. 3).

Retinal Capillaries Degenerate in Opsin Mutants, Especially Before Photoreceptor Degeneration

Nondiabetic C57Bl/6J mice had only a few degenerated capillaries at 10 months of age, whereas nondiabetic mice either deficient in opsin or expressing the P23H mutant opsin exhibited extensive degeneration of the retinal vasculature (Fig. 4; both $P < 0.001$). The vasodegenerative process began before appreciable photoreceptor cell loss, because nondiabetic P23H mutants that were 3 weeks old (when photoreceptor cells were still present) had almost twice as many degenerate capillaries as found in comparably aged nondiabetic WT C57Bl/6J mice (16 ± 2 degenerated capillaries/mm² retina in WT controls versus 29 ± 2 in P23H knock-in mice; $P < 0.05$). Evidence in Figure 4c demonstrates that degeneration of the retinal vasculature in opsin mutant mice was initiated before photoreceptors had degenerated, and that the capillary degeneration slowed down after approximately 3 months of age in these mutants (after the photoreceptors had largely degenerated).

This association between vascular degeneration and photoreceptor health has relevance for diabetes. Diabetes is

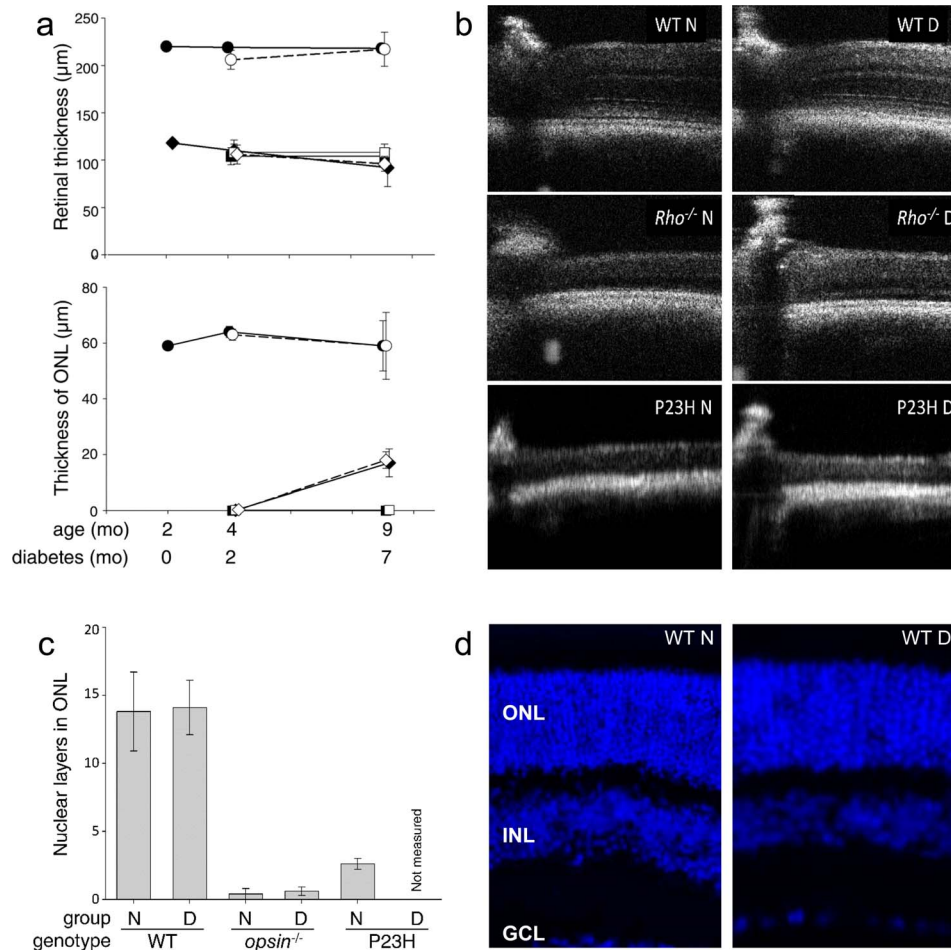


FIGURE 2. Essentially all photoreceptors in *opsin*^{-/-} and P23H knock-in mice degenerated by 2 to 4 months of age, whereas diabetes did not cause photoreceptor loss in wild-type (WT) mice for at least 10 months. (a) Graphical summary of OCT data at 2, 4, and 9 months of age in all groups. Circle: C57Bl/6j; square: *opsin*^{-/-}; diamond: *Rho*^{P23H/P23H}. Filled symbols: nondiabetic; hollow symbols: diabetic. (b) OCT images of representative animals at 9 months of age (7 months of diabetes). (c) Histologic quantitation of number of layers of nuclei in the ONL confirms no loss of photoreceptors in WT (C57Bl/6j) mice diabetic for 8 months (10 months of age) compared to nondiabetic controls, but shows extensive loss of photoreceptors in *opsin*^{-/-} and *Rho*^{P23H/P23H} mutants. (d) Immunofluorescence images of retinas from nondiabetic (left) and diabetic (right) C57Bl/6j mice, again showing no loss of photoreceptors in C57Bl/6j mice diabetic for 8 months (10 months of age). Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI). N, nondiabetic; D, diabetic. *n* = 4 to 7 per group.

recognized to increase degeneration of the retinal vasculature, and diabetes 8 months in duration (10 months of age) in the present study almost doubled the number of degenerated capillaries in WT C57Bl/6j mice compared to age-matched nondiabetic controls (Fig. 4; $P < 0.05$). In contrast, retinal capillaries from mice lacking photoreceptors (*opsin*-deficient mice and P23H knock-in mice) made diabetic for 8 months had the same amount of capillary degeneration as their age-matched nondiabetic controls. Since most of the photoreceptors had already degenerated by the time diabetes was induced in these mice (2 months of age; Fig. 2), the data are consistent with the premise that disease-stressed photoreceptors are involved in the degeneration of retinal capillaries that is critical in the development and progression of diabetic retinopathy.

Potential Contributions of Oxidative Stress, Inflammation, and Leukocytes in the Observed Degeneration of Retinal Capillaries

In light of the vaso-obliteration that developed in diabetic C57Bl/6j mice and *opsin*-mutant mice, we investigated potential causes of the capillary degeneration, focusing on

several molecular abnormalities that previously had been implicated in vaso-obliteration in diabetic retinopathy.

Oxidative Stress. Superoxide generation by retinas from nondiabetic mice having the P23H mutant gene knocked in showed greater than normal generation of superoxide at 2, 4, and 10 months of age compared to nondiabetic C57Bl/6j mice (Fig. 5). In contrast, superoxide generation by retinas from nondiabetic *opsin*^{-/-} mice was not significantly greater than in control WT C57Bl/6j mice at 2 or 4 months of age, but was significant at 10 months of age (Fig. 5).

Diabetes significantly increased retinal superoxide generation in WT C57Bl/6j mice at both 2 and 8 months of diabetes (4 and 10 months of age; Fig. 5). *Opsin*-deficient diabetics were protected from the diabetes-induced increase in retinal superoxide at both durations of diabetes (Fig. 5). Diabetes significantly increased superoxide generation in P23H knock-in mice at both durations of diabetes. Thus, increased retinal production of superoxide is associated with the degeneration of retinal capillaries in the P23H mutant model, but not in *opsin* deficiency.

Inflammation. Inflammatory conditions commonly are characterized by leukocyte activation, as well as the induction

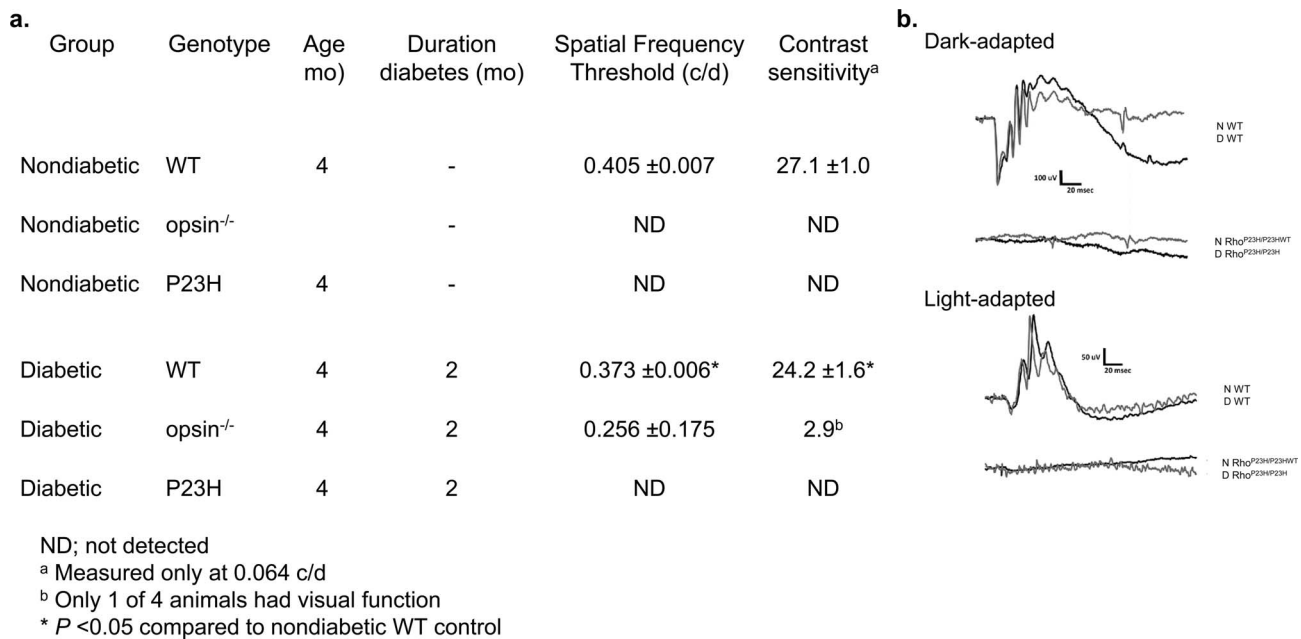


FIGURE 3. Impairment of visual function in nondiabetic and diabetic C57Bl/6J mice, *opsin*^{-/-} mice, and P23H mutant mice compared to C57Bl/6J control mice at 9 months of age (7 months of diabetes). **(a)** Spatial frequency threshold and contrast sensitivity were significantly reduced in diabetic C57Bl/6J mice compared to nondiabetic controls, whereas responses in both opsin mutant groups were largely absent. **(b)** ERG responses were partially (but not significantly) reduced in diabetic C57Bl/6J mice compared to nondiabetic controls, whereas responses in *Rho*^{P23H/P23H} mutants were largely absent. *Opsin*^{-/-} mice were not tested. The representative waveforms for both the dark- and light-adapted responses are in response to a 1.4 log cd s/m² stimulus. $n = 5$ to 7 per group.

of proinflammatory proteins. Thus we studied both of these alterations in nondiabetic and diabetic opsin mutants and C57Bl/6J controls.

We previously implicated leukocytes in the degeneration of retinal capillaries from diabetic mice *in vivo*,^{33,34,36,37} and provided evidence that impaired metabolism or degeneration of the photoreceptor cells/RPE layer could initiate leukocyte-mediated cytotoxicity against endothelial cells.⁴⁰ In the present study, we examined leukocyte-mediated cytotoxicity against retinal endothelial cells and its relationship with the presence or absence of photoreceptor cells. Compared with leukocytes from nondiabetic C57Bl/6J mice, leukocytes from nondiabetic opsin-deficient mice and P23H knock-in mutants killed significantly more endothelial cells at 4 months of age (both $P < 0.05$), but not at 2 or 10 months of age (Fig. 6).

In diabetic mice, leukocytes from WT C57Bl/6J mice showed a significant increase in endothelial cell cytotoxicity compared to nondiabetic C57Bl/6J mice at both durations of disease studied (2 and 8 months of diabetes) (Fig. 6; $P < 0.05$ and < 0.0001 , respectively). Leukocytes from opsin-deficient mice and P23H mutant mice diabetic for 2 months also displayed significant cytotoxicity against endothelial cells. At 8 months of diabetes, however, leukocytes from opsin-deficient diabetics and P23H knock-in diabetics were less cytotoxic ($P < 0.05$ and < 0.0001 , respectively) than cells from WT diabetics. We conclude that leukocyte-mediated cytotoxicity against endothelial cells diminished in both mutants after the photoreceptor cells had degenerated, but did not diminish in WT diabetics (in which photoreceptors remained intact).

Diabetes significantly increased the retinal expression of inflammatory proteins at all durations of study in C57Bl/6J mice (Fig. 7), but had no similar effect in either of the opsin mutants (at the times studied before or after photoreceptor degeneration). Levels of proinflammatory ICAM1 and iNOS in the mutants remained comparable to or less than those in nondiabetic C57Bl/6 controls at 2, 4, and 10 months of age,

except for a significant increase in ICAM in P23H mutants at 2 months of diabetes. To estimate NF- κ B (nuclear factor kappa-light-chain-enhancer of activated B cells) activation, we also evaluated the ratio of phosphorylated to total I κ B α . In nondiabetic or diabetic opsin mutants lacking photoreceptor cells, not only were these inflammation markers generally not increased at the times evaluated, but most were significantly decreased compared to the C57Bl/6J controls. Both opsin mutants differed from C57Bl/6J controls in that neither diabetes nor photoreceptor degeneration caused a local inflammatory response in their retinas at the times evaluated. Thus, inflammatory proteins seem unlikely to contribute to the capillary degeneration that developed in retinas of the opsin mutants, although we have not excluded the possibility that inflammatory changes were present transiently just after the onset of diabetes.

DISCUSSION

The present study was conducted to investigate how the absence of photoreceptors could cause degeneration of the retinal vasculature (in retinal degeneration models) while also inhibiting the vascular degeneration in diabetic retinopathy. We found that degeneration of retinal capillaries is extensive as a result of deletion or mutation of opsin (a gene expressed only in photoreceptor cells), clearly showing that photoreceptor cells can initiate retinal capillary degeneration. Moreover, our data show that the rate of degeneration of the capillaries diminished in P23H mutants after the photoreceptors degenerated. Thus, a significant new conclusion of the present study is that the vaso-obliteration that occurs in *opsin*^{-/-} and P23H knock-in models of photoreceptor degeneration is not caused by the absence of the photoreceptor cells, but instead is initiated by “debilitated” photoreceptors in the interval before those photoreceptors degenerate. Evidence in support of this conclusion is the appreciable degeneration of retinal capillaries

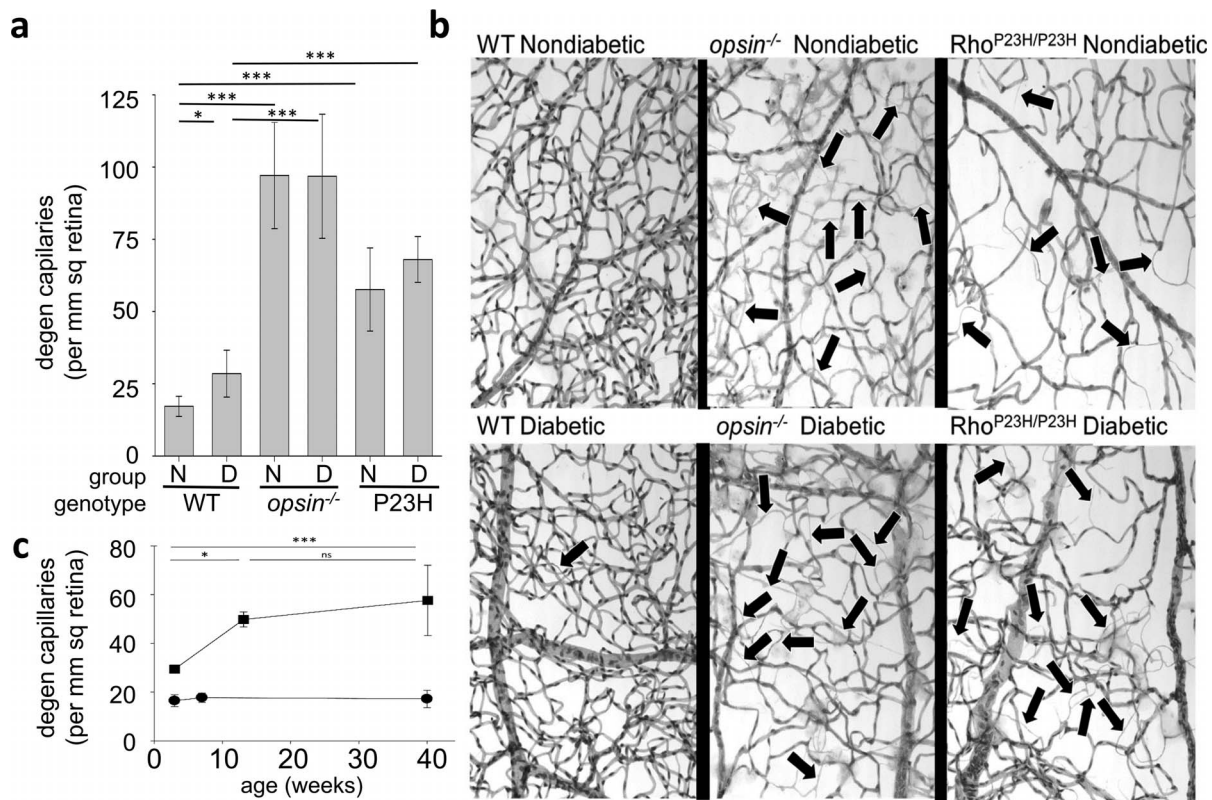


FIGURE 4. Effect of diabetes and opsin mutations on the retinal vasculature. (a) A graphical summary of the number of degenerate (degen) capillaries in retinas from nondiabetic and diabetic C57Bl/6J (WT), *opsin*^{-/-}, and *Rho*^{P23H/P23H} (P23H) mice at 10 months of age (8 months of diabetes; *n* = 5–14 in all groups). (b) Representative photomicrographs demonstrating the capillary degeneration at the same age. Degenerated retinal capillaries (acellular capillaries; illustrated by *black arrows*) are significantly more numerous in WT diabetic mice compared to WT nondiabetic mice, but the capillary degeneration is substantially exacerbated in nondiabetic or diabetic mice deficient in opsin or expressing the P23H mutant opsin. Unlike what was seen in WT mice, diabetes did not exacerbate retinal capillary degeneration in *opsin*^{-/-} and P23H mice compared to their appropriate nondiabetic controls. (c) Degeneration of retinal capillaries is accelerated during the first 3 months of life (while photoreceptors are present but degenerating) in nondiabetic P23H mice (*squares*) compared to nondiabetic WT controls (*circles*). Vascular degeneration slows after photoreceptors have degenerated (after 3–4 months); *n* = 3 to 14 in all groups. Vascular histopathology was quantitated microscopically following isolation of the vasculature by the elastase digestion method. *Horizontal lines* indicate statistically significant differences between groups; *absence of a bar* indicates no statistically significant difference. N, nondiabetic; D, diabetic. **P* < 0.05; ****P* < 0.001. Mean ± SD.

by only 3 weeks of age in P23H knock-in mice, when photoreceptor cells are still largely present.

The observed vascular pathology detected by us in these opsin mutants reflects damage to existing, mature capillaries, because immature capillaries that lack a complete basement membrane would not have been preserved (or detected) by our proteolytic vascular isolation technique. Moreover, retinas from mice deficient in opsin or having mutant opsin develop normally until around postnatal day 14 (P14), (when opsin begins to be expressed), and then degenerate over the next period of weeks.⁴¹ Since development of the retinal vasculature is largely completed by this time (the deep plexus reaches the retinal periphery at approximately P12, followed by the intermediate plexus between P12 and P15⁴²), altered vessel development seems unlikely to be a major cause of the vascular pathology addressed in the current study. Nevertheless, the observed paucity of capillaries in the retinas from the mutant mice suggests that capillary formation or remodeling in our opsin mutants also might have been impaired.

Photoreceptor-mediated degeneration of the retinal vasculature has implications for diabetic retinopathy, because diabetes likewise causes stress in the photoreceptor cells.^{6,14,28} The evidence suggests that diabetes causes a chronic stress within photoreceptor cells that may be similar to the stress that develops in photoreceptors of nondiabetic opsin mutants

(before photoreceptors of opsin mutants degenerate), and that this stress in photoreceptors directly or indirectly contributes to the degeneration of retinal capillaries. Apparently, the photoreceptor stress induced by diabetes in WT mice is less severe than that in opsin mutants, because little or no photoreceptor degeneration occurred in diabetic WT mice (present data and Refs. 28, 43), and the retinal capillary degeneration proceeds at a much slower pace in WT diabetics than in the opsin mutants.

De Gooyer et al.^{6,14} previously reported that retinal capillaries from animals deficient in photoreceptor cells were protected from diabetes-induced capillary degeneration. Our present results with diabetic opsin-deficient and P23H knock-in mice confirm this observation, thus clearly showing the important role of photoreceptor cells in the diabetes-induced degeneration of retinal microvessels.

A previous survey of diabetic patients having retinitis pigmentosa (and thus photoreceptor degeneration) reported that they had less diabetic retinopathy compared to normal diabetic patients.¹⁵ The finding that these diabetics were protected from development of diabetic retinopathy was interpreted as being secondary to the photoreceptor degeneration, but results of the present report suggest that diabetic retinopathy would be inhibited only in those patients with retinitis pigmentosa who had appreciable photoreceptor

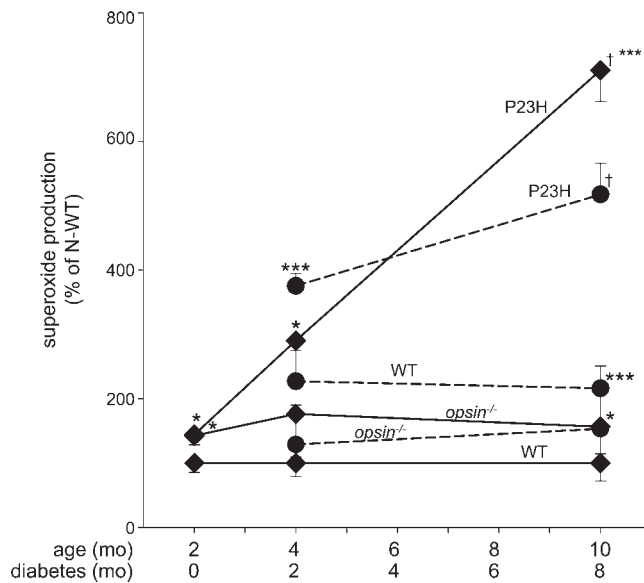


FIGURE 5. Effect of opsin deletion and P23H mutant knock-in on retinal generation of superoxide in mice at 2, 4, and 10 months of age (0, 2, or 8 months of diabetes, respectively). Data are expressed relative to age-matched nondiabetic WT (C57Bl/6J) mice. Data used to calculate superoxide values by the retinas of opsin-deficient mice nondiabetic and diabetic for 2 months (4 months of age) were reported previously,²⁸ but are regraphed here to show the results relative to age-matched nondiabetic C57Bl/6J mice. Nondiabetic mice, *solid lines with squares*; diabetic mice, *dashed lines with circles*. $n = 3$ to 10 in all groups. * $P < 0.05$ or *** $P < 0.001$ compared to N C57Bl/6J controls; † $P < 0.05$ or ††† $P < 0.001$ compared to D C57Bl/6J controls. N, nondiabetic; D, diabetic.

degeneration before diabetes developed. On the other hand, some diabetics having retinitis pigmentosa might have more retinopathy and vaso-obliteration if their photoreceptors were stressed but degenerating only slowly. In contrast to our interpretations, others^{44,45} have interpreted the colocalization of local photoreceptor loss with areas of capillary degeneration in diabetic patients as indicating that diabetes-induced defects in the retinal vasculature caused local photoreceptor degeneration. Integration of these seemingly opposite mechanisms will require further study.

How might photoreceptors cause (directly or indirectly) degeneration of retinal capillaries? We have generated evidence that photoreceptor sheets incubated under diabetes-like conditions release soluble factors that can activate nearby endothelial cells and leukocytes to generate cytokines such as $\text{TNF}\alpha$ ⁴⁶ suggesting that stressed photoreceptors can release factors that affect the microvasculature. Alternatively, the reduced metabolic need of the retina secondary to photoreceptor loss might result in the loss of a different signaling molecule. For example, the loss of photoreceptor cells reduces retinal O_2 consumption, thus increasing pO_2 in the remaining retina, but whether this suffices to contribute to alter relevant signaling molecules and cause the degeneration of retinal capillaries is not yet known.

Oxidative stress has been implicated in the development of tissue pathology, including diabetic retinopathy.⁴⁷ Consistent with this, we found that retinas from nondiabetic and diabetic P23H knock-in mice generated significantly more superoxide compared to C57Bl/6J controls at all ages tested, and retinas from these mice continued to generate superoxide even after photoreceptor cells were absent (perhaps indicating that the knock-in of the mutant opsin indirectly caused oxidative stress in other retinal cell types as well). Retinas from nondiabetic

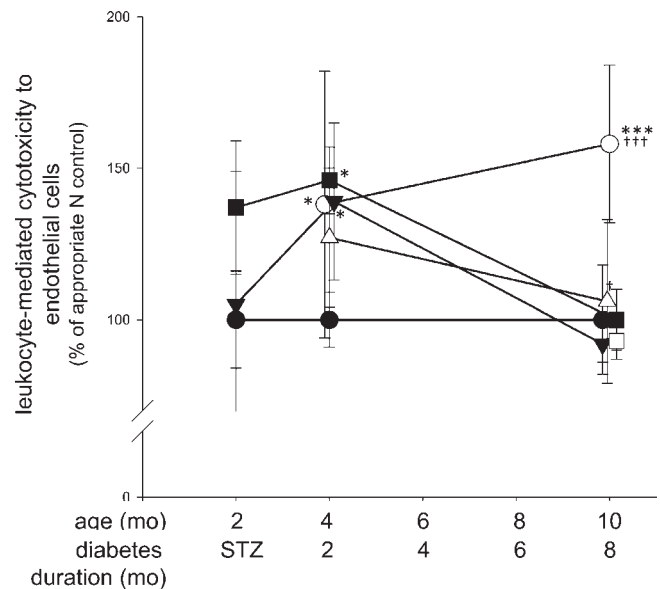


FIGURE 6. Leukocytes isolated from diabetic mice (diabetic 2 or 8 months) caused more cytotoxicity to retinal endothelial cells than did leukocytes from age-matched nondiabetic mice. Leukocytes isolated from mice deficient in opsin or containing the P23H mutation caused cytotoxicity to endothelial cells while photoreceptors were present and degenerating (age 2 and 4 months), but not after the photoreceptors were absent (10 months). *Circles: C57Bl/6J; triangles: opsin^{-/-}; squares: P23H.* Nondiabetic, *solid symbols*; diabetic, *hollow symbols*. Symbols have been offset slightly to allow visualization of data. * $P < 0.05$ or *** $P < 0.001$ compared to N C57Bl/6J controls; † $P < 0.05$ or ††† $P < 0.001$ compared to D C57Bl/6J controls. $n = 3$ to 5 in all groups. N, nondiabetic; D, diabetic.

opsin-deficient mice did not generate significantly elevated levels of superoxide until 10 months of age, whereas diabetic *opsin^{-/-}* mice never showed any significant increase. This difference between the two opsin mutant strains suggests that increased superoxide generation seems not to explain the capillary degeneration in these mutant strains.

Inflammatory changes also have been implicated in the development of vascular lesions of diabetic retinopathy, notably secondary to the interaction of leukocytes with the vascular endothelium. We previously implicated leukocytes in the diabetes-induced degeneration of retinal capillaries *in vivo*,^{33,34,36,37} and demonstrated that leukocytes from diabetic patients³⁴ or animals³⁷ (as in the present study also) were more toxic to retinal endothelial cells than were leukocytes from nondiabetics. Thus, the observed activation of circulating leukocytes by photoreceptor stress or degeneration could contribute to vascular degeneration. In the present study, deletion or mutation of a single protein (opsin) in photoreceptor cells led to activation of circulating leukocytes, leading to excessive killing of retinal endothelial cells at 4 months of age but not at 10 months of age, indicating that leukocyte activation persisted in those opsin mutants until the photoreceptor cells had degenerated. The stimulus for the leukocyte-mediated cytotoxicity diminished thereafter. Ongoing studies are beginning to identify soluble factors released from stressed photoreceptor cells that can activate leukocytes and other cell types.⁴⁶ Thus, induction of leukocyte-mediated cytotoxicity against retinal endothelial cells might have contributed to the observed capillary degeneration in both of these opsin mutants and in the WT diabetic mice.

Proinflammatory signaling pathways can have important effects on the vasculature,^{48,49} so we examined also the association between the expression of inflammatory molecules

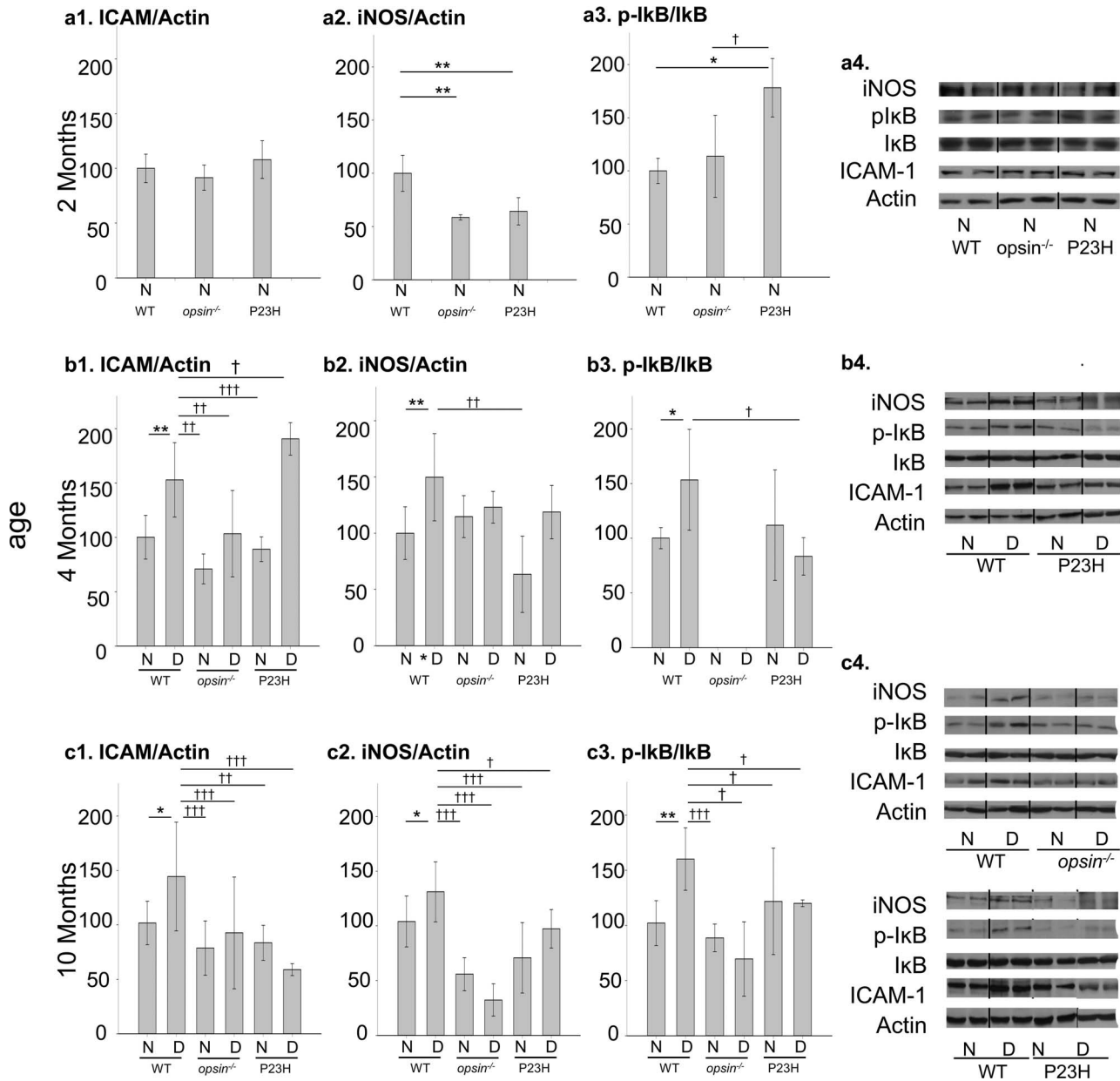


FIGURE 7. Effect of opsin deletion and the knock-in of P23H mutant opsin on retinal expression of proinflammatory proteins in mice. Data summarize expression of ICAM, iNOS, and the ratio of phosphorylated IκBα to total IκBα at 2 (**a1–4**), 4 (**b1–4**), and 10 (**c1–4**) months of age (0, 2, and 8 months of diabetes). Data at 2 months of age do not include diabetic animals because diabetes was not induced until 2 months of age. Data used to calculate expression of ICAM1 and iNOS by the retinas of *opsin*^{-/-} mice nondiabetic and diabetic for 2 months (4 months of age) were published previously²⁸ but are regraphed here to show the expression patterns relative to the WT nondiabetic C57Bl/6J group. Figures **a4**, **b4**, and **c4** show representative immunoblots. Areas where the membranes were cut to remove omitted lanes are indicated by vertical lines. Inflammatory proteins were quantitated by immunoblots of retinal homogenates, and expressed relative to actin in the same sample. *n* = 3 to 7 in all groups. **P* < 0.05 or ***P* < 0.01 or ****P* < 0.001 compared to N C57Bl/6J controls; †*P* < 0.05 or ††*P* < 0.01 or †††*P* < 0.001 compared to D C57Bl/6J controls. N, nondiabetic; D, diabetic.

and the capillary degeneration observed in diabetes and in the opsin mutants. Unlike what is seen in diabetes,^{21,34,36,37,47,49–52} markers of inflammation showed no consistent increase in either model of photoreceptor cell degeneration at the durations of diabetes studied, thus providing no evidence that they play a role in the retinal capillary degeneration manifested in these models.

In summary, findings presented here suggest that molecular abnormalities within stressed photoreceptor cells play a major role in the deterioration of the retinal vasculature in the opsin mutant mice, and that the capillary degeneration greatly

slows if those photoreceptors have degenerated. Diabetes did not exacerbate the capillary degeneration in the retinal degeneration models studied, likely because the photoreceptor cells had largely degenerated by the time diabetes was experimentally induced in these studies. The data are consistent with photoreceptor cells playing a role in the capillary degeneration that occurs in diabetic retinopathy in WT animals, however. The persistence of affected photoreceptor cells in WT diabetic animals results in long-term exposure of the retinal microvasculature to an abnormal local milieu that is generated or regulated by photoreceptor cells.

Factors released from these stressed photoreceptors still need to be identified, but the data are consistent with the postulate that preserving normal photoreceptor cell metabolism could be a novel strategy to inhibit the microvascular lesions of diabetic retinopathy.

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