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Article

Rodent Hyperglycemia-Induced Inner Retinal Deficits are Mirrored in Human Diabetes

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Purpose: To evaluate the utility of low luminance stimuli to functionally probe inner retinal rod pathways in the context of diabetes mellitus in both rat and human subjects.

Methods: Inner retinal dysfunction was assessed using oscillatory potential (OP) delays in diabetic rats. Scotopic electroretinograms (ERGs) in response to a series of increasing flash luminances were recorded from streptozotocin (STZ)-treated and control Sprague-Dawley rats after 7, 14, 20, and 29 weeks of hyperglycemia. We then evaluated OP delays in human diabetic subjects with (DR) and without (DM) diabetic retinopathy using the International Society for Clinical Electrophysiology in Vision (ISCEV) standard scotopic protocol and two additional dim test flashes.

Results: Beginning 7 weeks after STZ, OP implicit times in diabetic rats were progressively delayed in response to dim, but not bright stimuli. In many diabetic subjects the standard ISCEV dim flash failed to illicit measureable OPs. However, OPs became measurable using a brighter, nonstandard dim flash (Test Flash 1, −1.43 log cd s/m^2), and exhibited prolonged implicit times in the DM group compared with control subjects (CTRL).

Conclusions: Delays in scotopic OP implicit times are an early response to hyperglycemia in diabetic rats. A similar, inner retinal, rod-driven response was detected in diabetic human subjects without diabetic retinopathy, only when a nonstandard ISCEV flash intensity was employed during ERG testing.

Translational Relevance: The addition of a dim stimulus to standard ISCEV flashes with assessment of OP latency during ERG testing may provide a detection method for early retinal dysfunction in diabetic patients.

Introduction

Diabetic retinopathy is the leading cause of blindness in the United States among people between 20 and 74 years of age.[1] More than 80% of people with diabetes for over 15 years have some degree of diabetic retinopathy,[2] making it one of the most common complications of diabetes mellitus. Worldwide, by 2030 over 360 million people will develop diabetes,[3] effectively doubling the incidence of diabetic retinopathy associated vision loss.[4]

Despite its devastating impact, the pathophysiology of diabetic retinopathy remains poorly understood. The presence and extent of vascular malformations and permeability changes provide diagnostic and staging criteria, allowing division of both nonproliferative and proliferative diabetic retinopathy into mild, moderate, and severe disease.[5–8] Hyperglycemia typically takes years to produce visually evident vascular changes.[9] However, it is increasingly recognized that hyperglycemia also affects most nonvascular retinal cell types, including amacrine cells, Müller cells, photoreceptors, and ganglion cells, possibly before the appearance of

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clinically-relevant vascular lesions.[10,11] Consequently, the process of retinopathy may begin long before clinical diagnosis.

Nonvascular retinal manifestations of hyperglycemia have been observed using electroretinography (ERG), a tracing of the electrical retinal response to a flash of light. Each portion of the ERG waveform is generated by specific retinal cell types. The first negative wave (a-wave) is generated by photoreceptors,[12] the large positive b-wave reflects activity of depolarizing bipolar cells,[13–18] and the oscillatory potentials (OPs, high frequency wavelets on the rising phase of the b-wave) are generated by amacrine cells.[19] Thus, the ERG provides a noninvasive method to detect functional changes in retinas of diabetic patients. As early as the 1960s, Yonemura et al.[20] reported delayed OP latency in subjects with diabetes (see Ref. 21 for review). Since then numerous studies have reported an array of ERG changes in diabetic subjects, including delayed OPs,[22–26] delayed 30-Hz flicker implicit time,[25,27] decreased a- and b-wave amplitudes,[28,29] and delayed a- and b-wave implicit times.[25,30–33] Similar changes have been reported in diabetic animal models, including delayed OPs,[34–36] decreased a- and b-wave amplitudes,[34,37–41] and delays in a- and/or b-wave implicit times.[35,41,42] However, reported ERG changes in both humans and animals have been inconsistent across studies, likely due to different techniques, time-points measured, and diabetic animal models. Even ERG studies within apparently uniform model systems, such as streptozotocin (STZ)-induced diabetes, can result in variable phenotypes due to different STZ dosing and the divergent administration of insulin replacement.[43] In STZ-treated animals, delayed OP implicit times in response to bright-flash stimuli under dark-adapted conditions are the most consistently altered ERG parameters.[44–48] Indeed, bright stimuli are recommended by the International Society for Clinical Electrophysiology in Vision (ISCEV) for recording and assessing OPs in the course of clinical ERG testing.[49] However, studies in both animals and humans suggest that rod pathways probed with dim stimuli may more reliably detect early inner retinal dysfunction resulting from diabetes.[35,36,50,20]

The goal of this study was to determine which ERG flash stimuli would most robustly detect diabetes-induced retinal dysfunction and to determine if ERG abnormalities persist or worsen with increasing diabetes duration. We examined the retinal function of STZ-treated rat retinas from 7 to 29 weeks post hyperglycemia. Our data indicate that OP delays in response to dim stimuli are the earliest and most persistent deficit. This finding was then applied in a pilot human study, which revealed similar defects in diabetic patients. By demonstrating that dim-flash stimuli reveal rod-based retinal dysfunction in diabetic patients both with and without diabetic retinopathy, this study suggests that OP recordings may be useful in screening for diabetic retinopathy, and that clinical evaluation with ERG may be improved by adding dim flash stimuli to the standard bright flashes used in clinical ERG protocols.

Methods

STZ-Induced Rat Studies

We recorded ERGs from male Sprague-Dawley rats (150–175 g, Charles River, Wilmington, MA) in a cross-sectional design. Rats were housed in shoe-box cages on a 12:12 light:dark cycle at approximately 10 lux with chow and water provided ad libitum. All procedures were approved by the Atlanta VA Institutional Animal Care and Use Committee and conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Hyperglycemia was induced with intravenous injection of STZ (100 mg/kg; Ferro Pfanstiehl Laboratories, Waukegan, IL) dissolved in citrate buffer (pH 4.0), and control rats were injected with vehicle alone (at 1.5 months of age). Diabetes was defined as two successive daily blood glucose levels greater than 250 mg/dL (freestyle hand-held blood glucose meter from tail-prick blood using 29-G needle).[51] Body weights and blood glucose were monitored three times per week. Diabetic rats were treated with sustained-release subcutaneous insulin (1/4–2/3 pellet of Linplant; Linshin Canada, Scarborough, ON, Canada) at a dose sufficient to prevent weight loss but insufficient to ameliorate hyperglycemia. At 7, 14, 20, and 29 weeks, ERGs were recorded from subsets of STZ- and vehicle-injected rats. Animal numbers are indicated in the figure legends for each time point.

ERG Testing and Analysis in Rats

Rats were dark-adapted overnight and then prepared under dim red light as previously described.[52–54] Responses were recorded to flash stimuli presented in order of increasing intensity using a signal averaging system (UTAS 3000; LKC Technologies, Gaithersburg, MD). ERG stimuli consisted of a 10-step dark-adapted series (−3.4 to 2.1 log cd s/m^2) to isolate rod-dominated[55] and rod/cone mixed

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responses. After testing, rats received yohimbine (2.1 mg/kg) to reverse the effects of xylazine and prevent corneal ulcers.[56]

Amplitudes and implicit times were measured for both a- and b-waves (Supplementary Fig. S1). A-wave amplitudes were measured at decreasing times from 25 to 9 ms after flash onset for −1.8 log to 2.1 log cd s/m^2 flash stimuli to avoid the effects of b-wave intrusion on the a-wave trough.[57] OPs were digitally filtered using the ERG system software (75–500 Hz). Amplitudes and implicit times for OP1 to OP4 were measured. Two-way repeated measures ANOVA (rmANOVA) analysis was performed using statistical analysis software (SigmaStat 3.5; Aspire Software International, Ashburn, VA, or IBM SPSS 19.0; IBM Corporation, Armonk, NY) to compare the treatment groups across flash stimuli. Two-way ANOVA was also used to compare the response of a single ERG parameter between control and diabetic rats across time. Post-hoc multiple comparisons were performed using the Holm-Sidak method. Analysis was performed with significance set at P less than 0.05. Since the ERG values from the control rats across all ages were statistically similar, they are shown in figures using a 95% confidence interval (CI). All statistics reported are the ANOVA interaction effect, unless otherwise noted.

Human Studies

All subjects were recruited from the Atlanta VA Medical Center (VAMC). Sixteen subjects with diabetes mellitus type 2, with (DR) or without (DM) diabetic retinopathy, and eight control subjects (CTRL), met the inclusion criteria and agreed to participate. Three of the CTRL subjects and one DR subject were female. Subjects ranged in age from 25 to 65 years. Inclusion criteria included age from 18 to 80 years, and either no diabetes (CTRL) or a diagnosis of diabetes mellitus. The duration of diabetes was estimated based on the reported onset in the medical record, the first hemoglobin A_{1c} greater than 6.5%, or the first random blood glucose greater than 200 mg/dL documented in the clinical record, which ever was longer. Exclusion criteria included: a history of eye injury or surgery, existing diagnoses of eye disease (except nonproliferative diabetic retinopathy [NPDR]), proliferative diabetic retinopathy or a history of panretinal photocoagulation, dementia, cerebrovascular accident or acute myocardial infarction within the past three months, or a known seizure disorder. This study adhered to the tenets of the Declaration of Helsinki, and was approved by the institutional review board of Emory University (Atlanta, GA) and the Atlanta VAMC Research and Development Committee. Informed consent was obtained from all participants after the nature and possible consequences of the study had been explained.

Human Retinal Imaging

In order to confirm the classification of the subjects and check for other existing eye disease, standard seven-field fundus photographs were taken of CTRL and DM subjects. Photographs were reviewed by an ophthalmologist at the Atlanta VAMC blinded to group assignment who scored the findings as positive or negative for diabetic retinopathy signs such as microaneurysms and/or hard and soft exudates.[7] In addition, DM subjects were imaged with optical coherence tomography (OCT) to detect diabetic macular edema (DME; OCT3; Carl Zeiss Meditec, Inc., Dublin, OH).[58,59] DR was classified as mild, moderate, or severe based on established criteria.[60] Any participant originally recruited in the DM group who was discovered to have diabetic retinopathy was re-assigned to the DR group.

Human ERG Testing and Analysis

Full-field ERGs were recorded using an LKC UTAS system (LKC Technologies, Inc., Gaithersburg, MD) with DTL-Plus eye electrodes[61] (Diagnosys LLC, Lowell, MA), reference electrodes on the temples, and a ground electrode on the forehead. Eye drops (2.5% phenylephrine hydrochloride, 1% tropicamide, and 0.5% proparacaine hydrochloride) and electrodes were applied in the light, prior to 20-minute dark-adaptation. Methylcellulose was applied to the DTL electrodes before insertion. The ISCEV standard protocol was performed with the addition of two additional test flashes.

Under dark-adapted conditions, the flash stimuli consisted of ISCEV dim (−2.02 cd s/m^2, ISCEV "dark-adapted 0.01 ERG"), Test Flash 1 (−1.43 cd s/m^2), Test Flash 2 (−0.82 cd s/m^2), and ISCEV bright (0.39 log cd s/m^2, ISCEV dark-adapted 3.0 ERG). Interstimulus intervals began at 10 seconds, and increased to 12 seconds with increasing flash luminance (note that this deviates from the ISCEV standard, which recommends a 15-second interval for the bright-flash ERG). For each luminance, 10 to 12 responses were averaged with a sampling interval of 256 ms. The filter settings were from 0.3 to 300 Hz for all recordings. The LKC was calibrated quarterly

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Table 1. Weight and Blood Glucose in STZ and Control Rats Over the Experimental Period

	Weight, g		Blood Glucose, mg/dL	
	Control	Diabetic	Control	Diabetic
Baseline	156 ± 27	162 ± 25	102 ± 17	97 ± 14
7 wk	490 ± 48	355 ± 37*	86 ± 13	363 ± 56*
14 wk	573 ± 96	402 ± 22*	81 ± 12	349 ± 67*
20 wk	654 ± 88	443 ± 26*	85 ± 10	325 ± 29*
29 wk	711 ± 93	465 ± 21*	84 ± 8	361 ± 100*

Diabetic rats differed significantly from controls for both parameters (blood glucose: ANOVA main effect $F[4, 110] = 92.9$; weight: ANOVA main effect of treatment $F[4, 110] = 25.9$).

*$P < 0.001$.

with a radiometer/photometer (DR 2550; EG&G Gamma Scientific, Inc., San Diego, CA).

Binocular ERG recordings were obtained for all subjects, except for a DR subject for whom there were technical difficulties with his left-eye recording. No statistically significant differences were found between right and left eyes (paired *t*-tests with Bonferroni corrections), so only the data from the right eyes are shown. A- and b-wave amplitudes and implicit times were measured for each flash condition. The OPs (OP1–OP3) were extracted from the averaged waveforms off-line on the LKC, using bandpass settings of 75 to 300 Hz. Amplitudes greater than baseline noise (>0.5 μV) were measured and analyzed for all stimuli.

rmANOVA with Holm Sidak post-hoc comparisons was performed with statistical analysis software (SigmaStat 3.5; Aspire Software International, Ashburn, VA) to compare groups across flash stimuli using P less than 0.05 for statistical significance. The interaction term is reported, unless otherwise stated. Since the main objective was to determine retinal dysfunction prior to clinically detectable retinopathy, the comparisons between CTRL and DM groups are emphasized.

Results

Diabetic Rat Model

Weight and Blood Glucose in Control Versus Diabetic Rats

Average daily blood glucose in control rats was 90 ± 16 mg/dL compared with 347 ± 62 mg/dL for diabetic rats (Table 1: ANOVA main effect of treatment $F[4, 110] = 92.9$, $P < 0.001$). Weight of the control rats at each time point increased from baseline to 29 weeks. Although diabetic rats gained weight throughout the study, they remained smaller than control rats at each time point after hyperglycemia induction (Table 1: ANOVA main effect of treatment $F[4, 110] = 25.9$, $P < 0.001$).

Delays in Retinal Function in Diabetic Rats

Multiple ERG changes were observed in diabetic rats. Representative ERG waveforms from control and diabetic rats are shown in Figure 1. No differences between the control and diabetic rats were found in a-wave implicit time or amplitude (data not shown). However, implicit times of b-waves elicited with dim stimuli were delayed at 14 weeks post-STZ (−3.4 and −3.0 log cd s/m^2; rmANOVA $F[9, 155] = 6.91$, $P < 0.001$: Supplementary Fig. S1). The b-wave delays in diabetic rats persisted at 20 and 29 weeks. While initially only elicited in response to dim stimuli, b-wave delays were subsequently noted in responses to progressively brighter stimuli (−3.4 to −2.4 log cd s/m^2; 20 weeks: rmANOVA $F[9, 177] = 3.59$, $P < 0.001$; 29 weeks: rmANOVA $F[9, 153] = 3.18$, $P = 0.002$). In contrast, b-wave amplitudes were first reduced at 29 weeks in diabetic rats in response to

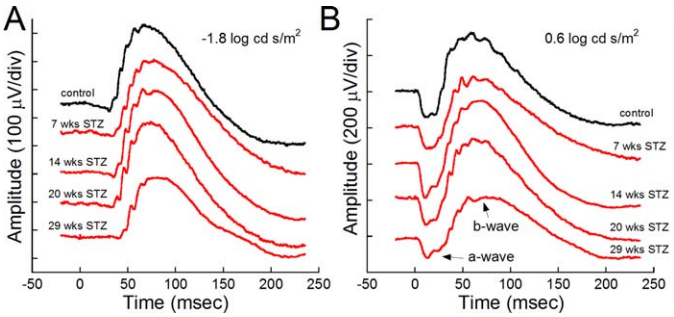

Figure 1. Representative ERG waveforms from control and diabetic rats in response to dim (A) and bright (B) flash stimuli from each post-STZ time point. The diabetic rat responses (*red lines*) are generally similar to the control rats (*black lines*) except at 29 weeks when the diabetic waveforms have smaller a- and b-wave amplitudes (indicated by *black arrows*).

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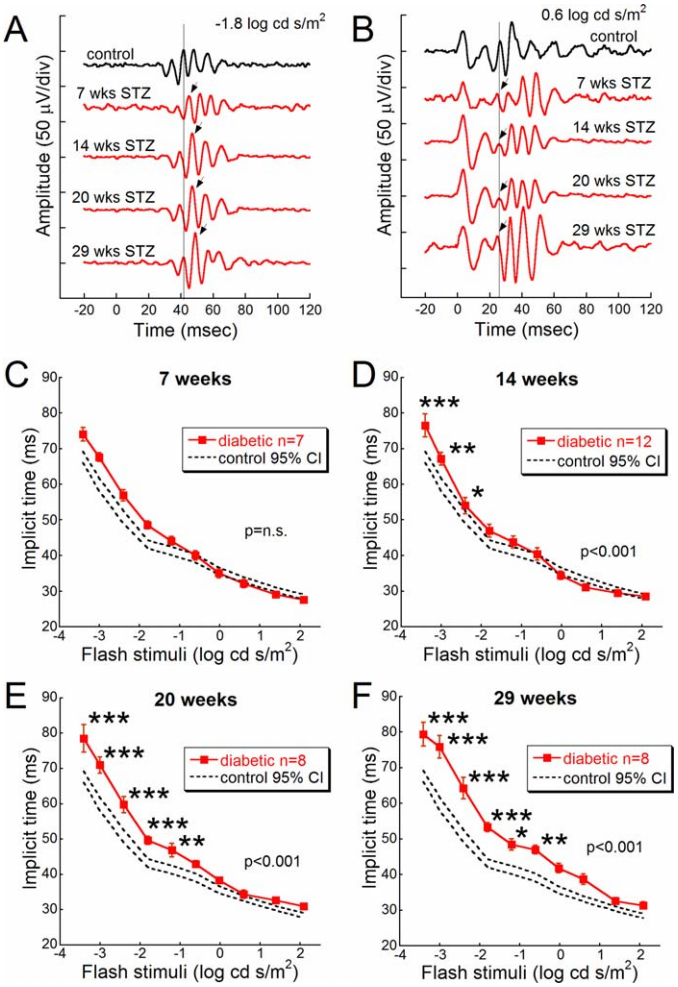

Figure 2. Representative OP waveforms from control (*black lines*) and diabetic (*red lines*) rats at each time point in response to dim (A) and bright (B) flash stimuli. The *vertical line* indicates the implicit time of OP2 in the control trace. The *arrows* indicated OP2 in each diabetic waveform. (A) The OPs are progressively more delayed within weeks after STZ exposure in response to a rod-stimulating dim flash. (B) No obvious differences in OP implicit time are detected in response to a bright flash in diabetic rats when compared with controls. OP2 implicit times across flash stimuli are shown in *panels C–F*. OP2 implicit times showed a trend for selective delays to dim stimuli at 7 weeks (C) and became statistically significant at 14 weeks ([D]; $F(9, 161) = 3.42$, $P < 0.001$], 20 weeks [E]; $F(9, 189) = 9.28$, $P < 0.001$), and 29 weeks ([F]; $F(9, 162) = 5.92$, $P < 0.001$) post-STZ. Note the progressive difference and delays for increasing number of flash stimuli with longer duration of diabetes. Control data is plotted as the 95% CI. Holm-Sidak post hoc comparisons $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

bright stimuli (rmANOVA main effect $F[1, 153] = 10.03$, $P = 0.007$; Supplementary Fig. S1).

OP delays in response to dim stimuli were observed as early as 7 weeks post-STZ and became significant by 14 weeks post-STZ (Fig. 2A), whereas no changes were seen in OP latencies in response to bright stimuli (>–0.6 cd s/m^2; Fig. 2B) at any time point. Quantitative changes in OP2 were representative of all four OP wavelets across the range of stimuli at different time points (Figs. 2C–F). In diabetic rats, the delay in OP2 implicit times in response to dim stimuli was detectable, but remained nonsignificant, at 7 weeks post-STZ (Fig. 2C). At 14 weeks, OP2 delays in response to dim stimuli (<–2.4 log cd s/m^2) became significant (Fig. 2D: rmANOVA $F[9, 161] = 3.42$, $P < 0.001$). At 20 weeks, delays in OP2 latency continued to increase and were evident at brighter stimuli (<–1.2 log cd s/m^2; Fig. 2E: rmANOVA $F[9, 189] = 9.28$, $P < 0.001$). By 29 weeks, OP2 implicit times were delayed at all flash intensities less than –0.6 cd s/m^2 (Fig. 2F; rmANOVA $F[9, 162] = 5.92$, $P < 0.001$). OP amplitudes were similar among STZ and control rats across the study period (data not shown).

Human Subjects

Characteristics of DM and DR subjects are shown in Table 2. One subject classified at enrollment as DM was re-assigned to the DR group after fundus photography revealed mild to moderate NPDR, producing nine subjects in the DM group, seven in the DR group, and eight in the CTRL group. OCT detected no abnormalities in any subject. Retinopathy in the DR group ranged from mild ($n = 3$), to moderate ($n = 2$), to severe ($n = 2$) NPDR. The average age was significantly higher for the DR group than the CTRL group (one-way ANOVA $F[2, 23] = 7.726$, $P = 0.003$; Table 2], but was not significantly different between the DM and DR groups, or the DM and CTRL groups. The duration of diabetes was significantly different between the DM and DR groups (4.2 ± 1.9 vs. 13.1 ± 6.8 years, Student's *t*-test, $P = 0.01$; Table 2).

Retinal Function Reflected by A- and B-Waves

All flash stimuli elicited detectable ERG responses in all subjects (Fig. 3). In addition to the standard dark-adapted ISCEV dim and bright stimuli, ERGs were recorded using two intermediate Test Flashes. The Test Flashes did not reveal a- and b-wave changes in the DM or DR group that were not also present in the responses evoked with standard ISCEV dim and/or bright stimuli (Supplementary Fig. S2). Compared with controls, a-wave implicit times were delayed in both DM and DR across all flash stimuli (Supplementary Fig. S2B; rmANOVA main effect

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Table 2. Characteristics of the Diabetic Subjects

	DM ($n = 9$)	DR ($n = 7$)
Age, y; mean ± SD†	49.9 ± 11.2	60.3 ± 4.6
Hypertension, % of subjects	89%	75%
Body mass index, mean ± SD	30.6 ± 6.48	32.1 ± 4.9
HbA_{1c}, %; mean ± SD	9.2 ± 3.0	7.2 ± 1.5
Duration of diabetes, y; mean ± SD	4.2 ± 1.9	13.1 ± 6.8*
Diabetes treatment:		
Oral medication alone	6	0
Insulin alone	2	6
Oral medication and insulin	0	1
None	1	0

*$P = 0.01$

†CTRLs: 41.4 ± 9.7 years old ($n = 8$)

$F[2, 80] = 7.84$, $P = 0.004$). The a-wave amplitude in response to the ISCEV bright flash was diminished in the DM group compared to controls and the DR group was decreased compared to DM (Supplementary Figure 2A; rmANOVA, $F[6, 80] = 2.44$, $P < 0.05$). The DR group showed delayed b-wave implicit time in response to standard ISCEV dim stimulus and Test Flashes 1 and 2 compared with both control and DM groups (Supplementary Fig. S2D; rmANOVA, $F[6, 83] = 5.13$, $P < 0.001$). No significant differences in b-wave amplitudes were observed between groups (Supplementary Fig. S2C).

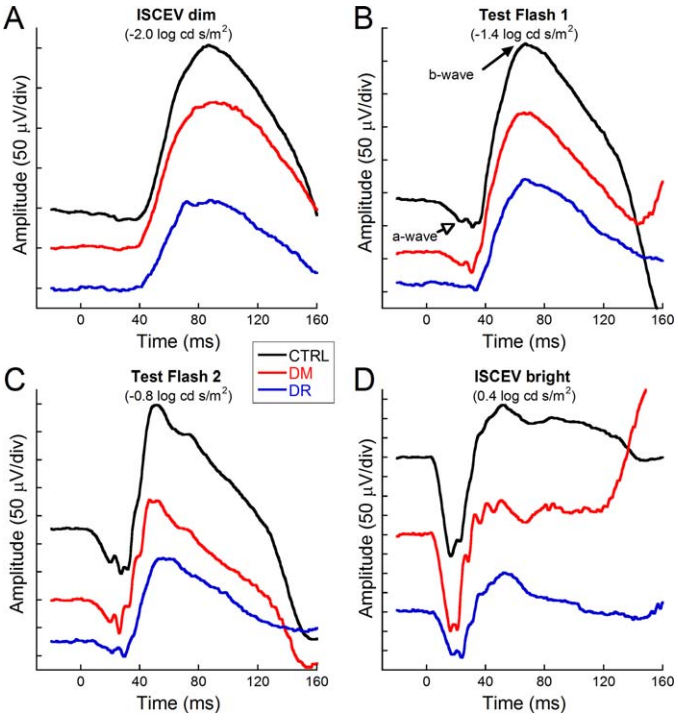

Figure 3. Representative ERG waveforms from CTRL subjects and diabetic DR or DM subjects for each flash stimulus tested. *Open arrow* indicates the a-wave and *closed arrow* indicates the b-wave.

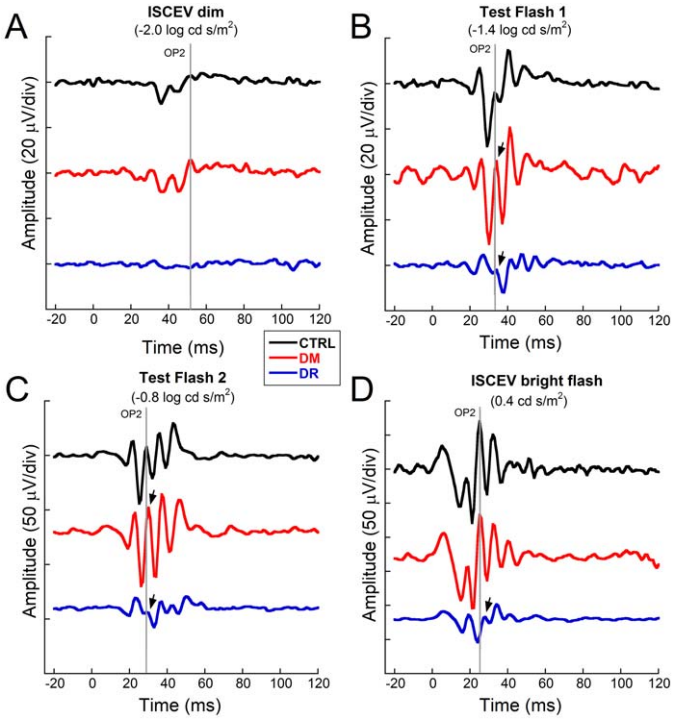

Figure 4. Representative OP waveforms from CTRL, DR, and DM subjects. Waveforms were filtered from dark-adapted ERGs in response to different flash stimuli. (A) OP waveforms in response to ISCEV dim flashes (–2.0 log cd s/m^2) were not easily discernible in the recording from this DR subject. (B) OPs to Test Flash 1 (–1.4 log cd s/m^2) showed delays in both the DM and DR subjects, as indicated by the *arrows* for OP2. (C) Test Flash 2 (0.8 log cd s/m^2) also elicited OP delays in both the DM and DR subjects. (D) OP delays were found in DR, but not DM, subjects in response to ISCEV bright flashes (0.4 cd s/m^2). The *gray vertical line* indicates the peak of OP2 in the control waveform. *Arrows* indicate delays in OP2.

Inner Retinal Function Measured by OPs

Representative OP waveforms in response to the four flash stimuli under dark-adapted conditions are shown in Figure 4. While several OP peaks were clearly visible in response to the standard ISCEV bright stimulus, the OP amplitudes were substantially reduced in response to both Test Flash stimuli and the standard ISCEV dim stimulus. Indeed, only three of nine DM subjects and four of seven DR subjects had

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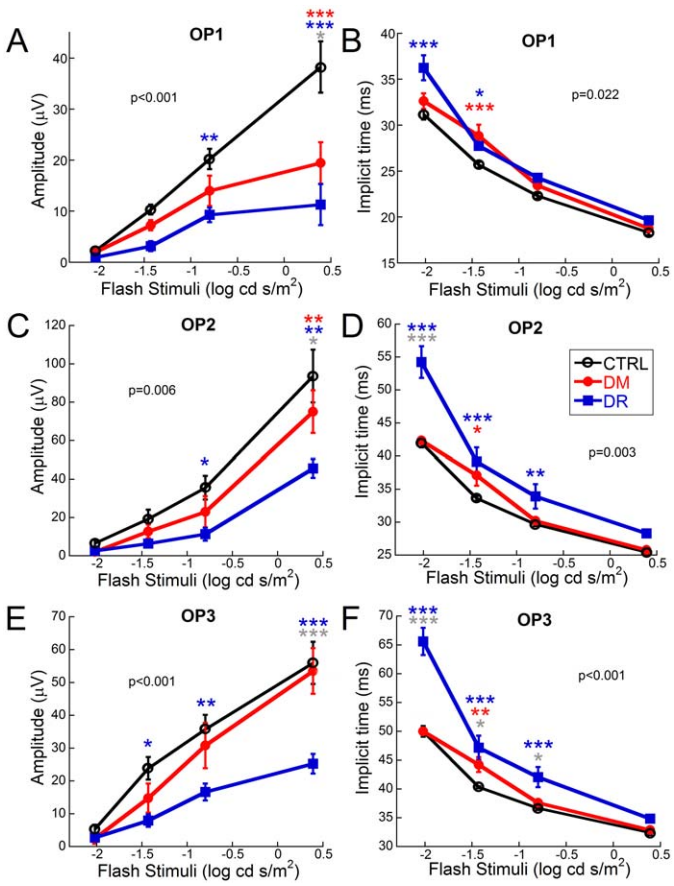

Figure 5. Average OP amplitudes and implicit times in CTRL and diabetic DR or DM subjects in response to ISCEV dim (−2.0 log cd s/m^2), Test Flash 1 (−1.4 log cd s/m^2), Test Flash 2 (−0.8 log cd s/m^2), and ISCEV bright (0.39 log cd s/m^2). (A) OP1 amplitudes were significantly reduced compared with CTRL (two-way rmANOVA $F(6, 94) = 7.39$, $P < 0.001$) for the DR group with Test Flash 2. With ISCEV bright flashes, OP1 amplitudes showed reductions for DM and DR versus CTRL, and for DM versus DR. (B) OP1 implicit times were significantly delayed compared with CTRL (rmANOVA $F[6, 86] = 2.73$, $P < 0.02$) for the DR group with ISCEV dim and for DM and DR versus CTRL for Test Flash 1. (C) OP2 amplitudes were reduced compared with CTRL (rmANOVA $F[6, 94] = 3.42$, $P = 0.006$) for DR group with Test Flash 2, and with ISCEV bright for the DM and DR groups versus CTRL and DR versus DM. (D) OP2 implicit times were delayed compared with CTRL and DM (rmANOVA, $F[6, 85] = 3.82$, $P < 0.003$) for DR with ISCEV dim, for DM and DR versus CTRL with Test Flash 1, and for DR versus CTRL with Test Flash 2. (E) OP3 amplitudes were reduced compared with CTRL (rmANOVA $F[6, 94] = 4.94$, $P < 0.001$) for DR with Test Flashes 1 and 2, and for DR versus both DM and CTRL with ISCEV bright. (F) OP3 implicit times were delayed compared with CTRL and DM (rmANOVA $F[6, 85] = 6.90$, $P < 0.001$) for DR with ISCEV dim; for DR and DM versus CTRL and DR versus DM for Test Flash 1; and for DR versus CTRL and DM for Test Flash 2. *Asterisks* indicate post-hoc comparisons for DM versus CTRL (*red*), DR versus CTRL (*blue*), and DR versus DM (*gray*). Holm-Sidak post hoc comparisons *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

measurable OPs in response to the standard ISCEV dim stimulus. In contrast, despite reduced amplitudes, both Test Flashes elicited measureable OPs in all subjects. OP4 was absent in many waveforms and, thus, further analysis was not performed for OP4.

OP amplitudes and implicit times for the three subject groups are shown in Figure 5. Test Flash 1 produced implicit times for OP1 to 3 in the DM group that were prolonged compared with CTRL group (OP1: Fig. 5B, rmANOVA $F[6, 86] = 2.73$, $P = 0.022$; OP2: Fig. 5D, rmANOVA, $F[6, 85] = 3.82$, $P < 0.003$; OP3; Fig. 5F, rmANOVA $F[6, 85] = 6.90$, $P < 0.001$). We observed even more prominent delays in response to dim stimuli in the DR group, with prolonged OP1 to OP3 implicit times in response to both Test Flash 1 and the standard ISCEV dim stimulus (Figs. 5B, 5D, 5F). OP2 and OP3 in response to Test Flash 2 also showed delays (Figs. 5D, 5F). In contrast to these findings for the dim flash and Test Flashes, the standard ISCEV bright flash did not elicit significant delays in OP1 to OP3 implicit times in either DR or DM compared with CTRL group (Figs. 5B, 5D, 5F).

OP1 and OP2 amplitudes after a standard ISCEV bright flash were reduced in the DM group compared with controls (OP1: Fig. 5A, rmANOVA $F[6, 94] = 7.39$, $P < 0.001$; OP2: Fig. 5C, $F[6, 94] = 3.42$, $P = 0.006$). The Test Flashes did not reveal OP amplitude reductions in DM. However, OP3 amplitude in response to Test Flash 1, and OP1 to OP3 amplitudes in response to Test Flash 2 and the standard ISCEV bright stimulus were reduced in DR compared with CTRL (Figs. 5A, 5C, 5E; OP3: rmANOVA $F[6, 94] = 4.94$, $P < 0.001$).

Ability of Dim Flash Stimuli to Detect Early DR in Rats and Humans

Figure 6A illustrates the percent difference in OP2 implicit times between control and diabetic rats in response to representative dim (−1.8 log cd s/m^2) and bright (0.6 log cd s/m^2) flashes from 7 to 29 weeks after hyperglycemia. OP2 implicit times to bright stimuli showed progressive delays starting at 20 weeks. In contrast, the onset for delays in response to dim stimuli occurred earlier, starting at 7 weeks (12.37 ± 2.52%), and progressing to 29 weeks post-STZ (25.11 ± 4.36%), to produce a significant main effect between flash stimuli (two-way ANOVA, $F[1,63] = 20.41$, $P < 0.001$).

Figure 6B shows the OP2 responses to the different ERG flash stimuli in human subjects. Significant delays of all three OPs (12.2 ± 4.8% for OP1, 10.4 ±

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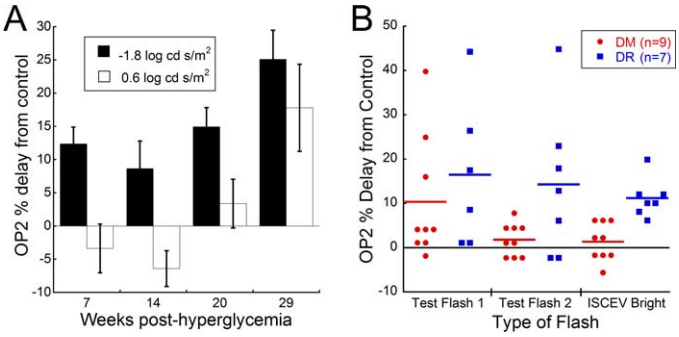

Figure 6. OP implicit time delays in response to dim and bright flash stimuli in rats (A) and diabetic subjects (B). (A) OP2 percent difference in implicit times (diabetic-control) across weeks of hyperglycemia in response to representative dim (−1.8 log cd s/m^2) and bright (0.6 log cd s/m^2) flash stimuli in STZ-treated rats. OP2 implicit times became significantly and progressively delayed compared with bright stimuli across all time points, as indicated by the significant main effect of flash stimuli (two-way ANOVA, $F[1, 63] = 20.41$, $P < 0.001$). (B) OP2 percent difference in implicit time (diabetic-control) in DR or DM subjects, plotted as individual points with the mean indicated by the horizontal line in each column. Test Flash 1 (−1.4 log cd s/m^2) revealed a 10% increase in OP2 implicit time in DM subjects. In DR subjects, all flash intensities elicited an OP2 delay of > 12%. Note that one DR subject had no OPs at Test Flash 1.

4.6% for OP2 [Fig. 4B], and 9.7 ± 3.2% for OP3) in DM compared with the controls were observed only in response to Test Flash 1. Less than 2% delay was measured for any OP in response to Test Flash 2 and for the standard ISCEV bright flash. In contrast, DR exhibited delays in OP1 through OP3 (7.2%–17.0% compared with CTRL) in response to Test Flashes 1 and 2, and the standard ISCEV bright flash. ISCEV dim stimuli were not included in this analysis since OPs were not elicited with this luminance in most subjects.

Discussion

In this study, OP delays consistent with early diabetic retinopathy were most evident in response to dim flash stimuli in both rats and humans. Dark-adapted ERGs recorded in diabetic rats showed that dim stimuli elicited OP delays that were detectable soon after the onset of hyperglycemia, and were progressive with increasing duration of diabetes. This result suggested that assessing OP delays in response to dim stimuli may provide a means to accelerate detection of retinal dysfunction in human DM patients. We found evidence of this in our human study. Through the addition of dim test flashes to the recommended ISCEV stimulation protocol, we detected significant delays in OP implicit time among DM subjects without clinically recognized retinopathy.

Selective Scotopic Dysfunction in Diabetic Rat Retinas

Prior studies in diabetic rodents have shown changes in OPs[35,36,42,47] and scotopic threshold response[36] that would suggest inner retinal dysfunction. In addition, rods[42,62] or rod-driven pathways (e.g., reflected in the scotopic threshold response[36]) have been reported to be more sensitive than cone pathways to hyperglycemic insult in these rodent models. The current results reinforce such findings as we found delays in OP implicit time in diabetic rats that were first observed with stimuli that isolate rod pathways (Figs. 2, 6).[55] Similar OP delays were not detected in response to bright stimuli predicted to elicit a mixed rod/cone response. The scotopic OP delays were progressive out to 29 weeks post-STZ when pericyte drop out and other late stage vascular changes have been reported.[63–65] Thus, these findings in our diabetic rat model provide additional evidence of progressive rod-driven inner retinal dysfunction with hyperglycemia.

The reason for rod sensitivity to high glucose is not known. Hyperglycemia alters numerous retinal proteins, including synaptic proteins, neurotransmitters and their receptors, and visual cycle proteins.[43,66–68] Hyperglycemia also increases retinal cell apoptosis,[69,70] induces glial cell changes,[71–73] and diminishes function of photoreceptors and ganglion cells.[11,74] Thus, in rod dominated retinas (97% rods in rodents[75]; 96% rods in humans[76]) functional deficits may be most easily detected under scotopic conditions that selectively measure rod responses.[55]

ERG Changes in Diabetic Subjects

Noninvasive measurements of retinal function with ERGs have shown functional changes in diabetic patients with clinically recognized vascular changes.[21,24–26,28,33,77–79] Notably, studies have found diminished dark-adapted threshold[80–83] and abnormal dark-adapted OPs[20,50] in human subjects with diabetic retinopathy, indicative of rod pathway dysfunction. In this study as well, ERG changes were detectable in the DR group, as indicated by decreased a-wave amplitudes after a bright stimulus and delayed b-wave implicit times in response to a standard dim stimulus or Test Flashes (Supplementary Fig. S2). Our results

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are also consistent with previous studies in demonstrating that OP amplitudes were diminished and implicit times prolonged in DR[20,24] (Fig. 5). The ERG changes reported in DR are consistent with the vascular pathology used to diagnose retinopathy in these diabetic subjects.

More importantly, the ERG has also been useful in detecting early functional changes in retinal neurons in diabetic patients prior to clinically recognized vascular changes such as macular edema, vessel leakage, and neovascularization. We observed decreased a-wave (Supplementary Fig. S2) and OP amplitudes (Fig. 5) in response to bright flash stimuli in the DM group. Other studies have also shown decreased ERG a- and b-wave, and OP amplitudes in diabetic patients without retinopathy with bright-flash stimuli or under light-adapted conditions.[23–26,28–30] In addition, several studies have described abnormal multifocal ERG (mfERG) responses in subjects with diabetes both with and without apparent retinopathy.[77,84] In these studies, delayed implicit times appear to be the most sensitive parameter to detect early vascular changes in the same retinal regions in diabetic retinopathy.[77,85–87] While these studies also show early retinal dysfunction that is predictive for developing clinically recognized diabetic retinopathy,[86] the mfERG is performed under photopic conditions, thus limiting the ability to probe the scotopic pathways that may show even earlier dysfunction in diabetes, as suggested by the current results. Although these results provide evidence for the sensitivity of ERGs to detect early diabetic retinopathy, they also illustrate that the inner retinal responses to scotopic stimuli in diabetic patients without retinopathy have not been systematically examined. Additional research is also needed to determine if delayed OPs under scotopic conditions predict vascular pathologies in later stages of the diabetic retinopathy.

Scotopic OPs Detect Preclinical DR

This study provides further evidence that the inner retinal ERG defects, demonstrable in patients without diabetic retinopathy, develop prior to retinal vascular disease. Previous studies have reported rod-driven bipolar cell dysfunction, as measured by b-wave delays in response to dim flash luminance in patients with no or minimal retinopathy.[25,33,81] However, few studies have examined inner retinal function reflected in OP wavelets under a range of dim luminances. The ISCEV ERG standard[48] recommends recording dark-adapted OPs in response to bright flashes of 3.0 log cd s/m^2. This may reflect the fact that OPs were first described in response to bright stimuli,[20,88] and were difficult to record in response to dim stimuli.[21] Similar to this study, Hancock and Kraft[89] were able to measure OPs down to the ISCEV dim-flash luminance (−1.9 log cd s/m^2) in their study of healthy subjects. While the current study was able to consistently detect OP1 to OP3 in control subjects with the ISCEV dim flash (−2.0 log cd s/m^2), OPs were undetectable in the ERGs of many DM and DR subjects at that luminance. Thus, diabetes status may contribute to the smaller number of detectable OPs at this lowest luminance. By contrast, only one DR subject entirely lacked OPs at the next highest luminance (Test Flash 1; −1.43 log cd s/m^2). Thus, Test Flash 1 seems well-suited for quantifying OPs in response to dim stimuli and potentially detecting rod-driven dysfunction in early diabetic retinopathy. Aside from serving as a potential screening tool, addition of these test flashes may specify which retinal neurons are affected in patients with early-stage diabetic retinopathy.

Contribution of Vascular Pathology to Rod-Pathway Dysfunction in Early Diabetes

The current results support the hypothesis that rod pathway dysfunction is among the earliest retinal responses to hyperglycemia. The debate remains as to whether diabetic retinopathy is strictly a vascular disease, as clinically assessed, or a combination of neuronal and vascular changes.[10,11,69] It is unknown whether neuronal or vascular changes precede the other in the setting of diabetes, if hyperglycemia affects one more than the other, or if neurovascular coupling defects[90–92] underlie diabetic retinopathy, in whole or in part. While ERGs reflect neuronal responses, our results do not exclude the possibility that vascular changes could underlie or exacerbate neuronal dysfunction. It is currently unknown if prolonged OP implicit times are manifestations of a reversible defect, subject to improved glycaemia, or if they represent irreversible damage. Thus, further research on the pathogenesis of scotopic dysfunction in diabetes may not only reveal why the rod-driven pathway is particularly susceptible to hyperglycemic insult but also identify potential molecular targets for intervention.

Limitations of the Human Study

Retinopathy takes several years to develop in diabetic patients. Thus, it is difficult to perform longitudinal studies that would demonstrate that

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dysfunction in rod-driven pathways is predictive of clinically-recognized retinopathy. In this study, we tested three groups of subjects: healthy CTRL, DM, and DR, with the goal of finding robust ERG parameter(s) that could detect functional abnormalities in the DM group in comparison with CTRL. Even with these broad categories and few subjects in each group, we were able to show that the analysis of OP implicit time in response to scotopic stimuli revealed delays in the DM group. As shown in Figure 6, there was variability amongst subjects in our DM and DR groups, with some subjects having near-normal OP implicit times. With the small number of diabetic subjects, we did not perform further analysis on the influence of medications, blood pressure, or a number of other potential clinical measures. Future studies, using multivariate modeling and a larger number of subjects, are needed to determine why some diabetic patients are more susceptible to hyperglycemic insult while others are spared. In addition, more sophisticated analysis (such as Fourier analysis of the OPs[93,94]) could be retrospectively performed on the stored ERGs recorded from these diabetic subjects without retinopathy to determine if functional abnormalities are present; however, that is beyond the scope of this paper. We have limited our analysis of OPs to the measurement of amplitude and implicit time, so that it might be adopted more easily for clinical screening. It is encouraging that we did find differences between different flash stimuli without more sophisticated analysis, suggesting that these differences are meaningful in detecting early functional changes in the diabetic retina.

Summary

These results suggest that adding dim stimuli to the standard ISCEV flashes for OP analysis may be a novel noninvasive method for detecting early hyperglycemia-induced retinal dysfunction. This study used a cross-sectional design for both rats and human subjects, similar to many clinical studies. However, longitudinal monitoring of retinal function will be needed to examine the predictive power of the scotopic ERG changes for subsequent diagnosis of diabetic retinopathy. Therefore, clinical ERG exams supplemented with lower intensity stimuli may permit identification of early neuronal pathology and provide rationale for more cost effective follow-up, as well as providing a tool to monitor neuroprotective strategies that could prevent diabetic retinopathy (see Ref. 95 for a recent review of potential drug therapies). In addition, combination testing to detect both neuronal and vascular dysfunction in the retina may provide increased sensitivity for identifying retinopathy before the current clinically recognizable vascular pathologies.

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