A Role for Omega-3 Polyunsaturated Fatty Acid Supplements in Diabetic Neuropathy

Peter Yee,1 Anne E. Weymouth,2 Erica L. Fletcher,1 and Algis J. Vingrys2

PURPOSE. Diabetes results in an insulin-related disorder of lipid metabolism that reduces production of long-chain polyunsaturated fatty acids (PUFAs; e.g., docosahexaenoic acid, DHA). This study considers the role that this lipid change has on retinal function.

METHODS. From conception, rats (n = 56) were fed diets either balanced (n = 32) in PUFAs or deficient in omega-3 (n = 24). Half were assigned to control (n = 28) or streptozotocin (STZ: n = 28) treatment at 7 weeks of age. Key metabolic indices were assayed at 19 weeks, and retinal function was determined by electroretinogram (ERG) at 20 weeks. Retinal anatomy and lipid assays of 20-week-old animals were used to identify structural changes and tissue PUFA content.

RESULTS. The systemic indices of diabetic rats were not affected by diet. Lipid composition of retinal membranes reflected the dietary manipulation, and diabetes amplified some fatty acid changes consistent with reduced desaturase activity. Diabetes produced significant reduction in rod function (−35%) only in the absence of fish oil, whereas cone responses (−46%) and inner retinal oscillatory potentials (−47%) showed either no effect of diet or a partial diet effect with a significant diabetes effect. Anatomic analysis revealed no disorder in the retinal neurons, although changes in the Müller glia were noted in diabetes, regardless of diet.

CONCLUSIONS. A diet balanced in long-chain PUFAs modifies retinal lipid membranes in diabetes and prevents rod dysfunction. Dietary modification was not found in the cone or glial response but a partial improvement was evident in the OPs, most likely secondary to the larger photoreceptor output. (Invest Ophthalmol Vis Sci. 2010;51:1755–1764) DOI:10.1167/iovs.09-3792

Diabetic retinopathy is a major complication for long-term management of diabetes mellitus. The majority (99%) of individuals with type 1 diabetes show some retinopathy, with 60% developing proliferative retinopathy in 15 to 20 years,1 indicating the limited success of present management methods. Current treatments target vascular changes, but there is growing evidence that neuronal changes take place either before or in conjunction with vascular abnormalities2–4 and may require an alternative approach to treating early diabetic retinopathy.

Neural dysfunction in the diabetic retina is well recognized in humans and animals.5–15 The electroretinogram (ERG) finds inner retinal dysfunction early9–12 and these changes can be useful predictors of progression.5,13 Indeed, the local ERG response can be abnormal in the absence of clinical signs of diabetic retinopathy2–3 and can predict the future development of vascular complications.3 More recently, photoreceptor abnormalities have been described in both diabetic patients14 and animals,2,8–10,12 In the streptozotocin (STZ) rat model, a photoreceptor and inner retinal dysfunction becomes evident 2 days after the induction of diabetes.9 Although the inner retinal response remains abnormal, the rod photoreceptor response returns normal waveforms with time, to become abnormal again 12 weeks after diabetes onset.8,9,10,12 Apart from these functional changes, the effect of diabetes on lipid metabolism and its implication for neural function has received little attention. The diabetic insulin deficiency results in reduced desaturase activity,15,16 an important enzyme in forming long-chain polyunsaturated fatty acid (PUFA) metabolites, of which docosahexaenoic acid (DHA, Fig. 1) is important for retinal function. Given that low levels of DHA are known to promote retinal dysfunction,17,18 it might be expected that the lower desaturase activity in diabetes would lead to retinal dysfunction in diets lacking long-chain substrates.

Indeed, studies have shown that DHA supplementation of STZ-treated rats can have beneficial effects on sciatic nerve function19,20 and can reduce oxidative damage to the heart and liver.21 Dietary omega-3 PUFA supplementation has also been reported to be beneficial in systemic diseases, such as hypertension,22 cardiovascular disease,23 and cancer.24 However, little is known about the possible benefits that dietary omega-3 PUFA may have for the diabetic retina. The findings of Hammes et al.25 indicate that such supplementation might place the retina under greater oxidative challenge, promoting the progression of diabetic eye disease. Later, we will argue that the lack of antioxidant cover used in that study (see the Discussion section) influences the interpretation of these findings. That intakes of these PUFAs and adequate antioxidant cover in that study (see the Discussion section) influences the interpretation of these findings. That intakes of these PUFAs and adequate antioxidant cover can reduce oxidative damage21 and modify retinal angiogenesis26 implies that these substrates could be of some benefit in the management of diabetes and that this issue should be revisited, given the negative finding of Hammes et al.25

In this article, we consider the role that balanced diets high in PUFA and adequate antioxidants can have in modulating the retinal function of diabetic animals. We propose that the lipid changes associated with diabetes (reduced DHA) might partially explain the reported neural dysfunction.3–14 If so, adequate dietary intakes of fish oil during diabetes should preserve retinal function. The purpose of our study was to evaluate this possibility.

METHODS

Animals and Diets

All procedures were conducted in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes set out by the National Health and Medical Research Council of Aus-

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tralia (2004) and the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, as approved in our institutional ethics clearance (A0/001). Six pregnant Sprague-Dawley dams (Monash University Animal House, Clayton, VIC, Australia) were introduced to diets based on a standard rodent Chow premix that complied with AIN93,27 the constituents of which have been listed elsewhere.28 To this premix, lipid (7% wt/wt) was added, either from an omega-6-rich oil (SO, 7% safflower oil) or a mix of oils (FO: 0.5% fish oil, 1.5% flaxseed oil, and 5% safflower oil). This latter diet, was called FO because of its fish oil content, but it provides a well-balanced PUFA oil, 1.5% flaxseed oil, and 5% safflower oil. This latter diet, was called FO because of its fish oil content, but it provides a well-balanced PUFA intake of both short- and long-chain omega-6 and -3 substrates.28 The premix and diets were made by Specialty Feed Services (Glen Forest, WA, Australia) and had a minimum vitamin E content of 75 IU/kg. The diet macronutrient composition, oil additive, and independent lipid assay outcomes are listed in Supplementary Table S1, http://www.iovs.org/cgi/content/full/51/3/1755/DC1.

The logic underlying our experimental design is that the FO diet comprises a sufficient intake of both omega-6 and omega-3 PUFA, whereas the SO diet represents an extreme omega-3 deficiency (ω-6/ω-3 = 234), beyond that found in a typical Western diet (ω-6/ω-3 = 17).28,29 However, adoption of such extreme PUFA manipulation is needed to produce rapid tissue lipid changes and expose the mechanisms underlying the physiological processes modulated by omega-3 deprivation in rats. Moreover, it has been argued that it is not the diet composition per se but the tissue effects that have to be considered in determining health outcomes.29,30 In support of our logic, we note that the effect that Western diets have on tissue lipid content over the long durations of a human lifetime is not insignificant29 and that human clinical trials show that a modest dietary increase in omega-3 intake by Western populations would lead to substantial health benefits.25,39

Pups were weaned onto the mother’s diet and were assigned to one of four cohorts (D, diabetic; C, control; DSO, n = 12; CSO, n = 12; DFO, n = 16; and CFO, n = 16) that were balanced for maternal source, weight, and sex. At 7 weeks of age (150–250 g) diabetes was induced with an injection of STZ (55 mg/kg injected into the tail vein). Diabetes was identified by elevated blood glucose (>15 mM) and glycosylated hemoglobin (HbA1c > 7.0%). As in past studies,12 STZ animals were given insulin infrequently (1 unit protophane), depending on their condition, and returned high HbA1c levels (Table 2) consistent with a poorly controlled diabetic state. Twelve weeks after diabetogenesis (age 19 weeks), the animals were housed in metabolic cages (24 hours) to measure urine and fecal output and water intake.

Electroretinograms

The ERG was used to assess retinal function. The initial 200 ms of a typical rod waveform after a bright flash is shown in Figure 2. The first, negative-going potential is known as the a-wave (Fig. 2), the initial leading edge of which represents photoreceptor activation. The subsequent positive-going potential is called the b-wave (Fig. 2) and reflects the function of ON-bipolar cells. The small ripples seen on the leading edge of the b-wave (Fig 2) are known as oscillatory potentials (OPs). These wavelets reflect the activity of inner retinal neurons, particularly amacrine and ganglion cells. The recording procedures and our step-wise analysis allows extraction of the neural generators (PIII, PI) of these waveforms (a- and b-wave) in terms of different retinal populations as will be described later and detailed elsewhere.30

A twin-flash paradigm was used to identify the cone contribution to the waveform.30,31 Cone responses were identified with a probe presented 800 ms after a rod-saturating exposure. The putative rod component was returned by digital subtraction of the cone signal from the mixed (rod/cone) response.30,31

Animals were dark adapted (>12 hours) before recording, to ensure maximum sensitivity.30 General anesthesia was achieved with an intramuscular injection of 60 mg/kg ketamine and 5 mg/kg xylazine (Troy Laboratories, Smithfield, NSW, Australia). Corneal anesthesis and mydriasis were induced with 1 drop of 0.5% proxymetacaine hydrochloride (Ophthetic; Allergan, Frenchs Forest, NSW, Australia) and 0.5% tropicamide (Mydriacyl; Alcon Laboratories). General anesthesia was achieved with an intramuscular injection of 60 mg/kg ketamine and 5 mg/kg xylazine (Troy Laboratories, Smithfield, NSW, Australia). Corneal anesthesis and mydriasis were induced with 1 drop of 0.5% proxymetacaine hydrochloride (Ophthetic; Allergan, Frenchs Forest, NSW, Australia).

A calibrated flash (60CT4 Mecablitz; Metz-Werke GmbH, Zirndorf, Germany) was presented via a Ganzfeld bowl (LKC Technologies, Gaithersburg, MD) to yield a maximum energy of 1.8 log cd · s · m−2 (1.65 × 105 photoisomerizations/rod−1); all calibrations were per-

![Figure 1](https://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/933247/)
formed with an integrating photometer (IL-1700; International Light Technologies Inc., Newburyport, MA). Calibrated neutral-density filters were used to attenuate the flash.

Chlorided silver wire formed the active (cornea) and inactive (incisor) electrodes. These were referenced to a ground (27-gauge needle; Terumo Corp., Tokyo, Japan) inserted in the animal’s tail. Signals were amplified (×5000, 1 Hz−1 kHz, −3 dB; ML132 BioAmp; ADInstruments, Castle Hill, NSW, Australia) over a 200-ms epoch with data sampled at 10 kHz (ML785, Powerlab/8sp amplifier, with Scope software, ver. 3.6.10; ADInstruments). Line noise (50 Hz) was minimized by a noise-reduction system (Humbug; Quest Scientific Instruments Inc., North Vancouver, BC, Canada).

The rod photoreceptor a-wave (PIII) was quantified as a function of time (t, in seconds) and luminous energy (E, in cd·s·m−2) by its saturated amplitude (Eamp, in microvolts) and sensitivity (σ, in m2·cd−1·s−1) after a small delay (τ0, in seconds). Parameter optimization was achieved by minimizing a sum-of-square (SS) merit function over an ensemble of three energies (0.8, 1.1, and 1.8 log cd·s·m−2; e.g., Ref. 30, using the Solver module of Excel; Microsoft, Redmond, WA).

The b-wave generator (PII) was exposed after digital subtraction of the PIII from the raw waveform. The oscillatory potentials were extracted by minimizing a sum-of-square (SS) merit function over an ensemble of three energies (0.8, 1.1, and 1.8 log cd·s·m−2; thin lines: control; thick lines: STZ treated). An implicit time analysis of these experimental groups is given in Table 3. The number of animals in each group was CSO, 12; DSO, 12; CFO, 16; and DFO, 16. *Significant differences (P < 0.05) between control and STZ-treated groups. Scale bars: (horizontal) 30 ms; (vertical) 300 μV.

Immunofluorescence
Eye cups were dissected as described (n = 6/group) and fixed in 4% PF for 30 minutes. After fixation, the retinas were cryoprotected in graded sucrose solutions (10%, 20%, and 30%) and sectioned vertically (12 μm). Retinal sections were blocked for 1 hour (10% normal goat serum [NGS], 1% bovine serum albumin [BSA], and 0.05% Triton X-100 in 0.1 M PB). The primary antibodies (diluted in 3% NGS, 1% BSA, and 0.05% Triton X-100 in 0.1 M PB) were applied and left overnight at room temperature. Primary antibodies used in this study were rabbit anti-GFAP (1:40,000; Dako, Carpinteria, CA) and mouse anti-β-III tubulin (1:200; donated by Robert Molday, University of British Colombia, Vancouver, Canada). A secondary antibody (diluted 1:500 goat anti-rabbit AlexaFluor 488 or goat anti-mouse AlexaFluor 488; Molecular Probes, Eugene, OR) was applied for 1 hour. Peanut agglutinin (PNA conjugated with FITC, 1:100; Vector Laboratories, Burlington, CA) was applied for 1 hour to identify the cone photoreceptors. The sections were coverslipped and imaged with a microscope (×40 oil objective, Axioplan II; Carl Zeiss Meditec, Oberkochen, Germany). Thickness was measured for specific retinal layers and for the total retina (distance from the inner limiting membrane to photoreceptor outer segments) from the digital images. Six measurements were made on each image, with a minimum of 30 images analyzed from each retina (n = 6/group). Oblique sections were excluded from the analysis.

Histologic Analysis
After ERG recording, pentobarbitone (60 mg/kg IM; Merial, Parramatta, NSW, Australia) was administered to ensure deep anesthesia. The animals were decapitated before enucleation, and the eye cup formed by careful dissection of the cornea and lens. This preparation was fixed in 1% paraformaldehyde (PF)/2.5% glutaraldehyde overnight. The eye cups were rinsed with 0.1 M phosphate buffer (PB) and dehydrated in a graded series of cold methanol washes, ending with acetone. The tissue was embedded in Epon resin and incubated at 60°C overnight. The resin blocks were sectioned vertically (1 mm) within 5 mm of the optic nerve head and the sections etched in a sodium ethoxide-ethanol solution (1:5), rinsed in a graded series of methanol, and washed in 0.1 M phosphate buffer (PB). Sections where applied with toluidine blue (1:2) for 30 seconds, washed with dH2O, and coverslipped. The images were captured with a high-resolution camera (Megaplus; Eastman Kodak, Rochester, NY) mounted on a microscope (×40 oil objective, Axioplan II; Carl Zeiss Meditec, Oberkochen, Germany). Thickness was measured for specific retinal layers and for the total retina (distance from the inner limiting membrane to photoreceptor outer segments) from the digital images. Six measurements were made on each image, with a minimum of 30 images analyzed from each retina (n = 6/group). Oblique sections were excluded from the analysis.

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**Figure 2.** The initial 200 ms of representative rod ERG waveforms (left) for SO (top) and FO (bottom) animals at a bright flash exposure (1.8 log cd·s·m−2; thin lines: control; thick lines: STZ treated. Top left: a- and b-wave and OPs. Average a-wave amplitudes (absolute value, mean ± SEM) are shown over a range of exposures (right) for SO (top) and FO (bottom) groups (i.e., control; STZ treated). An implicit time analysis of these experimental groups is given in Table 3. The number of animals in each group was CSO, 12; DSO, 12; CFO, 16; and DFO, 16. *Significant differences (P < 0.05) between control and STZ-treated groups. Scale bars: (horizontal) 30 ms; (vertical) 300 μV.
Table 1. Average Retinal Concentrations of Select Omega-6 and -3 Long-Chain PUFA

<table>
<thead>
<tr>
<th></th>
<th>Controls</th>
<th>STZ Treated</th>
<th>Experimental Effect (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SAfflower Oil (n = 4)</td>
<td>Fish Oil (n = 4)</td>
<td>Diet (SO - FO)/SO</td>
</tr>
<tr>
<td></td>
<td>Fish Oil (n = 4)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>18:2 n-6 (LA)</td>
<td>0.57 ± 0.03</td>
<td>0.21 ± 0.05</td>
<td>—</td>
</tr>
<tr>
<td>20:4 n-6 (AA)</td>
<td>11.01 ± 0.5</td>
<td>9.51 ± 1.1</td>
<td>8.08 ± 2.3</td>
</tr>
<tr>
<td>22:4 n-6 (DPA n-6)</td>
<td>17.85 ± 1.00†</td>
<td>0.51 ± 1.7†</td>
<td>10.90 ± 2.70†</td>
</tr>
<tr>
<td>22:5 n-3 (DPA n-3)</td>
<td>0.24 ± 0.12‡</td>
<td>0.45 ± 0.07†</td>
<td>0.57 ± 0.11†</td>
</tr>
<tr>
<td>22:6 n-3 (DHA)</td>
<td>13.41 ± 1.44‡</td>
<td>33.40 ± 4.44</td>
<td>10.14 ± 2.54†</td>
</tr>
<tr>
<td>n-6/n-3</td>
<td>2.67 ± 0.19*</td>
<td>0.58 ± 0.14§</td>
<td>2.26 ± 0.26</td>
</tr>
</tbody>
</table>

Retinal Fatty Acid Analysis

Retinal phospholipids (n = 4/group) were quantified as described by Sinclair et al. After isolation, retinal tissue was immersed in chloroform-methanol 2:1 (containing 10 mg/L of butylated hydroxytoluene as an antioxidant) and stored at −20°C. Phospholipids were separated from the neutral lipids by thin-layer chromatography. The methyl esters of the phospholipids were formed from saponification and the fatty acids separated by gas chromatography (Shimadzu, Kyoto, Japan) with a 60 × 0.25-mm fused, silica-bonded phase column (BPX 70 SGE column; Shimadzu). The column oven was programmed from 125°C for 3 minutes, rising to 220°C at 8°C/min with helium (43 cm/s flow) used as a carrier. Fatty acids were identified from standards of known composition (Nuchek Prep, Elysian, MN).

Statistics

Significance was determined with a two-way ANOVA (SigmaStat, ver. 3.11, Systat Software, Inc., San Jose, CA) with diet (SO and FO) and treatment (control and diabetic) being the dependant variables. In the presence of a significant interaction or main effect, simple comparisons were performed with a Holm-Sidak test. α = 0.05 was adopted for all statistical evaluations. The experimental effects of diet and diabetes (percentage change) were identified by their relative contributions as: (1) a diabetes effect [(STZ - control)/control] for a given diet and (2) a diet effect [(SO - FO)/SO] across treatment groups (diabetes and control). Correlations were determined by nonparametric (Spearman) correlation coefficient (r).
metabolic index, apart from a small reduction in blood glucose in the FO groups (14%, \( P < 0.05 \)).

**Rod Function**

Having established that lipid composition of retinal membranes was altered by dietary intake of PUFA, we evaluated the effect that diet and diabetes had on retinal function. Representative rod ERG waveforms are shown in Figure 2. They indicate a general reduction in rod amplitudes in STZ-treated animals fed SO, which was particularly evident in signals obtained at higher exposures (Fig. 2, top right). In contrast, minimal change was found between rod responses in FO-fed animals (Fig. 2, bottom).

Notably, a statistically significant reduction in \( R_{\text{amp}} \) was not observed in all diabetic animals (Table 3). A significant decrease was evident in mean rod \( R_{\text{amp}} \) in SO-fed diabetic animals compared with the control diet-fed animals (−351 ± 38 vs. −474 ± 36, \( P < 0.05 \); Table 3, Fig. 3). This a-wave deficit was not present in diabetic animals fed FO, resulting in a significant diet effect (Table 3; \( P < 0.05 \)). A similar diet but not diabetes effect was also found for the sensitivity parameter (\( \sigma \), −57%, \( P < 0.000 \)) with FO-fed animals producing larger values. In contrast, diabetes produced a significant timing (\( t_{\text{delay}} \)) delay of 15.8% across both diets beyond the 12% delay found for the diet effect across both treatment groups (Table 3).

The b-wave was significantly reduced and delayed by both experimental manipulations (Figs. 3G, 3H). On average, amplitudes were reduced by diet (−32%) and diabetes (−24%: Table 3). Although FO significantly improved the b-wave amplitudes of diabetic animals (+62%; 528 µV vs. 857 µV; Table 3) it failed to return control values (857 µV vs. 973 µV; \( P < 0.05 \)). Implicit time showed significant diet (17.4%) and diabetes (14.6%)-related delays.

OP amplitudes were unaffected, but their dynamics were altered by diet to give significant reductions in frequency (−24.7%) and delayed implicit times (23.5%) across both treatment groups. Diabetes significantly decreased amplitudes (−42.1%), reduced frequency (−13.8%), and delayed implicit times (30.4%; Table 3) across both diets. In particular, STZ rats fed SO had decreased OP amplitudes (−52%, 75.2 vs. 155.8 µV, Table 3, \( P < 0.000 \)) and frequency (−12%, 108.5 vs. 95.3 Hz, \( P < 0.000 \)) and a timing delay (+31%, 41.7 vs. 31.7 ms; \( P < 0.000 \)) compared with their dietary controls. Significant improvements in OP frequencies (22%, 116.5 µV vs. 95.3 µV; \( P < 0.05 \)) and implicit times (24%, 31.7 ms vs. 41.7 ms; \( P < 0.000 \)) were observed in STZ rats fed FO compared with the SO group (Table 3) but these parameters remained significantly smaller than in FO controls (frequency −15%, 137.3 Hz vs. 116.5 Hz; \( P < 0.000 \); implicit time +29%, 31.7 ms vs. 24.6 ms; \( P < 0.000 \)). Although FO diets improved OP amplitudes of STZ rats compared with the SO group (+45%, 75.2 µV vs. 108.8 µV; \( P = 0.004 \)) they failed to reach control amplitudes (Table 3, 162 µV) and might simply reflect downstream improvements in PII (+61%) or PII (+62%, Table 3).

A significant correlation was present between retinal DHA and both the PII (Fig. 3: \( r_c = 0.48 \), \( P < 0.05 \)) and PII (\( r_c = 0.42 \), \( P < 0.05 \)) amplitudes, indicating photoreceptor and bipolar cell dependency on DHA. There was no significant correlation between these ERG parameters and blood glucose (PII, \( r_c = −0.15 \), \( P = NS \); PII, \( r_c = −0.29 \), \( P = NS \)). This lack of correlation for PII is consistent with the previous observation of a significant diabetes effect, as Figure 3J shows that most of this group effect was driven by the low amplitudes in the SO group. In contrast, OP amplitudes correlated significantly with blood glucose alone (\( r_c = −0.53 \), \( P < 0.01 \)), confirming that OP inner retinal generators were influenced by hyperglycemia rather than by DHA.

**Cone Function**

Representative cone waveforms are shown in Figure 4, where a reduction in cone amplitude is evident in STZ animals, irrespective of diet (Figs. 4B, 4D). This is confirmed by the lack of a diet effect on cone b-wave amplitudes and a significant diabetes effect (Table 3; −42.9%); implicit times were increased uniformly (Table 3, 6.7%−7.1%) by both treatments. Reduced cone amplitudes (DSO −43%, \( P < 0.05 \); DFO −42%, \( P < 0.05 \)) and implicit time delays (DSO +4% \( P = 0.04 \); DFO +9% \( P < 0.000 \)) were found in STZ animals relative to the control diet subjects. As with the OPs a significant correlation was found only between cone amplitude and blood glucose (\( r_c = −0.64 \), \( P < 0.01 \)), but not DHA.

**Histologic Analysis**

Having established that the extent of retinal dysfunction during diabetes is dependent on diet, we examined whether this correlates with a change in cellular integrity. For example, a reduction in photoreceptor function might be explained by a reduction in the number of photoreceptors or thickness of the

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**Table 3. ERG Parameters Measured at 13 Weeks after STZ Injection**

<table>
<thead>
<tr>
<th></th>
<th>Controls</th>
<th>Fish Oil</th>
<th>STZ Treated</th>
<th>Experimental Effect (%)</th>
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<tbody>
<tr>
<td></td>
<td>(n = 12)</td>
<td>(n = 16)</td>
<td>(n = 12)</td>
<td>(n = 16)</td>
</tr>
<tr>
<td>Rod a-wave</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>( R_{\text{amp}} ), µV</td>
<td>−474 ± 36†</td>
<td>−560 ± 30.6†</td>
<td>−351 ± 38.0†</td>
<td>−567 ± 31.5†</td>
</tr>
<tr>
<td>( \sigma ), mV · cd⁻¹ · s⁻³</td>
<td>1932 ± 258†</td>
<td>3528 ± 216†</td>
<td>2611 ± 260†</td>
<td>3600 ± 223†</td>
</tr>
<tr>
<td>( t_{\text{delay}} ), ms</td>
<td>3.28 ± 0.07†</td>
<td>2.75 ± 0.06†</td>
<td>3.62 ± 0.08†</td>
<td>3.34 ± 0.06†</td>
</tr>
<tr>
<td>Rod b-wave</td>
<td></td>
<td></td>
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<tr>
<td>Amplitude, µV</td>
<td>854.7 ± 51.3†</td>
<td>973.8 ± 43.1†</td>
<td>528.1 ± 53.5†</td>
<td>857.0 ± 44.4†</td>
</tr>
<tr>
<td>Implicit time, ms</td>
<td>71.9 ± 1.5†</td>
<td>60.0 ± 1.2†</td>
<td>83.1 ± 1.0†</td>
<td>68.0 ± 1.3†</td>
</tr>
<tr>
<td>Rod OPs</td>
<td></td>
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<tr>
<td>Amplitude, µV</td>
<td>155.8 ± 11.0°</td>
<td>162.0 ± 9.2°</td>
<td>75.2 ± 11.5°</td>
<td>108.8 ± 9.5°</td>
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<tr>
<td>Frequency, Hz</td>
<td>108.3 ± 3.35†</td>
<td>137.3 ± 2.82†</td>
<td>95.3 ± 3.50†</td>
<td>116.5 ± 2.90†</td>
</tr>
<tr>
<td>Implicit time, ms</td>
<td>31.7 ± 0.67†</td>
<td>24.6 ± 0.52†</td>
<td>41.7 ± 0.65†</td>
<td>31.7 ± 0.54†</td>
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<tr>
<td>Cone b-wave</td>
<td></td>
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<tr>
<td>Amplitude, µV</td>
<td>378.1 ± 20.5°</td>
<td>398.0 ± 17.2°</td>
<td>214.3 ± 21.4°</td>
<td>228.6 ± 17.8°</td>
</tr>
<tr>
<td>Implicit time, ms</td>
<td>59.7 ± 1.2°</td>
<td>54.2 ± 0.97†</td>
<td>62.5 ± 1.2°</td>
<td>59.2 ± 1.0°</td>
</tr>
</tbody>
</table>

* Significant (\( P < 0.05 \)) treatment effect (STZ vs. control) within a diet.
† Significant (\( P < 0.05 \)) difference between FO- and SO-fed animals within a treatment group (control vs. STZ).
outer nuclear layer. Figure 5A shows that dietary manipulation and hyperglycemia did not significantly (*P < 0.05, NS) alter the thickness of the outer nuclear, inner nuclear, or inner plexiform layers or total retinal thicknesses. We then used immunocytochemical labeling as a means of detecting gross abnormalities in photoreceptor integrity. As shown in Figure 5, no differences in the number of rods immunolabeled for rhodopsin (Fig. 5C) or cones labeled for cone-opsin (Fig. 5D) were observed between control and diabetic animals. These findings suggest that no significant loss of photoreceptors occurred in any of the experimental treatments examined. However, it should be noted, that we did not perform a quantitative analysis of the level of rhodopsin or cone opsin immunolabeling in photoreceptors and therefore cannot exclude the possibility that the level of opsin expression may have been altered in some of the experimental conditions.

We next examined whether gliosis, a sensitive marker of retinal stress, showed any differences across the four experimental groups. In the normal rodent retina, glial fibrillary acidic acid (GFAP) labels astrocytes within the nerve fiber layer,35 and Müller cells show only very low levels of labeling. In contrast, diabetes, like other retinal diseases, is associated with an increase in GFAP immunolabeling of Müller cells. Figure 5E shows vertical sections immunolabeled with GFAP. Although diabetes was associated with an increase in GFAP labeling in Müller cells, there was no discernable diet effect (not shown).

**DISCUSSION**

The main finding of this study is that omega-3 deficiency can modify some (but not all) biomarkers of diabetes. Rod photoreceptor dysfunction can be prevented by FO consumption. Our past work indicated that FO diets affect lipid profiles in other tissues, especially in the brain, heart, and liver, implying the potential for generalized systemic involvement. However, in our study, changes in rod function were found in the presence of normal systemic manifestations of diabetes (Table 2), indicating that the protective effect of FO was local to the retina.

**Rod Photoreceptor and Inner Retinal Dysfunction in Diabetes**

A large body of evidence indicates abnormal ERGs in diabetic patients and animal models of diabetes.2–5,8–13 It is well recognized that OPs are abnormal early in diabetes37 and can be useful predictors of progression.13,38 More recently, abnormalities in photoreceptor function have been characterized in both patients13 and animal models.8,10 Our previous work has
shown that diabetes causes a reduction in photoreceptor and inner retinal function from as early as 2 days after STZ injection.8

In the present study, animals fed SO showed significant diet-related reductions in rod photoreceptor amplitudes and sensitivity, consistent with the literature on DHA deprivation.18,39 Thus, it is not surprising that FO intake modified rod photoreceptor function. The fact that photoreceptor function mirrored retinal DHA profiles, indicates that the rod photoreceptors are more dependent on PUFA intake than the presence of hyperglycemia.

Given the potential for confounding the diet and diabetes effects, we have isolated these in our analysis and show key aspects in Figure 6. It is evident from the top panel that, although FO diets significantly improved control PIII and PII waveforms (Fig. 6B, striped bars), as reported elsewhere,17,18,39 the effect on the diabetic animals (Fig. 6B, gray bars) was four times greater and was particularly marked for the OPs. On the other hand, cone waveforms (Fig. 6) and GFAP labeling (Fig. 5) showed little dietary effect. In a similar manner, the bottom panel confirms the presence of a significant and selective diabetes effect in SO diets (Fig. 6B, black bars) for the PIII and PII which was expressed in both diets for OPs and cone amplitudes. From these findings we can conclude that: (1) rod PIII and PII amplitudes are diet dependent; (2) the cone b-wave amplitude is unaffected by diet and is entirely diabetes dependent; and (3) the OPs have a dietary overlay on a predominantly diabetic effect.

Although there were beneficial effects of diet on amplitude, the FO diet did not normalize α- or b-wave implicit times (Table 3), indicating that diabetes is associated with timing deficiencies in retinal processing, as reported by others.3,9 Timing abnormalities of multifocal ERG waveforms in humans have been shown to be predictive of serious retinal complications.3 Anatomic assessment failed to detect abnormality that could account for the functional changes. Neither hyperglycemia nor diet affected retinal thickness. There were no notable differences in rod and cone photoreceptor density or structure, indicating that the functional changes induced by diabetes and diet cannot be a direct consequence of anatomic alteration consistent with the literature on dietary effects,39,40 although the anatomic integrity of the diabetic retina is debatable. Some groups report a reduced number of photoreceptors and shorter outer segments5 or increased apoptosis,6 whereas others find minimal change.41 This disparity might reflect the variable duration or severity of diabetes and its hyperglycemic
control. Nevertheless, our findings indicate that the changes observed in the present study most likely reflect changes to retinal lipid profiles rather than being secondary to changes in neural morphology.

Oxidative Stress in Diabetes

To our knowledge, only one other group has considered the effect of FO supplementation in the retina of diabetic animals. Hammes et al.25 showed that FO administration after diabetogenesis in STZ rats exacerbated retinal pericyte loss and increased oxidative stress. It is possible that their finding reflects markers not affected by diet, as we show for the cone response and GFAP (see earlier), although this would conflict with findings made in the heart and liver by others for decreased oxidative stress in diabetic rats fed FOs.21 Alternatively, it might arise from the inadequate antioxidant cover provided in the study of Hammes et al.25 who recognized, in the discussion section of their study, that this limitation would have promoted oxidative processes. Adequate antioxidants were provided in the present study (see Supplementary Table S1) to ensure that the dietary effects would not be contaminated by oxidation.

Many studies implement FO diets immediately after or just before diabetogenesis, limiting the amount of DHA that can be incorporated into tissues by such short-term interventions.42 Our feeding paradigm (from conception) was adopted to ensure maximum membrane effects, to establish proof of principle that adequate omega-3 tissue levels could protect against diabetes. As such, our dietary intervention would replicate that of people who traditionally consume large amounts of fish over their lifetime (e.g., Greenland Eskimos), in whom a low prevalence of diabetes has been reported and the regular consumption of seal meat is protective against diabetes.43 However, we acknowledge that the benefits may have invoked adult onset as well as developmental changes (e.g., Ref. 22), and so these differences should be borne in mind when comparing our findings with those of others.

Table 1 shows a buildup of 22:5 n-3 in diabetes (89%). Figure 1 indicates that such a buildup would be found either in an elongase deficiency (22:5 to 24:5) or due to a diabetes-related Δ6 desaturase deficit that reduces the metabolism of 24:5 n-3, as evident in the 69% increase in 18:2 n-6. If this were so, then our finding indicates that consumption of a typical Western diet, low in long-chain omega-3 metabolites (EPA and DHA), promotes a relative omega-3 PUFAs deficiency in people who have diabetes. The present study shows that adequate dietary FO can prevent this problem for key long-chain substrates (Table 1), indicating that the uptake and trafficking of these substrates is unaffected by diabetes. Moreover, our data show that this diet has the beneficial effect of reducing blood glucose by 14% (Table 2).

In this study, rod photoreceptor function did not correlate with blood glucose but did correlate with DHA concentrations, indicating that the photoreceptor is highly dependent on DHA levels in retinal membranes. This result is not surprising, given that photoreceptors have one of the highest levels of DHA in the body, and a deficiency in DHA leads to retinal dysfunction.18,59

Numerous roles for DHA have been proposed in normal cellular physiology and disease. It has been shown to affect the content and activation of rhodopsin,39 decrease the activation of PKC,44 reduce oxidative damage,23 and downregulate VEGF expression.45 Moreover, dietary supplementation with FO can modify the microvascular response in a positive manner, as omega-3 PUFAs are well recognized for their antiplatelet action46 and other positive effects on vascular endothelium.47

The influence of DHA on plasma membranes and rhodopsin content has been well documented.59,68 Our FO group could be expected to have larger a-waves because their greater levels of retinal DHA will alter rhodopsin content, increasing photon absorption.39 However, whether this mechanism can express in diabetes is not known. On the other hand, Na/K ATPase is a rod transmembrane-bound protein involved in sustaining the
ATPase activity in diabetes, so some of the benefits noted in group. FO supplementation has been shown to improve Na/K would explain the smaller a-waves of our omega-3-deficient our FO-fed diabetic animals might be due to improved ATPase exclude the possibility that additional beneficial effects may arise solely from the dietary DHA in our FO diet, as the retinas of these animals had normal DHA levels (despite the diabetes) which would explain the smaller a-waves of our omega-3-deficient group. FO supplementation has been shown to improve Na/K ATPase activity in diabetes, so some of the benefits noted in our FO-fed diabetic animals might be due to improved ATPase activity (Phipps JA, et al. IOVS 2004;45:ARVO E-Abstract 3233), either alone or in concert with changes in rhodopsin levels. It is most likely that the beneficial effects found in this study arose solely from the dietary DHA in our FO diet, as the retinas of these animals had normal DHA levels (despite the diabetes) and these correlated with rod function. This finding does not exclude the possibility that additional beneficial effects may arise from the short-chain omega-3 substrate (α-linolenic acid) present in flaxseed oil.

**Cause of Retinal Dysfunction in Diabetes**

The persistence of a cone abnormality and an inner retinal dysfunction (OP), despite FO supplementation in diabetic animals indicates that these anomalies reflect the hyperglycemic milieu, rather than a PUFA anomaly. OP dysfunction has been established in diabetic patients and animals as one of the earliest retinal changes; however, the underlying mechanism is unknown. Multiple metabolic anomalies occur in the inner retina during diabetes, including hypoxia and alterations in GABA metabolism, and these can modify both cone and OP wavelets. However, the cone loss may also be associated with the glial dysfunction, as found in the present study and by others.

In this study, glial reactivity in diabetic animals was increased in the Müller cell processes. In particular, no differences were observed between the two diet groups, indicating that FO supplementation did not prevent the diabetes-induced glial change. Given that these glial changes mirror the functional losses of cones and in the inner retina (OPs), they might indicate an underlying disease in our animals. As glia support neuronal metabolism and function and have a role in buffering neural generated ions, they are well placed for mediating such changes, but the precise relationships between cone and OP function and glial dysfunction must be clarified in future studies.

In this study, FO supplementation was protective against diabetes-related rod photoreceptor dysfunction, and so previous reports of these losses (e.g., Refs. 8, 10) might reflect a diabetes-induced change in DHA rather than hyperglycemia. In fact, the absence of this effect in STZ-treated rats might reflect either the diet used in those studies or the time of intervention, as retinal depletion of DHA requires a long period. However, as membrane DHA is essential in many biological processes, such as the generation of eicosanoids and not just retinal function, the possibility of delaying diabetes-related complications through dietary intervention seems promising. Furthermore, the intake of FOs may reduce the tendency toward diabetic retinopathy, given its beneficial effects on eicosanoid production as well as neural function. As an aside, that our irregular insulin injections failed to reverse the systemic indices (Table 2) and some neuronal dysfunction (Table 3) means that further work is needed to establish the dose-response relationships between these indices and insulin.

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**FIGURE 6.** Summary of diet (A) and treatment (B) effects for rod (Pill, a-wave; Pill, b-wave; and OPs) and cone waveform amplitudes expressed relative to a control condition (diet effect = [(SO – FO)/SO]; diabetic effect = (STZ – Con)/Con). (A) Relative benefits of FO intake for control animals (■) and STZ (□) animals relative to the SO diet. Left: rod-generated data; right: cone-generated data. (B) Relative effect of diabetes for FO-fed (□) and SO-fed (■) groups. Letters: significant (P < 0.05) differences within a treatment main effect and the abscissa within treatments. #Significant (P < 0.05) difference within a common component.
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