Remodeling of Retinal Fatty Acids in an Animal Model of Diabetes
A Decrease in Long-Chain Polyunsaturated Fatty Acids Is Associated With a Decrease in Fatty Acid Elongases Elovl2 and Elovl4

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OBJECTIVE—The results of the Diabetes Control and Complications Trial/Epidemiology of Diabetes Interventions and Complications cohort study revealed a strong association between dyslipidemia and the development of diabetic retinopathy. However, there are no experimental data on retinal fatty acid metabolism in diabetes. This study determined retinal-specific fatty acid metabolism in control and diabetic animals.

RESEARCH DESIGN AND METHODS—Tissue gene and protein expression profiles were determined by quantitative RT-PCR and Western blot in control and streptozotocin-induced diabetic rats at 3–6 weeks of diabetes. Fatty acid profiles were assessed by reverse-phase high-performance liquid chromatography, and phospholipid analysis was performed by nano-electrospray ionization tandem mass spectrometry.

RESULTS—We found a dramatic difference between retinal and liver elongase and desaturase profiles with high elongase and low desaturase gene expression in the retina compared with liver. Elovl4, an elongase expressed in the retina but not in the liver, showed the greatest expression level among retinal elongases, followed by Elovl2, Elovl1, and Elovl6. Importantly, early-stage diabetes induced a marked decrease in retinal expression profiles of Elovl4, Elovl2, and Elovl6. Diabetes-induced downregulation of retinal elongases translated into a significant decrease in total retinal docosahexaenoic acid, as well as decreased incorporation of very-long-chain polyunsaturated fatty acids (PUFAs), particularly 32:6n3, into retinal phosphatidylcholine. This decrease in n3 PUFAs was coupled with inflammatory status in diabetic retina, reflected by an increase in gene expression of proinflammatory markers interleukin-6, vascular endothelial growth factor, and intercellular adhesion molecule-1.

CONCLUSIONS—This is the first comprehensive study demonstrating diabetes-induced changes in retinal fatty acid metabolism. Normalization of retinal fatty acid levels by dietary means or and modulating expression of elongases could represent a potential therapeutic target for diabetes-induced retinal inflammation. Diabetes 59:219–227, 2010

Early diabetic retinopathy has been suggested to be a low-grade chronic inflammatory disease (1–3) with a number of inflammatory markers, such as vascular endothelial growth factor (VEGF) (4,5), intercellular adhesion molecule (ICAM)-1 (6,7), tumor necrosis factor (TNF)-α (8), and interleukin (IL)-6 (9), shown to be upregulated in diabetic retina. The individual molecular steps leading to inflammation in the retina are not well resolved but likely involve hyperglycemia and dyslipidemia associated with diabetes.

Dyslipidemia is a major metabolic disorder of diabetes, and the Diabetes Control and Complications Trial/Epidemiology of Diabetes Interventions and Complications cohort study revealed that dyslipidemia was significantly associated with the development of diabetic retinopathy (10). Diabetic dyslipidemia is the result of an imbalance in the complex regulation of lipid uptake, metabolism, release by adipocytes, and clearance from circulation (11,12). Fatty acid metabolism perturbation in diabetes is an important part of diabetic dyslipidemia (13).

To understand the effects of diabetes on plasma and tissue fatty acid composition, two metabolic routes have to be considered: de novo lipogenesis and the polyunsaturated fatty acid (PUFA) remodeling Sprecher pathway (14). Saturated fatty acids (SRAs), mono-unsaturated fatty acids (MUFA), and PUFAs are synthesized from dietary precursors (glucose, palmitic 16:0, oleic18:1n9, linoleic18:2n6, α-linolenic18:3n3, eicosapentaenoic acid [EPA20:5n3], and docosahexaenoic acid [DHA22:6n3]) through a series of desaturation (Δ5-desaturase [Δ5D], Δ6-desaturase [Δ6D], or Δ9-desaturase [Δ9D]) and elongation (Elovl1–7) reactions. In the recent work by Agbaga et al. (15), the Sprecher pathway was expanded to include very-long-chain PUFAs (VLCPUFAs), up to 38 carbon fatty acids.

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acids, in which elongation of shorter-chain fatty acids precursors is performed by Elovl4 (Fig. 1). Elovl4 has very limited tissue specificity. It is highly expressed in the retina (16–18), thymus, and skin (17), as well as at lesser levels in the brain (17,18) and testis (18). Elovl4 is not expressed in the liver (17,18). In human retina, Elovl4 was shown to be primarily expressed in the inner segment of photoreceptors extending to photoreceptor cell bodies in the outer nuclear layer (19). Moderate labeling was also observed in the ganglion cells (19). Elovl4 has received much attention recently, as an autosomal-dominant Stargardt-like macular dystrophy was linked to several dominant-negative mutations in Elovl4 (19–21). The role of VLCPUFAs produced by Elovl4 is not known, but because of their localization in retinal outer-segment membranes and their ability to span both leaflets of the lipid bilayer, they are suggested to play a role in stabilizing cellular membranes with high curvature, such as the rims of photoreceptor disks (15). Fatty acid desaturase enzymes are known to be inhibited in diabetes (22–24), and there is emerging information suggesting that certain elongases might also be affected (25). Thus, diabetes may result in reduced fatty acid remodeling and, consequently, lead to an accumulation of the substrates and depletion of the products. The elongases Elovl2 and Elovl6 are ubiquitously expressed in most tissues; however, retina expresses Elovl2 at a very high level. Elovl2 is involved in several steps of DHA\textsubscript{22:6n3} biosynthesis (26). Retina has a unique fatty acid profile with one of the highest levels of long-chain PUFAs, especially DHA\textsubscript{22:6n3}, in the body (27). We have previously demonstrated that Elovl4 is necessary for synthesis of C26 and C28 VLCPUFAs from 24:5n3 and 24:6n3 fatty acid precursors and suggests that Elovl4 is also required for synthesis of >C28 VLCPUFAs. There is no interconversion between n3, n6, and n9 fatty acids in animals.

FIG. 1. De novo lipogenesis and PUFA remodeling pathways. Fatty acids are synthesized from glucose through de novo lipogeneses or converted from dietary palmitic 16:0, oleic 18:1n9, linoleic 18:2n6, and \( \alpha \)-linolenic 18:3n3 acids to long-chain unsaturated fatty acids in vivo by a series of desaturation (\( \Delta 5 \)-desaturase \[ \Delta 5D \], \( \Delta 6 \)-desaturase \[ \Delta 6D \], or \( \Delta 9 \)-desaturase \[ \Delta 9D \]) and elongation (Elovl1–7) reactions. Fatty acids that accumulate in animal and human tissues are in solid boxes. Dietary linoleic 18:2n6 and \( \alpha \)-linolenic 18:3n3 acids are obtained from plants, and EPA\textsubscript{20:5n3} and DHA\textsubscript{22:6n3} are rich in fish oil. A recent study demonstrated that Elovl4 is necessary for synthesis of C26 and C28 VLCPUFAs from 24:5n3 and 24:6n3 fatty acid precursors and suggests that Elovl4 is also required for synthesis of >C28 VLCPUFAs. There is no interconversion between n3, n6, and n9 fatty acids in animals.
**RESEARCH DESIGN AND METHODS**

**Reagents.** High-performance liquid chromatography (HPLC)-grade acetonitrile, acetic acid, methanol, chloroform, streptozotocin (STZ), and commonly used chemicals and reagents were from Sigma-Aldrich Chemical (St. Louis, MO).

**Animals and induction of STZ-induced diabetes.** Male Sprague-Dawley rats weighing 250–290 g were made diabetic with a single intraperitoneal injection of 65 mg STZ per kg body wt. Body weight gains and blood glucose levels for the control and STZ-induced diabetic groups were monitored biweekly. At 3–6 weeks after STZ injection, the animals were killed and blood plasma, liver, and retina were recovered for analyses of fatty acid profiles and/or fatty acid elongase and desaturase expression levels. To isolate the retina, the optic nerve was cut; the eye was opened; the cornea, and lens were discarded; and the retina was separated from choroid, washed in PBS, and frozen. Rats were maintained on Harlan-Teklad laboratory diet (no. 8640) and water ad libitum. The fatty acid composition of the diet was analyzed by reverse-phase HPLC (RP-HPLC, see below) and found to be 16:0, 20.0%; 18:0, 1.8%; 18:1n9, 21.0%; 18:2n6, 50.8%; 18:3n3, 5.0%; 18:3n6, 0.1%; 20:4n6, 0.1%; 20:5n3, 0.4%; 22:5n3, 0.1%; and 22:6n3, 0.3%. Animal protocol was approved by the Michigan State University Institutional Animal Care and Use Committee. All experiments followed the guidelines set forth by the Association for Research in Vision and Ophthalmology Resolution on Treatment of Animals in Research.

**RNA and protein isolation.** Rat tissues were homogenized in Trizol reagent (Invitrogen), and RNA was isolated according to manufacturer instructions. After adding chloroform, the upper aqueous phase was separated and RNA was precipitated with isopropanol alcohol, washed with 75% ethanol, and redissolved in RNase-free water. Proteins from the same samples were isolated by washing in Tris buffer (30 mmol Tris-HCl, pH 6.8, with 0.1% SDS) followed by concentration on Amicon-15 (Millipore) centrifugal filters. After concentration, protease/phosphatase inhibitor cocktail was added and samples were frozen until further analysis.

**Real-time qRT-PCR.** Transcript-specific primers were designed using Primer3 software (available at http://frodo.wi.mit.edu/primer3/). First-strand cDNA was synthesized using the SuperScript II RNase H Reverse Transcriptase (Invitrogen, Carlsbad, CA), and PCRs were performed in triplicate as previously described (25). Transcripts of interest were normalized to the abundance of cyclophilin mRNA. Rat gene–specific primers used in this study were Elovl4: GAAGTGGATGAAAGACCGAGA (sense) and GGCCTTGATGATCCCATGAA (antisense); Elovl7: TGGGCTTGACGACATCT TAC and GATGATGTGGTGAGAGAG; IL-6: CCAGGAAATTTGCCTATTGA and GCTCTGAGAATGCCTCTTGTTT; and cyclophilin: GCCATTAGATCTCTTGTG TTG and VEGF A: GCCCTCTGTTGCACCTGG and CACCACTCATGGCCTTC; and ICAM-1: CCACCATACCTGTGAT TCGTCT and ACGCACAGACACTGAGA. All other primers for rat elongases were designed previously (25).

**Western blotting.** Protein concentration was determined by a Qubit fluorometer (Invitrogen), according to manufacturer’s instructions, and equivalent amounts of protein were loaded on the SDS-polyacrylamide (10%) minigels for Western blotting. Protein concentration was determined by a Qubit fluorometer (Invitrogen), according to manufacturer’s instructions. A decrease of arbitrary protein masses from that see below) and found to be 16:0, 20.0%; 18:0, 1.8%; 18:1n9, 21.0%; 18:2n6, 50.8%; 18:3n3, 5.0%; 18:3n6, 0.1%; 20:4n6, 0.1%; 20:5n3, 0.4%; 22:5n3, 0.1%; and 22:6n3, 0.3%. Animal protocol was approved by the Michigan State University Institutional Animal Care and Use Committee. All experiments followed the guidelines set forth by the Association for Research in Vision and Ophthalmology Resolution on Treatment of Animals in Research.

**Lipid extracts were introduced to a triple-quadrupole mass spectrometer (Thermo Scientific model TQF Quantum Ultra, San Jose, CA) for nano-electrospray ionization tandem mass spectrometry (nESI-MS/MS) analysis of lipid species as previously described (29). Identification of phospholipid species by precursor ion and neutral loss scan mode MS/MS was performed according to published methods (30,32). Assignment of phosphatidylycerol (GPCho) acyl substituents was achieved by negative ion mode mass analysis of corresponding GPCho [M + CI]– ions by product ion scan mode MS/MS, as well as by precursor ion scanning for m/z corresponding to specific deprotonated fatty acyl ions. Peak finding and correction for 13C isotope effects was performed using the Lipid Mass Spectrum Analysis (LIMSA) software version 1.0 peak model fit algorithm (33). Quantitative analysis of the relative changes in GPCho lipid abundances between control and diabetic samples was achieved by normalization of the peak area of each detected GPCho m/z to that of the GPCho(18:1/16:0) lipid present in each of the samples, after correction for 13C isotope contributions.

**Statistical analysis.** Data are expressed as the means ± SD. Student’s t test was used for comparing data obtained from independent samples. Significance was established at P < 0.05.

**RESULTS**

**Body weight gain and blood glucose concentrations of experimental animals.** As presented in Table 1, body weight gain was significantly slower in diabetic animals compared with control animals. Blood glucose levels were almost five times higher in diabetic animals compared with controls. As this was a short-term diabetes study, A1C levels were not measured.

| Control animals | n | Weight gain (g/day) | Blood glucose (mmol/l) |
|----------------|---|--------------------|------------------------|
| 4             |    | 4.05 ± 0.65        | 4.33 ± 0.29            |
| Diabetic animals | 7 | 2.32 ± 0.89        | 20.80 ± 1.16           |

Data are means ± SD.

**Lipid analysis by nano-electrospray ionization tandem mass spectrometry.** Lipid extracts were introduced to a triple-quadrupole mass spectrometer (Thermo Scientific model TQF Quantum Ultra, San Jose, CA) for nano-electrospray ionization tandem mass spectrometry (nESI-MS/MS) analysis of lipid species as previously described (29). Identification of phospholipid species by precursor ion and neutral loss scan mode MS/MS was performed according to published methods (30,32). Assignment of phosphatidylycerol (GPCho) acyl substituents was achieved by negative ion mode mass analysis of corresponding GPCho [M + CI]– ions by product ion scan mode MS/MS, as well as by precursor ion scanning for m/z corresponding to specific deprotonated fatty acyl ions. Peak finding and correction for 13C isotope effects was performed using the Lipid Mass Spectrum Analysis (LIMSA) software version 1.0 peak model fit algorithm (33). Quantitative analysis of the relative changes in GPCho lipid abundances between control and diabetic samples was achieved by normalization of the peak area of each detected GPCho m/z to that of the GPCho(18:1/16:0) lipid present in each of the samples, after correction for 13C isotope contributions.

**Statistical analysis.** Data are expressed as the means ± SD. Student’s t test was used for comparing data obtained from independent samples. Significance was established at P < 0.05.
Blood plasma fatty acid profiles of control and diabetic animals. The control and diabetic blood plasma fatty acid profiles 3 weeks after STZ injection are presented in Table 2. There was a tendency toward higher total fatty acids level in diabetic versus control blood plasma. We observed changes in the plasma fatty acid profile consistent with inhibition of fatty acid remodeling in diabetes that leads to a lower end product–to–precursor fatty acid ratio. There was a decrease in two major end products of the PUFA synthesis pathway, arachidonic acid and DHA22:6n3, relative to their precursors, linoleic acid and α-linolenic acid, respectively (Table 2). As a result of these changes, we observed a decrease in unsaturation index (the number of double bonds per fatty acyl residue) and a decrease in long-chain–to–short-chain PUFA ratio in diabetic versus control animals (Table 2).

Liver fatty acid profiles of control and diabetic animals. The control and diabetic liver fatty acid profiles 3 weeks after STZ injection are presented in Table 2. There was an increase in the linoleic acid level in the livers of diabetic versus control animals that led to a decrease in long-chain–to–short-chain PUFA ratio (Table 2). There were no other significant changes in liver fatty acid profiles in diabetic versus control animals. The liver unsaturation index and PUFA synthesis pathway end product–to–precursor ratios did not change in diabetic versus control animals (Table 2).

Retinal fatty acid profiles of control and diabetic animals. Retina has a unique fatty acid profile, with the highest content of long-chain PUFAs in the body. In agreement with other studies, retinal profiles were very rich in DHA22:6n3 and arachidonic acid (Table 4). The levels of linoleic acid and α-linolenic acid in the retina were very low; thus we did not calculate the PUFA synthesis pathway end product–to–precursor ratios. Importantly, the retinas of diabetic animals had 28% less DHA22:6n3 compared with controls. As a result, we observed a decrease in unsaturation index, a decrease in long-chain–to–short-chain PUFA ratio, and a decrease in the n3-to-n6 PUFA ratio in the retinas of diabetic versus control animals (Table 4). Representative RP-HPLC chromatograms of saponified fatty acids from control and diabetic retina are presented in Fig. 3A.

Retinal and erythrocyte phospholipid profiles of control and diabetic animals. In agreement with saponified fatty acid profile data, nESI-MS/MS analysis of the retinal lipid extracts of diabetic animals (n = 3) showed a significant (up to 34%) decrease in the abundance of glycerophospholipids containing DHA22:6n3 compared with the control animals (n = 3). For example, compared with the abundance of the GPCho(18:0/22:6) and GPCho(22:6/22:6) lipids in the ratiometric analysis shown in Fig. 3B. Similar decreases in the abundances of DHA22:6n3 containing lipids were also observed for glycerophosphoethanolamine and glycerophosphoserine lipids (data not shown). In contrast, an increase (37%) in the abundance of the linoleic acid–containing GPCho(16:0/18:2) lipid was observed for the same samples as shown in Fig. 3B. In addition to known fatty acids identified by HPLC analysis, nESI-MS/MS analysis revealed several VLCPUFAs, primarily 32:6n3 and 34:6n3, esterified to GPCho in the retina. Interestingly, GPCho(32:6/22:6) was significantly decreased (24%) in diabetic retinas compared with controls, and there was a nonsignificant decrease (9%) of GPCho(34:6/22:6).

In erythrocyte lipid extracts, an increase in the abundance of linoleic acid–containing lipids, namely GPCho(16:0/18:2) and GPCho(18:0/18:2), was observed between the diabetic and control samples, consistent with the changes in retina lipids, as shown in Fig. 3C. Erythrocytes had very low levels of DHA-containing phospholipid species, and there was no effect of diabetes on these species. There was no detectable GPCho(32:6/22:6) or GPCho(34:6/22:6).
TABLE 2
Blood plasma fatty acid profiles of control (n = 4) and diabetic (n = 7) animals

| Fatty acids                     | Control animals | Diabetic animals | Difference | P  |
|---------------------------------|-----------------|-----------------|------------|----|
| Total (nmol/mg protein)         | 2,262.55 ± 639.64 | 3,286.54 ± 766.69 |            | 0.0972 |
| Mole % of total fatty acids     |                 |                 |            |    |
| 16:0 (palmitic)                 | 2.19 ± 1.17        | 3.70 ± 0.46      | ↑          | 0.0362* |
| 18:0 (stearic)                  | 2.25 ± 0.34        | 2.08 ± 0.40      |            | 0.5639 |
| 18:1n9 (oleic)                  | 12.65 ± 1.03       | 13.43 ± 1.74     |            | 0.5126 |
| 18:2n6 (linoleic)               | 54.12 ± 2.31       | 57.44 ± 2.62     |            | 0.1207 |
| 18:3n3 (α-linolenic)            | 1.93 ± 0.32        | 2.71 ± 0.36      | ↑          | 0.0214* |
| 18:3n6 (γ-linolenic)            | 0.31 ± 0.13        | 0.51 ± 0.37      |            | 0.4028 |
| 20:3n6 (dihomo-γ-linolenic)     | 0.84 ± 0.12        | 0.87 ± 1.15      |            | 0.9631 |
| 20:3n9 (mead)                   | 0.52 ± 0.32        | 0.35 ± 0.30      |            | 0.4636 |
| 20:4n6 (arachidonic)            | 21.29 ± 2.70       | 15.90 ± 1.95     | ↓          | 0.0161* |
| 20:5n3 (eicosapentaenoic)       | 0.53 ± 0.08        | 0.51 ± 0.03      |            | 0.7449 |
| 22:5n3 (docosapentaenoic)       | 0.84 ± 0.22        | 0.54 ± 0.04      | ↓          | 0.0235* |
| 22:6n3 (docosahexaenoic)        | 2.78 ± 0.23        | 1.72 ± 0.25      | ↓          | 0.0010* |
| Fatty acid ratios               |                 |                 |            |    |
| Unsaturation index              | 61.58 ± 18.05     | 38.91 ± 5.56     | ↓          | 0.0342* |
| LCPUFAs/SCPUFA†                 | 0.49 ± 0.06       | 0.33 ± 0.05      | ↓          | 0.0107* |
| 20:4n6/18:2n6                   | 0.40 ± 0.07       | 0.28 ± 0.04      | ↓          | 0.0232* |
| 22:6n3/18:3n3                   | 1.46 ± 0.17       | 0.64 ± 0.13      | ↓          | 0.0002* |

Data are means ± SD. *P < 0.05. †Long-chain PUFAs/short-chain PUFAs.

GPCho 34:0 (34:0) in the erythrocytes or in liver and blood plasma (data not shown).

Inflammatory marker expression in control and diabetic retinas. As n3 PUFAs are known to have anti-inflammatory properties, we hypothesized that a decrease in n3 PUFAs would be associated with a proinflammatory state in diabetic retinas. As shown in Fig. 4, diabetic retinas had increased expression levels of several inflammatory markers including adhesion molecules (ICAM-1), cytokines (IL-6), and growth factors (VEGF).

DISCUSSION
The association of dyslipidemia with the development of diabetic retinopathy has been underscored by the Diabetes Control and Complications Trial/Epidemiology of Diabetes Interventions and Complications cohort study (8). Despite this evidence, the experimental data on diabetes-induced changes in lipid profile and lipid metabolism in the retina are not available. This is the first comprehensive study to analyze retinal-specific fatty acid profiles and metabolism and to compare them to liver and blood plasma in control and diabetic animals.

In this study utilizing STZ-induced diabetic rats, we found a decreased level of DHA 22:6n3, the major retinal long-chain PUFA, in diabetic retina. This finding confirmed earlier studies showing a decrease in relative percentage of DHA 22:6n3 in the diabetic retina (34,35). In addition to DHA 22:6n3, VLCPUFAs including 32:6n3 and 34:6n3 were

TABLE 3
Liver fatty acid profiles of control (n = 4) and diabetic (n = 7) animals

| Fatty acids                     | Control animals | Diabetic animals | Difference | P  |
|---------------------------------|-----------------|-----------------|------------|----|
| Total (nmol/mg protein)         | 1,762.82 ± 480.50 | 1,357.85 ± 241.19 |            | 0.1542 |
| Mole % of total fatty acids     |                 |                 |            |    |
| 16:0 (palmitic)                 | 26.73 ± 8.18     | 17.55 ± 3.25     |            | 0.0518 |
| 18:0 (stearic)                  | 11.25 ± 3.74     | 12.64 ± 3.55     |            | 0.9706 |
| 18:1n9 (oleic)                  | 3.11 ± 1.03      | 3.39 ± 1.04      |            | 0.4785 |
| 18:2n6 (linoleic)               | 33.14 ± 5.34     | 39.90 ± 2.71     | ↑          | 0.0161* |
| 18:3n3 (α-linolenic)            | 0.49 ± 0.11      | 0.59 ± 0.11      |            | 0.2101 |
| 18:3n6 (γ-linolenic)            | 0.13 ± 0.14      | 0.09 ± 0.04      |            | 0.2173 |
| 20:3n6 (dihomo-γ-linolenic)     | 0.34 ± 0.04      | 0.43 ± 0.15      |            | 0.3942 |
| 20:3n9 (mead)                   | 0.62 ± 0.24      | 0.51 ± 0.14      |            | 0.9340 |
| 20:4n6 (arachidonic)            | 19.87 ± 4.43     | 21.37 ± 2.50     |            | 0.4581 |
| 20:5n3 (eicosapentaenoic)       | 0.15 ± 0.03      | 0.15 ± 0.05      |            | 0.7777 |
| 22:5n3 (docosapentaenoic)       | 0.57 ± 0.15      | 0.44 ± 0.07      |            | 0.2388 |
| 22:6n3 (docosahexaenoic)        | 3.60 ± 0.92      | 2.94 ± 0.35      |            | 0.2294 |
| Fatty acid ratios               |                 |                 |            |    |
| Unsaturation index              | 5.05 ± 1.82      | 6.65 ± 1.31      |            | 0.0911 |
| LCPUFAs/SCPUFA†                 | 0.74 ± 0.07      | 0.64 ± 0.02      | ↓          | 0.0123* |
| 20:4n6/18:2n6                   | 0.60 ± 0.07      | 0.53 ± 0.03      |            | 0.1211 |
| 22:6n3/18:3n3                   | 7.74 ± 2.96      | 5.11 ± 1.05      |            | 0.0979 |

Data are means ± SD. *P < 0.05. †Long-chain PUFAs/short-chain PUFAs.
detected as substitutes of retina GPCho. VLCPUFAs were not detected in lipid classes other than GPCho and were only detected in retina. Three weeks of diabetes reduced retinal levels of 32:6n3-GPCho compared with controls. As a result of these changes, the diabetic retina had a lower unsaturation index and lower long-chain–to–short-chain PUFAs. Moreover, there was a shift toward n3 PUFAs–deficient, n6 PUFA–rich, profile in the diabetic retina.

In general, n6 PUFAs induce, while n3 PUFAs inhibit, inflammation, and the relative amount of these PUFAs plays an important role in the regulation of immunity (36). Our previous studies indicated that treatment of a cell type affected by diabetic retinopathy, HRECs, with n6 PUFA leads to a lipoxygenase-dependent increase in ICAM-1/vascular cell adhesion molecule-1 expression (37). Conversely, we have demonstrated that DHA22:6n3 inhibited cytokine-induced activation of the NFκB signaling pathway and adhesion molecule expression in HRECs (28). Thus, a decrease in the n3-to-n6 PUFAs ratio in the diabetic retina observed in this study would create proinflammatory conditions potentially contributing to the development of diabetic retinopathy. Indeed, previous studies demonstrated an upregulation in a number of inflammatory markers in the retina early in diabetes: VEGF (4,5), ICAM-1 (6,7), TNF-α (8), and IL-6 (9). ICAM-mediated leukostasis was detected within 1 week of diabetes in rats (38,39). VEGF was shown to increase ICAM expression in retinas of nondiabetic mice (40), and viretal PUFAs were found to be correlated with that of IL-6 and severity of diabetic retinopathy in diabetic patients (41).

In this study, we chose a cytokine (IL-6), a growth factor (VEGF), and an adhesion molecule (ICAM-1) as readout of an inflammatory status in the retinas of diabetic animals with decreased n3/n6 PUFAs. mRNA levels of all three markers were elevated in diabetic retinas compared with controls.

Importantly, diabetes induced the most pronounced changes in the retinal fatty acid profile, whereas liver fatty acid profile was only slightly affected, indicating that the disruption of retinal fatty acid metabolism in diabetes might not simply be a result of altered liver metabolism. Moreover, VLCPUFA-containing phospholipids detected in the retina were not present in the liver or erythrocyte total lipids. The fatty acid profile in a particular peripheral tissue depends on two factors: 1) the profile in circulation due to the diet and liver metabolism and 2) the ability of a local tissue to remodel fatty acids. Retina has a unique fatty acid profile characterized by one of the highest levels of DHA22:6n3 in the body and by the presence of VLCPUFAs (27,42). While the expression level of retinal desaturases was relatively low compared with retinal elongases, it has been reported that retina can synthesize DHA22:6n3 from α-linolenic, 18:3n3 acid and EPA20:5n3 (43). Although retina may obtain additional DHA22:6n3 by uptake from the circulation, changes in the fatty acid profiles of diabetic animals did not mirror changes observed in liver and plasma fatty acid profiles. Thus, a retina-specific decrease in DHA22:6n3 in diabetes is likely to be due to changes in retinal fatty acid metabolism.

To determine the effect of diabetes on retinal fatty acid metabolism, we analyzed the level of fatty acid elongase and desaturase gene expression in control and diabetic animals. Retinas had a very high expression level of the retinal-specific elongase, Elovl4, as well as high expression levels of long-chain PUFA elongase Elovl2. Δ5-, Δ6-, and Δ9-desaturase levels were low compared with the liver expression levels. The high levels of Elovl4 and Elovl2 and low levels of desaturases suggest that the retina is preferentially involved in production of very-long-chain fatty acids and exhibits a low level of de novo lipogenesis. The retinal elongase expression profile that we observed likely explains the high level of long-chain PUFAs in the retina compared with liver and blood plasma levels. Elovl2 elongates C20–22 fatty acids (44–46). Elovl4 was recently shown to be involved in VLCPUFA synthesis with substrate specificity for C26–36 fatty acids (15). The role of VLCPUFAs is not known. Because of their specific presence in tissues with high membrane curvature and their ability to span both leaflets of the lipid bilayer, VLCPUFAs are suggested to play the role of an anchor stabilizing high

**Table 4**

| Fatty acids                           | Control animals | Diabetic animals | Difference | P |
|---------------------------------------|-----------------|-----------------|------------|---|
| Total (nmol/mg protein)               | 488.96 ± 17.64  | 460.32 ± 27.82  | ↓          | 0.43 |
| n3 fatty acids                        | 225.1 ± 4.23    | 166.34 ± 20.66  | ↓          | 0.0495* |
| n6 fatty acids                        | 38.67 ± 4.71    | 40.59 ± 3.9     |            | 0.7693 |
| Mole % of total fatty acids           |                 |                 |            |     |
| 16:0 (palmitic)                       | 15.51 ± 0.8     | 18.32 ± 1.67    |            | 0.2046 |
| 18:0 (stearic)                        | 18.61 ± 0.97    | 21.98 ± 2.0     |            | 0.2046 |
| 18:1n9 (oleic)                        | 10.58 ± 0.2     | 12.9 ± 0.77     | ↑          | 0.0441* |
| 18:2n6 (linoleic)                     | 0.44 ± 0.06     | 0.88 ± 0.05     | ↑          | 0.0051* |
| 18:3n3 (α-linolenic)                  | 0.24 ± 0.06     | 0.17 ± 0.02     |            | 0.3566 |
| 20:3n6 (dihomo-γ-linolenic)           | 0.11 ± 0.02     | 0.04 ± 0.01     |            | 0.0819 |
| 20:3n9 (mead)                         | 0.97 ± 0.06     | 1.09 ± 0.2      |            | 0.6103 |
| 20:4n6 (arachidonic)                  | 7.33 ± 0.75     | 7.9 ± 0.64      |            | 0.6004 |
| 20:5n3 (eicosapentaenoic)             | 0.04 ± 0.02     | 0.01 ± 0.01     |            | 0.2910 |
| 22:5n3 (docosapentaenoic)             | 0.47 ± 0.08     | 0.28 ± 0.05     |            | 0.1129 |
| 22:6n3 (docosahexaenoic)              | 45.37 ± 1.32    | 35.47 ± 2.71    | ↓          | 0.0050* |
| Fatty acid ratios                     |                 |                 |            |     |
| Unsaturation index                    | 20.77 ± 1.63    | 14.9 ± 2.57     |            | 0.1250 |
| LCPUFA/SCPUFA†                        | 80.39 ± 4.86    | 42.53 ± 3.94    | ↓          | 0.0033* |
| % n3 fatty acids of total             | 46.12 ± 1.41    | 35.93 ± 2.77    | ↓          | 0.0036* |

Data are means ± SD. *P < 0.05. †Long-chain PUFAs/short-chain PUFAs.
In the retina, VCLPUFAs are mainly present in the rod outer-segment membrane, where they are suggested to play a role in stabilizing the rims of photoreceptor disks. This specific localization might explain low abundance of VCLPUFAs in the total retinal lipids extracted in this study. At the same time, specific localization suggests that VCLPUFAs might play an important role in photoreceptor function. This study provides the first direct evidence that a significant decrease in Elovl4 in diabetic retina is indeed associated with a decrease in VCLPUFA (i.e., 32:6n3) synthesis. Despite lower abundance, diabetes-induced decrease in 32:6n3 containing GPCho (24%) was similar to the decrease in DHA_{22:6n3} containing GPCho (15–34%). Elovl4 protein
expression in diabetic retina was inhibited to a higher degree (73%) compared with mRNA expression (40%), suggesting control of Elovl4 expression at both transcriptional and translational levels. Although decrease of VLCPUFAs is most likely to arise from Elovl4 loss, another plausible explanation could be that this reduction was due to reduction in VLCPUFA precursor lipids, EPA20:5n3, and/or DHA22:6n3. This possibility can be tested in the future by determining whether downregulation of VLCPUFAs in diabetes persists in animals supplemented with high-EPA20:5n3/DHA22:6n3 diet.

Another possibility could be that high level of reactive oxygen species in diabetic retina leads to degradation of a highly oxidation-prone DHA molecule. Previous studies using the same STZ-induced diabetic model of similar duration, however, did not find oxidized DHA products in diabetic retina (47).

Several Elovl4 gene mutations have been recently identified in pathogenesis of another retinal disease, Stargardt-like macular dystrophy (19–21). Stargardt-like macular dystrophy is an autosomal-dominant disorder due to a dominant-negative effect of the mutated Elovl4 on wild-type protein (19). As Elovl4 is highly expressed in the photoreceptors (19,21), it is not surprising that mutant Elovl4 transgenic mice are characterized by lipofuscin accumulation, abnormal electrophysiology, and photoreceptor degeneration (20). Although photoreceptors are not the primary site of diabetic retinopathy, several abnormalities in neural retina have been associated with the development of diabetic retinopathy (48,49). The decrease in Elovl4 observed in this study would not be expected to have as dramatic an effect on photoreceptor viability as the dominant-negative mutation in Elovl4. However, the reduction in Elovl4 in diabetic retina could be responsible for more subtle changes in photoreceptor/RPE cell function that could lead to metabolic changes in the whole retina and eventually contribute to the pathology characteristic of diabetic retinopathy.

In conclusion, a decrease in the expression level of retinal fatty acid elongases Elovl2 and Elovl4 and concomitant decrease in the major n3 PUFA, DHA22:6n3, as well as the VLCPUFA32:6n3, results in an increased n6-to-n3 PUFA ratio in the diabetic retina that likely creates a proinflammatory state contributing to the development of diabetic retinopathy. Increasing the gene expression of fatty acid elongases in the retina represents a potential therapeutic strategy for modulating fatty acid metabolism and altering the pathogenesis of diabetic retinopathy.

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REFERENCES

1. Schroder S, Palinski W, Schmid-Schonbein GW. Activated monocytes and granulocytes, capillary nonperfusion, and neovascularization in diabetic retinopathy. Am J Pathol 1991;139:81–100
2. Miyamoto K, Khosrof S, Bursell SE, Rohan R, Murata T, Clermont AC, Aiello LP, Ogura Y, Adamis AP. Prevention of leukostasis and vascular leakage in streptozotocin-induced diabetic retinopathy via intercellular adhesion molecule-1 inhibition. Proc Natl Acad Sci U S A 1999;96:10836–10841
3. Adamis AP. Is diabetic retinopathy an inflammatory disease? Br J Ophthalmol 2002;86:363–365
4. Yang LP, Sun HL, Wu LM, Guo XJ, Dou HL, Tso MO, Zhao L, Li SM. Baicalein reduces inflammatory process in a rodent model of diabetic retinopathy. Invest Ophthalmol Vis Sci 2000;41:2191–2197
5. Sone H, Kawakami Y, Okuda Y, Sekine Y, Honnmaura S, Matsuo K, Segawa T, Suzuki H, Yamashita K. Ocular vascular endothelial growth factor levels in diabetic rats are elevated before observable retinal proliferative changes. Diabetologia 1997;40:726–730
6. Nozaki M, Ogura Y, Hirabayashi Y, Saishin Y, Shimada S. Enhanced expression of adhesion molecules of the retinal vascular endothelium in spontaneous diabetic rats. Ophthalmic Res 2002;34:158–164
7. Al-Shabrawey M, Rojas M, Sanders T, Behzadian A, El-Remessy A, Bartoli M, Parpia AK, Liou G, Caldwell RB. Role of NADPH oxidase in retinal vascular inflammation. Invest Ophthalmol Vis Sci 2008;49:3239–3244
8. Joussen AM, Poulik V, Mitsiades N, Kirchhof B, Koizumi K, Dohmen S, Adams AP. Nonsteroidal anti-inflammatory drugs prevent early diabetic retinopathy via TNF-alpha suppression. FASEB J 2002;16:438–440
9. Gustavsson C, Agardh CD, Hagert P, Agardh E. Inflammatory markers in diabetic retinopathy via TNF-alpha suppression. FASEB J 2002;16:438–440
10. Lyons TJ, Jenkins AJ, Zheng D, Lackland DT, McGee D, Garvey WT, Klein...
11. Coppack SW, Evans RD, Fisher RM, Frayn KN, Gibbons GF, Humphreys SM, Kirk ML, Potts JL, Hockaday TD. Adipose tissue metabolism in obesity: lipase action in vivo before and after a mixed meal. Metabolism 1992;41:264–272

12. Weinstock PH, Levak-Frank S, Hudgins LC, Radner H, Friedman JM, Zechner R, Breslow JL. Lipoprotein lipase controls fatty acid entry into adipose tissue, but fat mass is preserved by endogenous synthesis in mice deficient in adipose tissue lipoprotein lipase. Proc Natl Acad Sci U S A 1997;94:10261–10266

13. Julius U. Influence of plasma free fatty acids on lipoprotein synthesis and diabetic dyslipidemia. Exp Clin Endocrinol Diabetes 2003;111:246–250

14. Sprecher H, Chen Q. Polyunsaturated fatty acid biosynthesis: a microsomal-peroxisomal process. Prostaglandins Leukot Essent Fatty Acids 1999;60:317–321

15. Agbaga MP, Brush RS, Mandal MN, Henry K, Elliott MH, Anderson RE. Role of Stargardt-3 macular dystrophy protein (ELOVL4) in the biosynthesis of very long chain fatty acids. Proc Natl Acad Sci U S A 2008;105:12843–12848

16. Lagali PS, Liu J, Ambasudhan R, Kakuk LE, Bernstein SL, Siegel GM, Wong PW, Ayyagari R. Evolutionarily conserved ELOVL4 gene expression in the vertebrate retina. Invest Ophthalmol Vis Sci 2003;44:2841–2850

17. Umeda S, Ayyagari R, Suzuki MT, Ono F, Iwata F, Fujiki K, Kanai A, Takada Y, Yoshikawa Y, Tanaka Y, Iwata T. Molecular cloning of ELOVL4 gene from cymolgus monkey (Macaca fascicularis). Exp Anim 2003;52:120–135

18. Zhang XM, Yang Z, Karan G, Hashimoto T, Baehr W, Yang XJ, Zhang K. Lipofuscinosis in ELOVL4 deficient in adipose tissue lipoprotein lipase. Proc Natl Acad Sci USA 2005;102:4164–4169

19. Grayson C, Molday RS. Dominant negative mechanism underlies autosomal dominant Stargardt-like macular dystrophy linked to mutations in ELOVL4. Mol Vis 2003;9:301–307

20. Karan G, Lillo C, Yang Z, Cameron DJ, Locke KG, Zhao Y, Thirumalaichary PW, Ayyagari R. Evolutionarily conserved ELOVL4 gene expression in the vertebrate retina. Invest Ophthalmol Vis Sci 2003;44:2841–2850

21. Umeda S, Ayyagari R, Suzuki MT, Ono F, Iwata F, Fujiki K, Kanai A, Takada Y, Yoshikawa Y, Tanaka Y, Iwata T. Molecular cloning of ELOVL4 gene from cymolgus monkey (Macaca fascicularis). Exp Anim 2003;52:120–135

22. Brenner RR. Hormonal modulation of delta6 and delta5 desaturases: case of macular degeneration. Mol Vis 2003;9:301–307

23. Nakamura MT, Nara TY. Gene regulation of mammalian desaturases. Biochem Soc Trans 2002;30:1076–1079

24. Rimoldi OJ, Finarelli GS, Brenner RR. Effects of diabetes and insulin on hepatic delta6 desaturase gene expression. Biochem Biophys Res Commun 2003;309:232–236

25. Wang Y, Botolin D, Xu J, Christian B, Mitchell E, Jayaprakasham B, Nair MG, Peters JM, Busik JV, Olson LD, Jump DB. Regulation of hepatic fatty acid elongase and desaturase expression in diabetes and obesity. J Lipid Res 2006;47:2028–2041

26. Meyer A, Kirsch H, Domergue F, Abbadi A, Sperling P, Bauer J, Cirpus P, Zank TK, Moreau H, Roscoe TJ, Zahringer U, Heinz E. Novel fatty acid elongases and their use for the reconstruction of docosahexaenoic acid biosynthesis. J Lipid Res 2004;45:1096–1099

27. Anderson RE. Lipids of ocular tissues: IV. A comparison of the phospholipids from the retina of six mammalian species. Exp Eye Res 1970;10:339–344

28. Chen W, Esselman WJ, Jump DB, Busik JV. Anti-inflammatory effect of docosahexaenoic acid on cytokine-induced adherence molecule expression in human retinal vascular endothelial cells. Invest Ophthalmol Vis Sci 2005;46:4342–4347

29. Lydic TA, Busik JV, Esselman WJ, Reid GE. Complementary precursor ion and neutral loss scan mode tandem mass spectrometry for the analysis of glycerophosphoethanolamine lipids from whole rat retina. Anal Bioanal Chem 2009;394:267–275

30. Murphy EJ, Stephens R, Jurkowitz-Alexander M, Horrocks LA. Acidic hydrolysis of photolipids followed by high-performance liquid chromatography. Lipids 1993;28:565–568

31. Rose H, Oklander M. An improved method for the extraction of lipids from human erythrocytes. J Lipid Res 1965;6:428–443

32. Han X, Gross RW. Shotgun lipidomics: electrospray ionization mass spectrometric analysis and quantitation of cellular lipolipides directly from crude extracts of biological samples. Mass Spectrom Rev 2005;24:367–412

33. Haimi P, Uphoff A, Hermansson M, Somerharju P. Software tools for analysis of mass spectrometric lipidome data. Anal Chem 2006;78:8324–8331

34. Puttermann S, Stettevant R, Kupfer C. Effect of alloxan diabetes on the fatty acid composition of the retina. Invest Ophthalmol Vis Sci 2008;53:545–554

35. Hegde KR, Varma SD. Electrophore impact mass spectrometric studies on mice retinal fatty acids: effect of diabetes. Ophthalmic Res 2009;42:9–14

36. Habige LS. Fatty acids, the immune response, and autoimmunity: a question of n-6 essentiality and the balance between n-6 and n-3. Lipids 2006;41:329–341

37. Chen W, Jump DB, Grant MB, Esselman WJ, Busik JV. Dyslipidemia, but not hyperglycemia, induces inflammatory adhesion molecules in human retinal vascular endothelial cells. Invest Ophthalmol Vis Sci 2003;44:5016–5022

38. Joussen AM, Poulaki V, Qin W, Kirchhoff B, Mitsiades N, Wiegand SJ, Rudge J, Yancopoulos GD, Adams AP. Retinal vascular endothelial growth factor induces intercellular adhesion molecule-1 and endothelial nitric oxide synthase expression and initiates early diabetic retinal leukocyte adhesion in vivo. Am J Pathol 2002;160:501–509

39. Joussen AM, Murata T, Tsujikawa A, Kirchhoff B, Bursell SE, Adams AP. Leukocyte-mediated endothelial cell injury and death in the diabetic retina. Am J Pathol 2001;158:147–152

40. Lu MP, Perez VL, Ma N, Miyamoto K, Peng HB, Liao JK, Adams AP, VEGF increases retinal vascular JCAM-1 expression in vivo. Invest Ophthalmol Vis Sci 1999;40:1898–1812

41. Funatsu H, Yamashita H, Shimizu E, Kojima R, Hori S. Relationship between vascular endothelial growth factor and intercellulin-6 in diabetic retinopathy. Retina 2001;21:469–477

42. Fliesler SJ, Anderson RE. Chemistry and metabolism of lipids in the vertebrate retina. Prog Lipid Res 1983;22:79–131

43. Delton-Vandenbroucke I, Grammas P, Anderson RE, Polynsaturated fatty acid metabolism in retinal and cerebral microvascular endothelial cells. J Lipid Res 1997;38:147–159

44. Moon YA, Shah NA, Mohapatra S, Warrington JA, Horton JD. Identification of a mammalian long chain fatty acid elongase regulated by sterol regulatory element-binding proteins. J Biol Chem 2001;276:45358–45366

45. Jakobsson A, Westerberg R, Jacobsson A. Protein-acylating activity of lipases in leukocytes. J Biol Chem 2001;276:45358–45366

46. Jakobsson A, Westerberg R, Jacobsson A. Fatty acid elongases in mammals: their regulation and roles in metabolism. Prog Lipid Res 2006;45:237–249

47. Leonard AE, Pereira SL, Sprecher H, Huang YS. Elongation of long-chain fatty acids. Prog Lipid Res 2004;43:36–54

48. Sunada S, Kiyose C, Kubo K, Takebayashi J, Sanada H, Saito M. Effect of diabetes on retinal fatty acid composition. Invest Ophthalmol Vis Sci 2005;46:4342–4347

49. Lydic TA, Busik JV, Esselman WJ, Reid GE. Complementary precursor ion and neutral loss scan mode tandem mass spectrometry for the analysis of glycerophosphoethanolamine lipids from whole rat retina. Anal Bioanal Chem 2006;394:267–275

50. Murphy EJ, Stephens R, Jurkowitz-Alexander M, Horrocks LA. Acidic hydrolysis of photolipids followed by high-performance liquid chromatography. Lipids 1993;28:565–568

51. Rose H, Oklander M. An improved method for the extraction of lipids from human erythrocytes. J Lipid Res 1965;6:428–443

52. Han X, Gross RW. Shotgun lipidomics: electrospray ionization mass spectrometric analysis and quantitation of cellular lipolipides directly from crude extracts of biological samples. Mass Spectrom Rev 2005;24:367–412

53. Haimi P, Uphoff A, Hermansson M, Somerharju P. Software tools for analysis of mass spectrometric lipidome data. Anal Chem 2006;78:8324–8331

54. Puttermann S, Stettevant R, Kupfer C. Effect of alloxan diabetes on the fatty acid composition of the retina. Invest Ophthalmol Vis Sci 2008;53:545–554
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