

# Diabetes Impairs the Aldehyde Detoxifying Capacity of the Retina

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Submitted: February 24, 2016  
Accepted: August 5, 2016

Citation: McDowell RE, McGahon MK, Augustine J, Chen M, McGeown JG, Curtis TM. Diabetes impairs the aldehyde detoxifying capacity of the retina. *Invest Ophthalmol Vis Sci*. 2016;57:4762-4771. DOI:10.1167/iops.16-19424

**PURPOSE.** We studied whether the accumulation of advanced lipoxidation end-products (ALEs) in the diabetic retina is linked to the impairment of lipid aldehyde detoxification mechanisms.

**METHODS.** Retinas were collected from nondiabetic and diabetic rats and processed for conventional and quantitative RT-PCR (qRT-PCR), Western blotting, immunohistochemistry, and aldehyde dehydrogenase (ALDH) activity assays. The effect of the ALDH1a1 inhibitor, NCT-501, on ALE accumulation and cell viability in cultured Müller glia also was investigated.

**RESULTS.** The rat retina expressed a range of lipid aldehyde detoxifying ALDH and aldo-keto reductase (AKR) genes. In diabetes, mRNA levels were reduced for 5 of 9 transcripts tested. These findings contrasted with those in the lens and cornea where many of these enzymes were upregulated. We have reported previously accumulation of the acrolein (ACR)-derived ALE, FDP-lysine, in retinal Müller glia during diabetes. In the present study, we show that the main ACR-detoxifying ALDH and AKR genes expressed in the retina, namely, *ALDH1a1*, *ALDH2*, and *AKR1b1*, are principally localized to Müller glia. Diabetes-induced FDP-lysine accumulation in Müller glia was associated with a reduction in *ALDH1a1* mRNA and protein expression in whole retina and a decrease in *ALDH1a1*-immunoreactivity specifically within these cells. No such changes were detected for *ALDH2* or *AKR1b1*. Activity of ALDH was suppressed in the diabetic retina and blockade of *ALDH1a1* in cultured Müller glia triggered FDP-lysine accumulation and reduced cell viability.

**CONCLUSIONS.** These findings suggest that downregulation of ALDH and AKR enzymes, particularly ALDH1a1, may contribute ALE accumulation in the diabetic retina.

**Keywords:** diabetic retinopathy, lipid peroxidation, aldehyde detoxification, aldehyde dehydrogenase, aldo-ketoreductase

Diabetic retinopathy is a leading cause of newly diagnosed blindness in people of working age in industrialized nations.<sup>1</sup> Hyperglycemia-induced oxidative stress is widely regarded as one of the key factors underlying the pathogenesis of diabetic retinopathy.<sup>2</sup> Numerous studies have demonstrated increased levels of reactive oxygen species (ROS) in the retinas of experimental animal models of diabetes as well as retinal cells maintained in high glucose conditions.<sup>3-5</sup> The elevated ROS levels in the diabetic retina are thought to arise through a number of mechanisms, including the autoxidation of glucose,<sup>6</sup> increased NADPH oxidase activity,<sup>7</sup> mitochondrial dysfunction,<sup>3</sup> and the impaired activity of antioxidant defense enzymes such as superoxide dismutase (SOD) and catalase.<sup>8</sup> Although ROS may directly affect cell function, in recent years increasing evidence has suggested that many of the detrimental effects of oxidative stress result from ROS-induced lipid peroxidation reactions.<sup>9-11</sup> Lipid peroxidation causes the formation of a number of lipid aldehyde byproducts, including malondialdehyde (MDA), 4-hydroxynonenal (4-HNE), and acrolein (ACR). These aldehydes are toxic, at least in part, due to their ability to react with cellular and tissue proteins leading to the formation of advanced lipoxidation end products (ALEs) which induce protein dysfunction and alter cellular responses.<sup>9-11</sup> They also are more stable than ROS, allowing them to propagate oxidative injury by diffusing far from their site of origin.<sup>12</sup>

Several studies have suggested that lipid aldehydes and ALEs contribute to the development of diabetic retinopathy. Patients with diabetic retinopathy show increased levels of MDA and 4-HNE in their blood when compared to diabetic subjects without retinopathy and healthy controls.<sup>13-15</sup> We also have shown that hemoglobin-associated levels of the ACR-derived ALE, N<sup>ε</sup>-(3-formyl-3,4-dehydropiperidino)lysine (FDP-lysine), are elevated in patients with type 1 and type 2 diabetes and correlate with the severity of retinopathy.<sup>16</sup> Higher lipid aldehyde levels also have been reported in the retinas of diabetic animal models<sup>17-19</sup> and in human postmortem retinas from diabetic donors.<sup>20</sup> More recently, we demonstrated that FDP-lysine preferentially accumulates in retinal Müller glia during diabetes and, when modelled in vitro, this accumulation induces dysfunction of these cells consistent with that observed in the diabetic retina.<sup>21,22</sup> Although it generally has been assumed that the raised levels of lipid aldehydes and ALEs observed in the retina during diabetes result from increased lipid peroxidation,<sup>10</sup> the possibility that impairment of lipid aldehyde defense mechanisms also may contribute has yet to be determined.

Aldehyde dehydrogenases (ALDHs) and aldo-ketoreductases (AKRs) represent two of the major enzymatic pathways through which lipid aldehydes are detoxified in vivo. The ALDH superfamily comprises a wide variety of NAD(P+)-



TABLE 1. RT-PCR Primer Sequences Used to Amplify ALDH and AKR Genes Studied in This Work

| Genes           | Forward                   | Reverse                   |
|-----------------|---------------------------|---------------------------|
| <i>β-actin</i>  | GCGCAAGTACTCTGTGTGGA      | AAGGGTGTAAAACGCAGCTC      |
| <i>ALDH1a1</i>  | CCCACAGTCTTCTCCAATGTGACCG | AAGGGGCACTGGGGCTGACAAGA   |
| <i>ALDH1a2</i>  | CCGTCACCCACGCCCAACC       | AGAACCAAGAGAGAAGGCCAGGCG  |
| <i>ALDH2</i>    | CAGCTACACCCGCCACGAGC      | GCGGTAGGGCCGAATCCAGG      |
| <i>ALDH3a1</i>  | CACCGTGAAACGGGCCAGGG      | CCACAGGCTCATCCTCAGCCCA    |
| <i>ALDH3a2</i>  | CGCGAAAAGCCCTGGCACT       | AACAAGCAGGGGCGTGATGA      |
| <i>ALDH9a1</i>  | CCTGGAGCGAGTCCTCGGGTT     | CCCGTCCAGAACCTCGGCT       |
| <i>ALDH18a1</i> | CCCAGTGATGGGCCACAGCG      | AGGCAAACCTGGGGCCCTGCG     |
| <i>ALDH1L1</i>  | GGATGGCCGTGCCGTGTTC       | AAGCCCCCTCGATGCCTGGGT     |
| <i>ALDH3b1</i>  | GGGGCTTCTGCGGGAACGAT      | TTGCGACTGGTGTAGGGCCGG     |
| <i>AKR1b1</i>   | CTCTTGCGGGTTCGTGTGCGT     | GGCGCTTACCACCTGTCTCC      |
| <i>AKR1b7</i>   | TCGCTTGATGAGATTCAAGCCGGG  | TCCCTGTGAGAGTCTGCACCTTGAT |
| <i>AKR1b8</i>   | CAGGCCTAGGGCCAAGCCAGAT    | AGGCAGGCCCTCCAGTTCTGTT    |
| <i>AKR1c15</i>  | AGAACCAAGAGAGAAGGCCAGGCG  | AGCGATCTCTCAGGCACTGACG    |
| <i>AKR1c19</i>  | AAGCTGGAGACCTCCCCAGGA     | GCGGAACCCAGCCTCTACAGCTAA  |
| <i>AKR7a1</i>   | CTGGTCTTGTCATGATGCGCCC    | CGCCGATCTCAGCCCCACTG      |
| <i>AKR7a2</i>   | CCTCTCGCCATGTCCCGGTCT     | GAAGGCCGTGTCCAGCTCGT      |
| <i>AKR7a3</i>   | ACGGTCACCATGTGCAAGCC      | GGGGCAGCCTTGGTGCCAAT      |

dependent enzymes that irreversibly catalyze the oxidation of lipid aldehydes to their respective carboxylic acids.<sup>23</sup> A significant amount of work remains to be done in assessing the substrate specificity of many of these enzymes. A large proportion of them, however, appear to have multiple roles in aldehyde processing with varying catalytic efficiency for the metabolism of different lipid aldehyde species.<sup>24</sup> Enzymes belonging to the AKR superfamily are NADPH-dependent oxidoreductases which catalyze the reduction of a wide range of aldehydes and ketones to primary and secondary alcohols, respectively.<sup>25</sup> These enzymes display very broad substrate specificities, although the vast majority have been shown to have a role in lipid aldehyde detoxification.<sup>26</sup>

In the present study, we examined the effects of diabetes on the mRNA expression of ALDH and AKR enzymes in the rat retina and contrasted our results with lens and cornea tissues collected from the same animals. Experiments also were performed to characterize the normal localization pattern of ACR detoxifying ALDH and AKR enzymes in the retina, and to determine whether impairment of these enzymes during diabetes might underlie the observed accumulation of FDP-lysine adducts in retinal Müller glia.

## METHODS

### Diabetic Rat Model

Animal use conformed to the standards in the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Male Sprague-Dawley (SD) rats (~200 g body weight) were rendered diabetic by a single intraperitoneal injection of streptozotocin (STZ; 65 mg/kg in 0.1 mol/L citrate buffer, pH 4.6; Sigma-Aldrich, Poole, UK). Citrate buffer-injected age-matched rats were used as controls. One week after STZ injection, blood glucose measurements were made by glucometric assessment of tail prick blood samples (Breeze2; Bayer Scientific, Leverkusen, Germany). Animals with blood glucose concentrations >15 mmol/L were enrolled in the study. Animal weights were monitored daily. At 3 months of diabetes duration, rats were euthanized by CO<sub>2</sub> asphyxiation and blood collected via cardiac puncture for measurement of hemoglobin A1c (HbA<sub>1c</sub>) levels (GLYCO-Tek kit; Helena Biosciences, Gateshead, UK).

### Primary Müller Glia Culture

Müller glia were isolated from 7-day-old C57BL/6 neonatal mice and cultured as described previously.<sup>27</sup> Cells were grown in 10% fetal calf serum/Dulbecco's modified Eagle Medium (DMEM) supplemented with 5% spleen conditioned media<sup>28</sup> and used between passages 3 and 6.

### Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

Total RNAs were extracted from the retina, lens, and cornea using an RNeasy Mini Kit (Qiagen, Crawley, UK). Total RNA also was extracted from liver samples to act as a positive control for the PCR primers. The RNA concentration and purity of each sample was determined using a NanoDrop2000 UV spectrometer (Thermo-Fisher Scientific, Waltham, MA, USA) and 500 ng of RNA reverse transcribed into cDNA using SuperScript III Reverse Transcriptase Kit (Life Technologies, Paisley, UK). Gene specific primers were designed using NCBI Primer Blast and synthesized by Eurogentec (Southampton, UK). Primer sequences are presented in Table 1. For traditional RT-PCR, PCR samples were run on 1.5% agarose gels stained with 0.1 µg/mL ethidium bromide, and PCR products visualized using a MultiDoc-It UV Imaging System (UVP, Upland, CA, USA). SYBR Green quantitative RT-PCR was performed using a ROCHE lightcycler480 (Roche, Basel, Switzerland). Relative gene expression was calculated using the comparative Ct method (2<sup>-ΔCt</sup>) with β-actin as the housekeeping gene.<sup>29</sup> Minus reverse transcriptase and no template controls were all negative.

### Confocal Immunolabeling

Immunofluorescent staining of retinal cryosections was performed as described previously.<sup>21,22</sup> A range of primary antibodies was used in combination with appropriate fluorescently labeled secondary antibodies (Table 2). In experiments examining colocalization of ALDH and AKR enzymes with the Müller glia marker, CRALBP, nuclei of retinal cells were counterstained with TO-PRO nuclear dye (1:1000; pseudo-colored blue; Life Technologies). Confocal images were captured with a Leica SP5 confocal microscope (HCX PL APO ×63 oil immersion lens; Leica Geosystems, Heerbrugg, Switzerland). For experiments comparing ALDH1a1, ALDH2,

TABLE 2. Primary and Secondary Antibodies Used for Confocal Immunolabeling Studies

| Antigen              | Primary Antibody (Dilution and Source)                             | Secondary Antibody   |
|----------------------|--|--|
| FDP-lysine           | Mouse monoclonal (mAb5F6) antibody (1:500; JaICA, Shizuoka, Japan) | Donkey anti-mouse AlexaFluor 488                             |
| ALDH1a1              | Rabbit polyclonal (1:200; Abcam, Cambridge, UK)                    | Donkey anti-rabbit AlexaFluor 488                            |
| ALDH2                | Rabbit polyclonal (1:100; Abcam)                                   | Donkey anti-rabbit AlexaFluor 488                            |
| AKR1b1               | Rabbit polyclonal (1:200; Abcam)                                   | Donkey anti-rabbit AlexaFluor 488                            |
| CRALBP               | Mouse monoclonal (1:100; Abcam)                                    | Donkey anti-mouse AlexaFluor 568                             |
| Kir4.1               | Rabbit polyclonal (1:100; Alomone, Israel),                        | Donkey anti-rabbit AlexaFluor 488<br>(Supplementary Fig. S3) |
| Glutamine synthetase | Rabbit polyclonal (1:1000; Sigma Aldrich)                          | Donkey anti-rabbit AlexaFluor 568 (Fig. 7)                   |
| Vimentin             | Mouse monoclonal (1:200; Dako, Ely, UK)                            | Donkey anti-rabbit AlexaFluor 488                            |
| GFAP                 | Rabbit polyclonal (1:200; Dako)                                    | Donkey anti-rabbit AlexaFluor 488                            |
| eNOS                 | Mouse monoclonal (1:200; BD Biosciences, London, UK)               | Donkey anti-mouse AlexaFluor 568                             |
| IBA1                 | Mouse monoclonal (1:500; Wako Chemicals GmbH, Neuss, Germany)      | Donkey anti-mouse AlexaFluor 568                             |
| PGP9.5               | Mouse monoclonal (1:100; Abcam)                                    | Donkey anti-mouse AlexaFluor 568                             |
| NG2                  | Mouse monoclonal (1:200; Millipore, Hertfordshire, UK)             | Donkey anti-mouse AlexaFluor 568                             |
| FGFR4                | Mouse monoclonal (1:200; Abcam)                                    | Donkey anti-mouse AlexaFluor 568                             |

All Secondary antibodies were used at a dilution of 1:200 and purchased from Life Technologies (Paisley, UK).

and AKR1b1 immunofluorescence levels between control and diabetic tissues, all detector and laser settings were held constant. Images were processed and analyzed using ImageJ software (National Institutes of Health [NIH], Bethesda, MD, USA). Images were imported, converted to 8-bit grayscale and background subtracted using measurements taken from tissue regions devoid of antibody staining. For each retinal section, regions of interest were drawn around individual Müller glia radial fibers in the inner plexiform layer (IPL) and the mean fluorescence intensity calculated. All secondary-only controls were negative for staining.

Immunocytochemistry was performed to characterize cultured mouse Müller glia and to investigate the effects of ALDH1a1 inhibition on FDP-lysine accumulation in these cells. For cell characterization, Müller glia were grown on coverslips for 2 days, fixed with 4% paraformaldehyde and incubated in permeabilization and blocking buffer (0.05% Triton-X 100 and 1% normal donkey serum) for 1 hour at 4°C. Cells subsequently were labeled for 1 to 3 days with antibodies against known markers of Müller glia as well as other markers to confirm that the cultures were not contaminated by other retinal cell types (Table 2). For ALDH1a1 inhibition studies, Müller glia were cultured on coverslips for 2 days and then grown for a further 4 days in the presence of the selective ALDH1a1 inhibitor, NCT-501<sup>30</sup> (10 µM; Tocris, Bristol, UK). Normal and vehicle (0.1% dimethyl sulfoxide [DMSO]) control experiments were run in parallel for comparison. Cells then were fixed and dual labeled with anti-FDP-lysine and anti-Kir4.1 antibodies (Table 2). In all immunocytochemistry experiments, cell nuclei were labeled with 4',6-diamidino-2-phenylindole (DAPI; 1:1000; Sigma-Aldrich) and cells imaged using a Leica TCS SP8 confocal microscope (HC PL APO ×20 lens; Leica Geosystems). For quantification of FDP-lysine staining, the same confocal settings were used for image acquisition across all experimental groups and captured images analyzed using Image J. Images were initially converted to 8-bit grayscale and background subtracted as described above. The Kir4.1 channel, which labeled the entire surface area of the Müller glia, then was used to digitally segment the cells from any background regions of the images. Following image segmentation, measurements of mean Müller glia FDP-lysine fluorescence intensity were made. No immunostaining was observed in cultured Müller glia incubated with secondary antibodies alone.

## Western Blotting

Retinas were homogenized in RIPA buffer and protein concentration determined using a BCA Assay Kit (Thermo-Fisher Scientific). Protein (15 µg) was loaded per lane and separated by SDS-PAGE on 10% polyacrylamide gels. Proteins then were transferred to polyvinylidene fluoride (PVDF) membranes and probed with the same primary antibodies used for immunohistochemistry (1:2000 anti-ALDH1a1 antibody, 1:500 anti-ALDH2 antibody, or 1:2000 anti-AKR1b1 antibody) along with mouse monoclonal β-actin antibodies (1:10,000; Life Technologies). After washing, membranes were incubated with appropriate IRDye 680 and IRDye 800 goat secondary antibodies against mouse or rabbit IgG (Li-COR Biosciences, Cambridge, UK). Membranes were imaged with an Odyssey Infrared Imaging System (Li-COR Biosciences) and the integrated density of protein bands determined using Image J.<sup>31</sup> Relative protein levels were normalized to β-actin loading controls.

## Aldehyde Dehydrogenase Activity Assay

Activity of ALDH in retinal lysates from individual animals was measured using the PicoProbe ALDH fluorometric assay kit (Biovision, San Francisco, CA, USA) according to the manufacturer's instructions. Retinas from each rat were lysed by 200 µl ALDH Assay Buffer, centrifuged for 5 minutes at 17,950g and 15 µl of the resulting supernatant used for the assay. Fluorescence measurements (Ex/Em = 535/587 nm) were made every 2 minutes using a Tecan Safire Microplate Reader (Tecan, Reading, UK). For purposes of statistical comparison, ALDH activity was normalized to the protein concentration of the samples (BCA assay).

## Müller Cell Viability

The effects of ALDH1a1 blockade on cultured Müller cell viability was quantified using the Promega RealTime-Glo MT assay kit (Promega, Southampton, UK). Müller cells were seeded into the bottom of 96-well plates (5 × 10<sup>3</sup> cells per well) and cultured for 96 hours under normal control conditions or in the presence of 10 µM NCT-501 or 0.1% DMSO (vehicle controls). Media then was exchanged for one containing RealTime-Glo MT and cell viability assessed using a FLUOstar Omega plate reader (BMG Labtech, Aylesbury, UK).

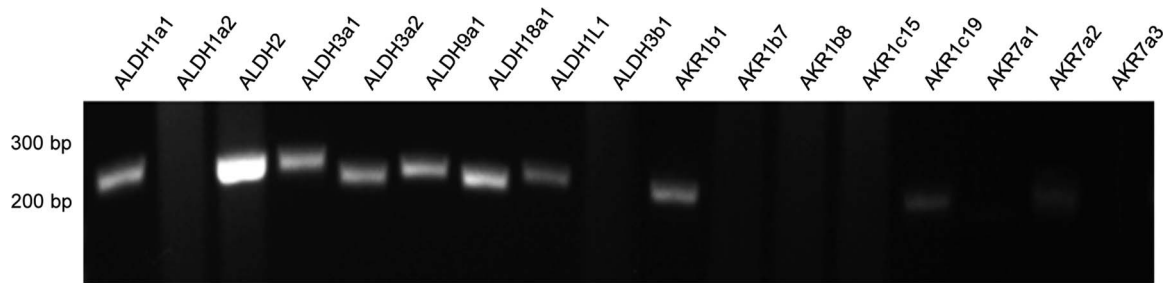


FIGURE 1. Representative RT-PCR gel showing the mRNA transcript expression of *ALDH* and *AKR* genes in the rat retina.

## Statistics

Data are presented as means  $\pm$  SEM. Statistical analyses were performed using Prism V5.03 (Graphpad Software, San Diego, CA, USA). Data normality was tested using Kolmogorov-Smirnov tests. Two-tailed Student's *t*-tests were used to assess statistical significance between data comprising two groups. Multiple comparisons were made by using the Kruskal-Wallis test followed by Dunn's post hoc analyses. In all graphic representations of the data, statistical significance is indicated as follows: \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ .

## RESULTS

### Experimental Animals

A total of 12 control, 19 sham-injected nondiabetic and 19 diabetic rats were used in these experiments. Mean HbA<sub>1c</sub> values were  $6.16 \pm 0.57\%$  and  $13.73 \pm 0.68\%$  for the nondiabetic and diabetic animals, respectively ( $P < 0.001$ ). All diabetic animals gained weight during the 3-month experimental period, but their growth was significantly reduced compared to that observed in nondiabetic animals (end weights were  $254 \pm 13$  and  $454 \pm 11$  g, respectively;  $P < 0.001$ ). In agreement with our previous findings,<sup>22</sup> diabetic animals displayed marked accumulation of FDP-lysine in retinal Müller glia after 3 months' disease duration. This was not evident in retinal sections from nondiabetic control animals (Supplementary Fig. S1).

### ALDH and AKR mRNA Expression in Retina

Presently, very little information is available regarding which ALDH and AKR enzymes are expressed in retinal tissue. Therefore, we began by screening for the mRNA transcript expression of these enzymes in retinas obtained from control Sprague Dawley rats. Genes of interest were selected primarily on the basis of those that had been shown previously to be involved in lipid aldehyde detoxification.<sup>32-43</sup> ALDH1L1 was included as a positive control, since its expression has been reported previously in the retina.<sup>44</sup> All primers used were first validated using rat liver which is known to express a broad range of ALDH and AKR enzymes<sup>45,46</sup> (Supplementary Fig. S2). The majority of the ALDH enzymes examined were present at the mRNA level in rat retina, including ALDH1a1, ALDH2, ALDH3a1, ALDH3a2, ALDH9a1, ALDH18a1, and ALDH1L1 (Fig. 1). In contrast, transcripts for ALDH1a2 and ALDH3b1 could not be detected. Fewer of the AKR enzymes examined were expressed in the retina. Of the 8 AKR enzymes investigated, only AKR1b1, AKR1c19, and AKR7a2 were detected (Fig. 1). Those which were not present were AKR1b7, AKR1b8, AKR1c15, AKR7a1, and AKR7a3.

### Diabetes-Induced Changes in ALDH and AKR mRNA Expression

Quantitative RT-PCR revealed that several of the ALDH and AKR enzymes expressed in the retina displayed changes in their mRNA transcript levels after 3 months of experimental diabetes. There was downregulation of mRNA for 5 of the 9 enzymes in the retinas of diabetic animals when compared to those of nondiabetic controls (Fig. 2), that is, ALDH1a1, ALDH3a2, ALDH18a1, AKR1c19, and AKR7a2. The only enzyme found significantly increased at the mRNA level was ALDH3a1, while the expression of ALDH2, ALDH9a1, and AKR1b1 remained unchanged (Fig. 2).

Since diabetes appeared to mediate downregulation of the mRNA expression of ALDH and AKR enzymes in retina, we were interested to establish whether this response also was common to other tissues in the eye. The lens and cornea were selected for comparison since diabetic patients are known to be at higher risk of development of vision-threatening cataracts and corneal complications than nondiabetic subjects.<sup>47</sup> Furthermore, gene knockout studies have shown previously that lens and corneal ALDH enzymes have a critical role in protecting against cataract formation and corneal damage comparable to those observed in diabetes.<sup>48-50</sup> Rat lens and corneal tissues expressed a greater number of ALDH and AKR enzymes compared to the retina (Supplementary Fig. S2; Table 3). Interestingly, however, none of the ALDH and AKR enzymes tested were downregulated at the mRNA level in lens and corneal tissue from diabetic animals; indeed, a large number appeared to be upregulated (Table 3). Taken together, these data suggested that diabetes induces differential effects on the

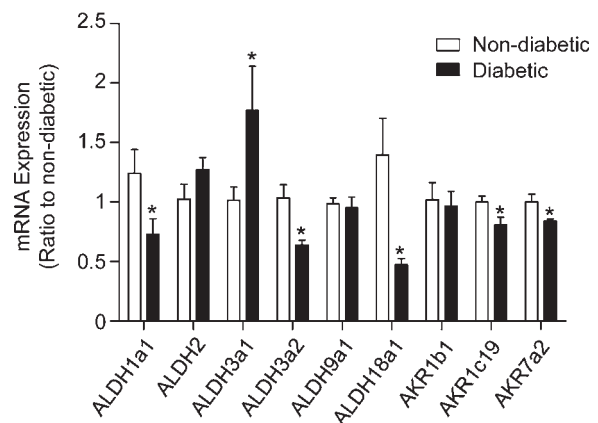


FIGURE 2. mRNA transcript expression of *ALDH* and *AKR* genes in nondiabetic and diabetic rat retinas. Results were averaged from retinas obtained from 6 nondiabetic and 6 diabetic animals. \* $P < 0.05$  for nondiabetic versus diabetic rats.

**TABLE 3.** Quantitative RT-PCR Data Detailing Normalized Average Fold Changes in *ALDH* and *AKR* Gene Expression in the Retina, Lens, and Cornea After 3 Months of Experimental Diabetes

| Enzyme   | Retina | Lens   | Cornea |
|----------|--------|--------|--------|
| ALDH1a1  | ↓ 0.6* | ↑ 2.1* | -      |
| ALDH1a2  | N/A    | N/A    | ↑ 6.5* |
| ALDH2    | -      | ↑ 1.5† | -      |
| ALDH3a1  | ↑ 1.5* | ↑ 3.4‡ | -      |
| ALDH3a2  | ↓ 0.7* | ↑ 2.3‡ | ↑ 1.4* |
| ALDH9a1  | -      | ↑ 1.6‡ | -      |
| ALDH18a1 | ↓ 0.5* | ↑ 2.8‡ | -      |
| ALDH3b1  | N/A    | ↑ 2.1‡ | ↑ 4.1† |
| AKR1b1   | -      | -      | -      |
| AKRc15   | N/A    | N/A    | ↑ 6.5† |
| AKRc19   | ↓ 0.8* | -      | ↑ 2.5* |
| AKR7a2   | ↓ 0.8* | ↑ 2.8* | -      |
| AKR7a3   | N/A    | -      | -      |

Upwards arrows, dashes, and downwards arrows indicate upregulation, no change in expression, and downregulation, respectively. N/A refers to genes that were not expressed within a particular tissue and, hence, not examined in diabetic animals.

\*  $P < 0.05$ .

†  $P < 0.01$ .

‡  $P < 0.001$  versus nondiabetic rats.

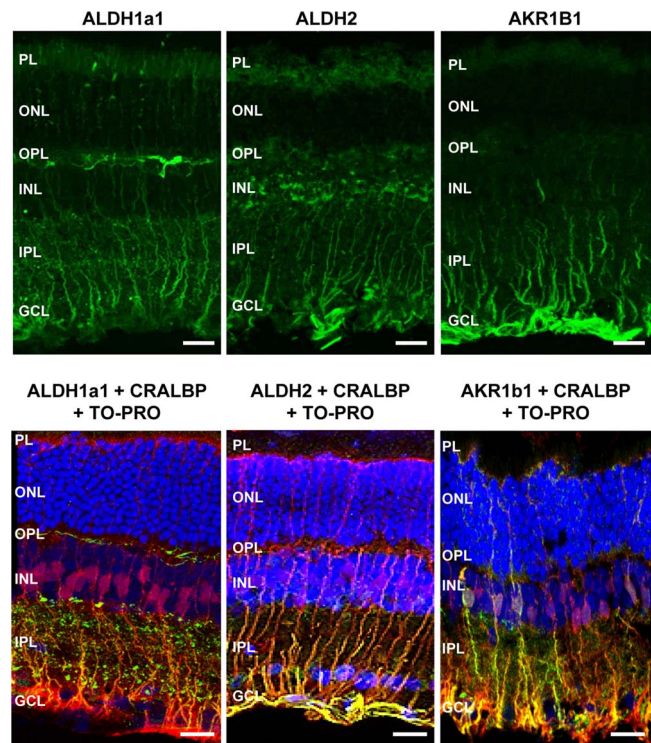
mRNA expression of lipid aldehyde detoxification enzymes in different tissues of the eye.

### Diabetes Downregulates Müller Glia ALDH1a1 Protein Expression

Presently, the reason(s) why the ACR-derived ALE, FDP-lysine, selectively accumulates in retinal Müller glia during diabetes remains unclear. However, this is unlikely to occur exclusively through elevated oxidative stress and lipoxidation since other lipid aldehydes and ALEs fail to accumulate in Müller cells during diabetes.<sup>10,22</sup> Therefore, we were interested in exploring whether the accumulation of FDP-lysine adducts in retinal Müller glia might be associated with the downregulation of specific ACR detoxifying ALDH and AKR enzymes in these cells. Of the *ALDH* and *AKR* genes found to be expressed in the retina (Fig. 1), three have been identified previously as key enzymes that contribute to ACR detoxification, namely, *ALDH1a1*,<sup>32,51</sup> *ALDH2*,<sup>32</sup> and *AKR1b1*.<sup>52</sup> Although *ALDH1a1* was the only one of these enzymes downregulated at the mRNA level in the whole retina during diabetes (Fig. 2), *ALDH2*, and *AKR1b1* also were included for subsequent analysis to validate our earlier findings at the protein level.

Confocal immunolabeling studies of retinal cryosections were initially performed to examine whether *ALDH1a1*, *ALDH2*, and *AKR1b1* are expressed in retinal Müller glia. As shown in Figure 3, all three enzymes were mainly localized to these cells. The localization of *ALDH1a1*, *ALDH2*, and *AKR1b1* in Müller glia was confirmed by colocalization with the Müller glia marker, *CRALBP* (Fig. 3). In addition to staining Müller glia, some punctate staining for *ALDH1a1* also was apparent in the IPL and in processes running along the outer plexiform layer.

Western blotting of whole retinal cell lysates obtained from nondiabetic and diabetic animals demonstrated that protein levels of *ALDH1a1*, *ALDH2*, and *AKR1b1* followed similar trends to those observed in our mRNA data. *ALDH1a1* protein was decreased by approximately 50% in retinas from diabetic animals, whereas no change in the protein expression of *ALDH2* or *AKR1b1* was evident between retinal lysates from nondiabetic and diabetic rats (Fig. 4).



**FIGURE 3.** Immunohistochemical staining for *ALDH1a1*, *ALDH2*, and *AKR1b1* in transverse retinal cryosections from normal rats. *Top*, *ALDH1a1*, *ALDH2*, and *AKR1b1* immunolabeling of the retina revealed staining patterns consistent with localization to Müller glia. *Bottom*, merged images from another set of retinal sections showing colocalization of *ALDH1a1*, *ALDH2*, and *AKR1b1* (green channel) with the Müller glia marker, *CRALBP* (red channel). Nuclei were counterstained with TO-PRO nuclear dye (blue channel). Scale bars: 20  $\mu\text{m}$ .

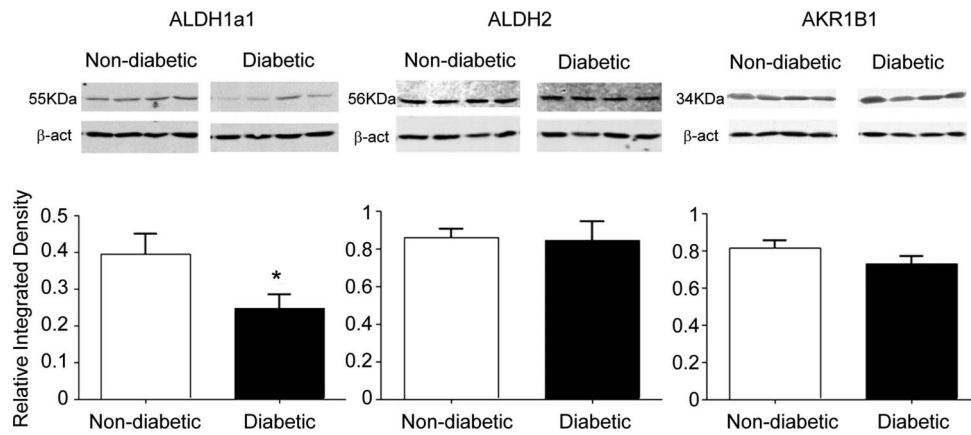
To confirm that diabetes specifically downregulates *ALDH1a1* protein expression in retinal Müller glia, semiquantitative analysis of immunofluorescence staining in Müller cell radial fibers in the IPL was performed. The staining intensity of the Müller cell processes for *ALDH1a1* was significantly reduced in retinal sections from diabetic animals compared to nondiabetic controls (Fig. 5). In contrast, no changes in staining intensity for *ALDH2* or *AKR1b1* were observed (Fig. 5).

### ALDH Activity Is Depressed in the Diabetic Retina

Our data supported the hypothesis that Müller glia FDP-lysine accumulation during diabetes may, at least in part, result from a reduction in ALDH-dependent detoxification of lipid aldehydes due to downregulation of *ALDH1a1*. To test this possibility further, experiments were performed to establish whether ALDH enzymatic activity is impaired in the diabetic retina. A commercially available PicoProbe Aldehyde Dehydrogenase Activity Assay Kit was used, which predominantly measures the rate of activity of *ALDH1a1* and *ALDH2*.<sup>53</sup> Consistent with our expression studies, ALDH enzymatic activity was appreciably lower in retinal lysates from diabetic rats when compared to those from the nondiabetic control group (Fig. 6).

### Effects of Reduced *ALDH1a1* Activity in Cultured Mouse Müller Glia

To examine whether there is a causal relationship between reduced *ALDH1a1* activity and FDP-lysine accumulation in



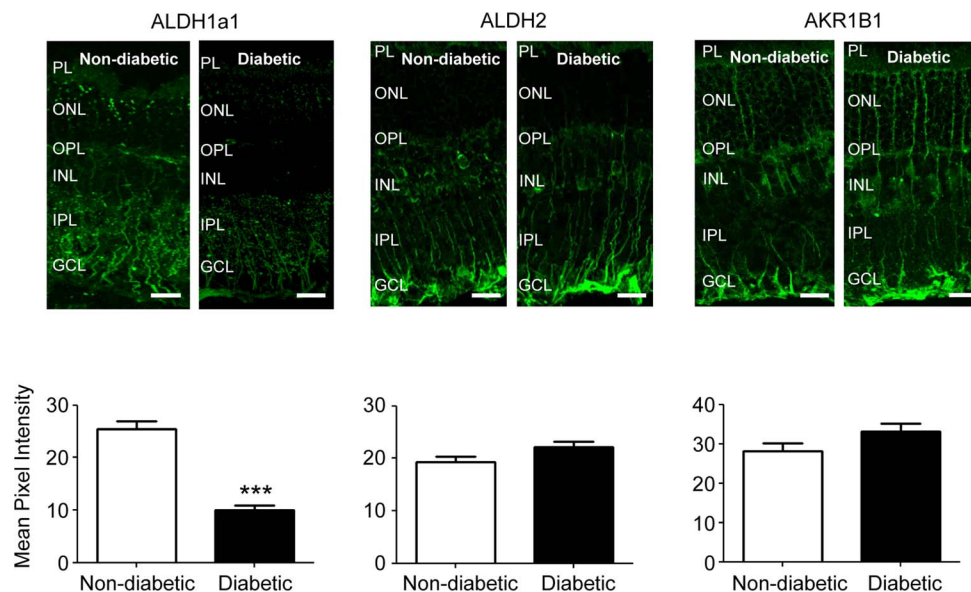
**FIGURE 4.** Effects of diabetes on retinal ALDH1a1, ALDH2, and AKR1b1 protein levels. *Top*, representative Western blot images showing ALDH1a1, ALDH2, and AKR1b1 protein expression in retinal extracts from nondiabetic and diabetic rats. Each *lane* shows retinal protein extracted from an individual animal. *Bottom*, bar charts showing the mean integrated density of ALDH1a1, ALDH2, and AKR1b1 protein bands in retinal samples from 7 nondiabetic and 7 diabetic animals normalized to  $\beta$ -actin loading controls. \* $P < 0.05$  for nondiabetic versus diabetic rats.

Müller glia we undertook cell culture studies using the ALDH1a1 inhibitor, NCT-501. Primary mouse Müller glia were cultured and their identity confirmed using several Müller glia markers, including CRALBP, Kir4.1, glutamine synthetase (GS), vimentin, and glial fibrillary acidic protein (GFAP; Supplementary Fig. S3). The cultures were negative for markers of vascular endothelial cells (eNOS), microglia (IBA-1), neurons (PGP9.5), pericytes (NG2), and fibroblasts (FGFR4; Supplementary Fig. S3). The expression of ALDH1a1 also was investigated and localized to nuclear and cytoplasmic regions of the cells (Supplementary Fig. S3). Exposure to 10  $\mu$ M NCT-501 for 96 hours produced an increase in FDP-lysine accumulation, particularly within the nuclei of the Müller glia (Fig. 7). However, some cytoplasmic staining for FDP-lysine also was observed that was not apparent in the normal and vehicle (DMSO) control groups (Fig. 7). Diabetes causes Müller cell loss,<sup>54</sup> an effect that we have shown previously to occur in

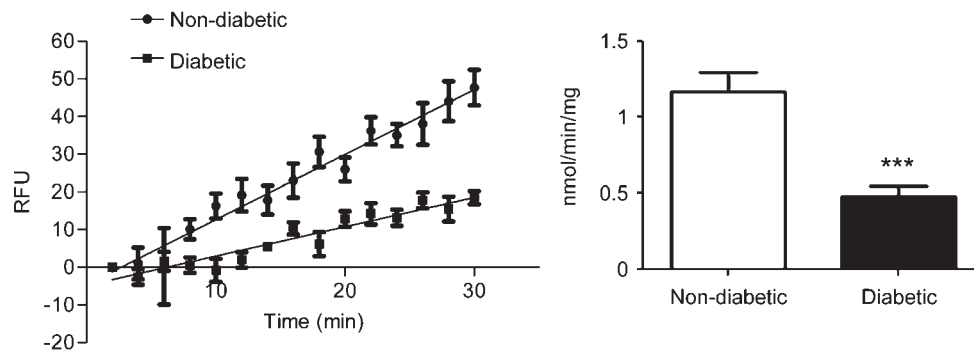
cultured Müller glia following accumulation FDP-lysine-modified protein.<sup>22</sup> To examine whether inhibition of ALDH1a1 activity in itself is sufficient to trigger cytotoxicity, we exposed cultured Müller glia to 10  $\mu$ M NCT-501 for 96 hours and undertook cell viability assays. NCT-501 reduced cell viability by approximately 30% when compared to the normal and vehicle control groups (Fig. 7).

### DISCUSSION

To the best of our knowledge, our results represented the first broad scale examination of the isoform expression of lipid aldehyde detoxifying ALDH and AKR enzymes in the retina and quantification of changes in their mRNA expression levels during diabetes. Our data have highlighted differences in the complement of ALDH and AKR enzymes expressed in the



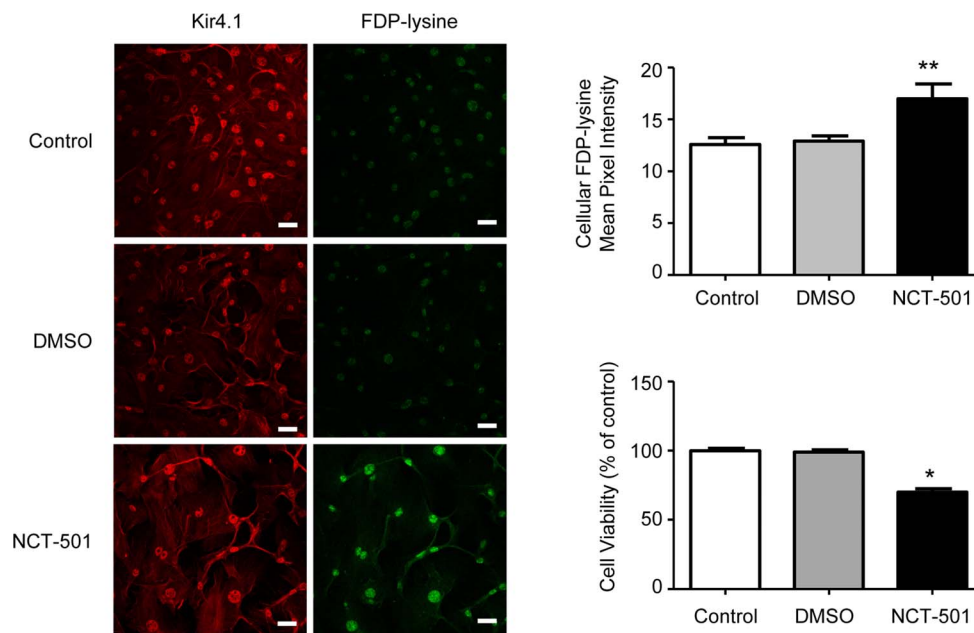
**FIGURE 5.** Immunohistochemical comparison of Müller glia ALDH1a1, ALDH2, and AKR1b1 expression in retinal sections from nondiabetic and diabetic rats. *Top*, representative confocal images showing reduced Müller glia immunoreactivity for ALDH1a1, but not ALDH2 or AKR1b1, in retinal sections from diabetic rats. *Scale bars*: 20  $\mu$ m. *Bottom*, summary data quantifying Müller glia ALDH1a1, ALDH2, and AKR1b1 immunofluorescence in retinas from 6 nondiabetic and 6 diabetic animals. Retinal sections used in these studies were from the same groups of animals used for the FDP-lysine immunoreactivity studies in Supplementary Figure S1. \*\*\* $P < 0.001$  versus nondiabetic.



**FIGURE 6.** ALDH activity in the nondiabetic and diabetic retina. *Left*, mean time course profiles of ALDH activity in retinal homogenates from 6 nondiabetic and 6 diabetic animals. RFU, relative fluorescence units. *Right*, bar charts showing the rate of ALDH activity converted to nmol NADH/min/mg protein. \*\*\* $P < 0.001$  versus nondiabetic.

retina when compared to lens and cornea. In particular, it was evident that the retina expresses a smaller number of these enzymes. This difference might reflect the fact that the lens and cornea are normally exposed to much higher levels of photooxidative stress than the retina. Indeed, the lens and cornea are known to have a key role in absorbing ultraviolet light and protecting the retina against photooxidative injury.<sup>55</sup> Therefore, the wider range of ALDH and AKR enzymes expressed in the lens and cornea may represent a protective mechanism through which these tissues are capable of withstanding relatively high levels of lipooxidative stress under physiologic conditions. It also should be emphasized that several members of the ALDH superfamily have been identified as lens and corneal crystallins and, therefore, also have an important function in maintaining the transparency of the anterior segment of the eye.<sup>56</sup> This also could explain why a broader range of these enzymes were expressed in the lens and cornea when compared to the retina.

We observed a general trend toward downregulation of *ALDH* and *AKR* gene expression in the retina during diabetes. These findings are consistent with the proposal that, in addition to increased oxidative stress and lipoxidation, the impairment of lipid aldehyde defense mechanisms also may contribute to the accumulation of toxic aldehydes and ALEs in the diabetic retina. These results are similar to those reported previously in the heart, where downregulation of certain ALDH enzymes has been implicated in the accumulation of lipid aldehydes and the development of cardiomyopathy during experimental diabetes.<sup>57</sup> Loss of ALDH activity also has been suggested to have a role in the diabetes-induced accumulation of lipid aldehydes in the liver,<sup>58</sup> although whether this occurs through a reduction in the expression of these enzymes or results from their direct inhibition by the diabetic milieu remains to be determined. In contrast to the retina, diabetes triggered upregulation of many of the ALDH and AKR enzymes examined in the lens and cornea. It is unclear why diabetes



**FIGURE 7.** Effects of the ALDH1a1 inhibitor, NCT-501, on FDP-lysine levels and cell viability in cultured retinal Müller glia. *Left*, representative confocal images showing Kir4.1 and FDP-lysine immunoreactivity following culture of Müller glia for 96 hours under normal conditions or in the presence of 0.1% DMSO (vehicle controls) or 10  $\mu$ M NCT-501. Scale bars: 50  $\mu$ m. *Top right*, summary data quantifying Müller glia FDP-lysine immunofluorescence levels across the three experimental groups. For these measurements, the Kir4.1 channel was used to digitally segment the Müller glia to ensure that any background regions between individual cells did not contribute to the analyses (see Methods). *Bottom right*, bar chart showing the effects of NCT-501 on Müller glia cell viability measured using the Promega RealTime-Glo MT assay. All experiments were performed in triplicate from three separate Müller glia isolations. \* $P < 0.05$ , \*\* $P < 0.01$  versus control.

exerts opposing actions on *ALDH* and *AKR* gene expression in different tissues of the eye. Previous studies have shown that a number of the *ALDH* and *AKR* genes are downstream targets of the transcription factor, Nrf2,<sup>59</sup> which regulates the constitutive and inducible transcription of a wide range of antioxidant defense genes.<sup>60</sup> During diabetes, however, Nrf2 activity has been reported to be repressed in the retina and the lens,<sup>61,62</sup> and, therefore, differential activation of this pathway in these tissues is unlikely to explain the differences in *ALDH* and *AKR* gene expression observed in the present study. Other studies have suggested that certain lipid aldehyde detoxifying *ALDH* and *AKR* genes may be regulated at the transcriptional level by the transcription factors, AP-1,<sup>63</sup> NFY,<sup>64</sup> NFAT-5,<sup>65</sup> and hStaf/ZNF143,<sup>66</sup> and posttranscriptionally by miR-28.<sup>67</sup> Clearly, it will be of interest in the future to examine whether variable activation/inhibition of these pathways underlies the heterogeneous effects of diabetes on *ALDH* and *AKR* gene expression in the retina when compared to the lens and cornea.

Recent evidence has suggested that Müller glia dysfunction has a critical role in the pathogenesis of diabetic retinopathy.<sup>1</sup> Furthermore, we have shown that accumulation of the ACR-derived ALE, FDP-lysine, is a key pathogenic process in the development of Müller glia abnormalities during diabetes.<sup>21,22</sup> In an effort to better understand why FDP-lysine selectively accumulates in Müller glia during diabetes, and in light of our gene expression findings in whole retina, we were interested in examining whether diabetes causes downregulation of specific ACR detoxifying ALDH and AKR enzymes in these cells. As a first step in addressing this issue, we sought to establish which of the ACR detoxifying ALDH and AKR enzymes detected at the mRNA level in whole retina are expressed in Müller glia. ALDH1a1, ALDH2, and AKR1b1 were identified as the main ACR detoxifying enzymes expressed in the retina and all three were localized to Müller glia. These results strongly suggested that Müller glia may act as primary sites for lipid aldehyde and ACR detoxification in the retina. When considered together with previous studies showing that other enzymes involved in detoxification processes, such as glutathione s-transferase<sup>68</sup> and glutamine synthase (glutamate and ammonia detoxification),<sup>69</sup> also are predominantly localized to retinal Müller cells, our findings add weight to the notion that one of the key functions of these cells is to protect the retina from endogenous and xenobiotic toxicants.<sup>69</sup>

Our results support the view that downregulation of ALDH1a1 may contribute to retinal Müller glia accumulation of FDP-lysine adducts in diabetes. In particular, our data have shown that ALDH1a1 expression is reduced at the mRNA and protein level in the whole retina, and, specifically, within Müller glia radial fibers during diabetes. These changes also were associated with a reduction in the combined activity of ALDH1a1/ALDH2 in the whole retina and blockade of ALDH1a1 activity was sufficient to cause FDP-lysine accumulation and reduced cell viability in cultured Müller glia. Despite this, and although we observed no alterations in the gene or protein expression of ALDH2 or AKR1b1 in the diabetic retina, at this stage we cannot fully rule out the possibility that changes in the activity or availability of these enzymes also might contribute to the diabetes-induced FDP-lysine accumulation in Müller glia.

In addition to their role in detoxifying lipid aldehydes, ALDH and AKR enzymes are known to be involved in a broader spectrum of biological processes of physiologic and pathophysiological significance. Thus, it seems likely that our data showing downregulation of a number of ALDH and AKR enzymes in the diabetic retina may have implications beyond those just relating to lipid aldehyde and ALE accumulation. ALDH1a1, for example, is known to be involved in the biosynthesis of retinoic acid (RA) from retinal (retinaldehyde),

a vitamin A metabolite.<sup>55</sup> In glial cells of the retina, RA signaling via the RAR $\alpha$  receptor, is known to have an important role in regulating the expression of glial-cell derived cytokines involved in maintaining inner blood retinal barrier (iBRB) function.<sup>70</sup> Hence, it is possible that ALDH1a1 downregulation in retinal Müller glia with a concomitant decrease in RA signaling could contribute to iBRB breakdown during diabetic retinopathy, a major cause of vision loss in this disease. ALDH18a1 is an enzyme that is known to convert glutamate to glutamate 5-semialdehyde, an intermediate in the biosynthesis of proline, ornithine, and arginine.<sup>71</sup> Glutamate accumulation is a recognized phenomenon in the diabetic retina that has been linked to the neurodegenerative changes seen in diabetic retinopathy.<sup>72</sup> The elevated glutamate levels in the diabetic retina are thought to arise through a number of mechanisms, including inhibition of glial glutamate transporters and downregulation of glutamine synthase.<sup>73</sup> Our data showing strong downregulation of ALDH18a1 in the diabetic retina suggests that changes in the activity of this enzyme also might be involved, and this possibility clearly warrants future investigation.

In summary, our data suggested that lipid aldehyde defense mechanisms are impaired in the diabetic retina and that this may represent a major mechanism through which toxic aldehydes and ALEs accumulate in this tissue during diabetic retinopathy. Therapeutic strategies aimed at enhancing the expression or activity of certain ALDH and AKR enzymes could have utility for the future treatment of this disease.

### Acknowledgments

Supported by Fight for Sight, UK and the National Eye Research Centre (grant number: SCIAD 076).

Disclosure: **R.E. McDowell**, None; **M.K. McGahon**, None; **J. Augustine**, None; **M. Chen**, None; **J.G. McGeown**, None; **T.M. Curtis**, None

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