

# Monocarboxylate Transporter Expression Remains Unchanged during the Development of Diabetic Retinal Neuropathy in the Rat

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**PURPOSE.** To determine the effect of diabetes on monocarboxylate transporter (MCT) expression in the rat retina.

**METHODS.** Diabetes was induced in Wistar rats by a single intraperitoneal injection of streptozotocin (62.5 mg/kg). Rats were killed after 10 weeks, and the retinal levels of PKC $\alpha$ , bFGF, glial fibrillary acidic protein (GFAP), caspase-3, and MCT1, -2, and -4 were assessed with immunoblot analysis and RT-PCR. Hippocampal samples were used as a comparison. In other animals, the retinas were processed histologically and sections stained for the breakdown of DNA (TUNEL procedure) and for the distribution of MCT1, -2, and -4.

**RESULTS.** Diabetic rats exhibited cataract formation, elevated blood glucose levels, and polydipsia. Retinal levels and distribution of MCT1, 2, and -4 were similar in the diabetic and age-matched control groups; however, the early retinopathy markers bFGF and PKC $\alpha$  were significantly elevated in the diabetic retinas but not in the hippocampal samples. ERG recordings showed decreased oscillatory potentials in the diabetic group, and TUNEL staining was most evident in the photoreceptor layer. Activated caspase-3 levels were elevated in the diabetic retina.

**CONCLUSIONS.** The expression of retinal MCT1, -2, and -4 is unaffected after 10 weeks of diabetes in rats. It appears unlikely that diabetic retinal neuropathy is a result of a hyperglycemic induction of altered MCT expression. (*Invest Ophthalmol Vis Sci.* 2005;46:2878–2885) DOI:10.1167/iovs.04-1458

Diabetic retinopathy has traditionally been considered a microvascular disease of the retina caused by excessive tissue glucose. However, recent work has shown that neuronal death in the retina predates the onset of microvascular changes by many years<sup>1</sup> and is not accompanied by similar changes in brain tissue. Decreased retinal function can be demonstrated early in diabetes by a loss of contrast sensitivity<sup>2</sup> and tritan changes in color vision that correlate with a selective loss of

S-type cones.<sup>3</sup> Oscillatory potentials (representing the function of the inner retina) are reduced on the electroretinogram (ERG) in early diabetes, and this decrease predicts deterioration caused by the disease in humans.<sup>4</sup> In humans and animal models, there is a matching increase in apoptotic activity in the diabetic retina, demonstrated by increased levels of activated caspases<sup>5,6</sup> and TUNEL staining<sup>7</sup> and a decreased thickness of the ganglion cell and inner plexiform layers in retinal sections.<sup>7</sup>

It has been proposed that diabetic retinal neuropathy is due either to secondary changes in the extracellular fluid after blood-retina barrier breakdown or to a direct effect of diabetes on neuronal metabolism.<sup>8</sup> The hyperglycemic changes in the diabetic retina are thought to lead to an oversupply of glucose in the retinal tissues and compensatory changes in other metabolites, with conflicting reports concerning changes in lactate, with *in vivo* investigations reporting both increases<sup>9,10</sup> and decreases (Ambrosio AF, et al. *IOVS* 2004;45:ARVO E-Abstract 3222) in the metabolite.

It is widely accepted that central nervous system (CNS) neurons rely on glucose to maintain metabolism, but several studies have shown that lactate, a product of the glycolytic pathway, acts as an energy source for rat brain neurons and astroglia *in vitro*.<sup>11</sup> Lactate can support synaptic function in the hippocampal slice<sup>12</sup> and appears to be necessary for recovery of this preparation from hypoxia.<sup>13</sup> Glia release lactate under conditions of hypoxia or hypoglycemia,<sup>14</sup> and lactate release has also been demonstrated in conscious rats in response to a variety of metabolic insults.<sup>15</sup> This finding has led to the suggestion that a “lactate shuttle” operates in the CNS, where lactate is released by glia for use by neurons in a variety of conditions.<sup>16,17</sup> Lactate therefore appears to be an important contributor to neural health and resistance to injury.

In the retina, the role of lactate is more controversial, with some investigators recently presenting work consistent with the notion that lactate is present in this tissue merely as a byproduct of metabolism.<sup>18</sup> However, lactate production is known to be significantly higher in the retina than in the brain,<sup>19</sup> and other investigators have shown that lactate assists in the maintenance of interactions between Müller cells, neurons, and photoreceptors.<sup>20,21</sup> Müller cells release lactate for metabolism by photoreceptors<sup>22</sup> and, in conditions of low glucose, by other retinal neurons.<sup>23</sup> It is a preferred substrate for the Krebs cycle in photoreceptors under conditions of intense activity<sup>19</sup> and in protecting the optic nerve from injury.<sup>24</sup> These data, together with the previously mentioned studies in other CNS tissue, suggest that lactate may also play an important role in maintaining retinal health.

Lactate is transported in and out of cells by proton-coupled monocarboxylate transporters (MCTs). Eight isoforms have been described; with MCT1, -2, and -4 present in the retina.<sup>25</sup> The regulation of these transporters is known to be an active process, and alterations have been reported in the brain during ischemia<sup>26</sup> and diet-induced ketosis,<sup>27</sup> two conditions known to be characteristic of chronic uncontrolled type 1 diabetes in

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TABLE 1. Sequences for Amplified mRNAs

mRNA	Primer Sequences	Product Size (bp)	Annealing Temp. (°C)	Cycles	Accession Number
Cyclophilin	5'-TGG TCA ACC CCA CCG TGT TCT TCG-3' 5'-TCC AGC ATT TGC CAT GGA CAA GA-3'	314 bp	52	26	M19533
MCT 1	5'-AGA AGT CAG CCT TCC TCC TTT-3' 5'-CCA CAA GCC CAG TAT GTG TAT-3'	394 bp	55	32	D63834
MCT 2	5'-GGC CTT CGG TAG GAT TAA TAG-3' 5'-ATG CCT GAT GAT AAC ACG ACT-3'	367 bp	55	34	X97445
MCT 4	5'-TGC GGC CCT ACT CTG TCT AC-3' 5'-TCT TCC CGA TGC AGA AGA AG-3'	369 bp	55	34	U87627
bFGF	5'-GCCTTCCGACCCGGCCACTTCAAG G-3' 5'-GCACACACTCCCTTGATGGACACAA-3'	179 bp	55	27	M22427
GFAP	5'-ATT CCG CGC CTC TCC CTG TCT C-3' 5'-GCT TCA TCC GCC TCC TGT CTG T-3'	437 bp	55	27	U03700

the retina.<sup>28</sup> In the retina, inhibiting MCTs alters the ERG and retinal metabolism.<sup>29</sup> Given these facts and the importance of glucose and lactate in neural metabolism, the main purpose of this study was to elucidate whether the altered loads of glucose and lactate present in diabetic retinal tissues alter the expression of these transporters, since any changes could contribute to diabetic retinal neuropathy by further altering the availability of energy substrates for neuronal metabolism.

## METHODS

### Animals

All experiments conformed to Principles of Laboratory Animal Care (NIH Publication 85-23) and adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Twenty-eight age-matched adult male Wistar rats (300–350 g) were housed in a temperature- and humidity-controlled room with a 1-hour light-dark cycle and were provided with food and water ad libitum. Rats were randomly assigned to diabetic and control groups of 14 rats each. Baseline random blood glucose concentrations were measured with a glucometer (Precision PCx; Medisense, Cambridge, UK). Randomly selected rats received an intraperitoneal injection of 62.5 mg/kg of streptozotocin in 10 mM citrate buffer, and the control group received an intraperitoneal injection of the citrate buffer alone and were then left untreated until the end of the experiment. Diabetes was allowed to continue for 10 weeks, to allow previously reported signs of diabetic retinal neuropathy to develop.<sup>30,31</sup> Rats were then killed and whole retina and hippocampal samples taken for analysis.

### Western Blot Analysis

Retinal proteins were isolated simultaneously with RNA (Tri-Reagent; Sigma-Aldrich, Poole, UK). After processing, samples were solubilized in buffer with protease inhibitors (20 mM Tris-HCl [pH 7.4], containing 2 mM EDTA, 0.5 mM EGTA, 1% SDS, 0.1 mM phenylmethylsulfonyl fluoride, 50 µg/mL aprotinin, 50 µg/mL leupeptin, and 50 µg/mL pepstatin A). An equal volume of sample buffer (62.5 mM Tris-HCl [pH 7.4] containing 4% SDS, 10% glycerol, 10% β-mercaptoethanol, and 0.002% bromophenol blue) was then added. Electrophoresis of samples was performed with 10% polyacrylamide gels, containing 0.1% SDS, and the proteins were blotted onto activated ECL paper (GE Healthcare, Little Chalfont, UK). Blots were incubated for 3 hours at room temperature with primary antibodies against actin (monoclonal antibody, 1:1,000; Chemicon, Chandler's Ford, UK), MCT1 (polyclonal anti-rabbit 1:10,000; Chemicon), MCT2 (polyclonal chicken, 1:1,000; Chemicon UK), MCT4 (1:100 polyclonal goat; Santa Cruz Biotechnology, Santa Cruz, CA), bFGF (polyclonal rabbit, 1:200; Santa Cruz Biotechnology), glial fibrillary acidic protein (GFAP; polyclonal rabbit, 1:400; Dako, Ely, UK), and activated caspase 3 (monoclonal antibody,

1:500; BD Biosciences, Cowley, UK). Blots were then incubated with appropriate secondary antibodies conjugated to horseradish peroxidase (1:5000, Sigma-Aldrich). Proteins were visualized by adding the chemiluminescent reagent (ECL; GE Healthcare) to the blots for 1 minute, and the intensity of the fluorescence recorded on high-sensitivity ECL film (GE Healthcare). The resultant bands' intensity was quantified by computer (LabWorks software; UVP, Inc., Upland, CA).

### Semiquantitative PCR

The levels of cyclophilin; MCT1, -2, and -4; bFGF; and GFAP were determined by semiquantitative RT-PCR, as described previously.<sup>32</sup> Primer sequences are listed in Table 1. Briefly, total RNA was isolated and first-strand cDNA synthesis performed on 2 µg of DNase treated RNA. Aliquots of the resultant cDNA species were amplified in PCR buffer with MgCl<sub>2</sub> (4 mM with MCT1, MCT2, bFGF, and GFAP and 4.5 mM with MCT4). Reactions were initiated by incubating at 94°C for 10 minutes, and PCRs (94°C, 15 seconds; 52°C, 30 seconds; 72°C, 30 seconds) were performed for a suitable number of cycles followed by a final extension at 72°C for 3 minutes. Amplifications of each cDNA species were performed in a single run to reduce variation. PCR reaction products were separated on 1.5% agarose gels using ethidium bromide for visualization. The relative abundance of each PCR product was determined by quantitative analysis of digital photographs of the gels viewed under UV light (LabWorks software; UVP, Inc.).

### Real-Time PCR

Real-time RT-PCR reactions were performed in 96-well optical reaction plates using the cDNA equivalent of 50 ng total RNA for each sample in a total volume of 25 µL (*TaqMan* Universal PCR Master Mix; Applied Biosystems, Inc. [ABI], Foster City, CA). The thermal cycling conditions of PCR were as follows: 50°C for 2 minutes and 95°C for 10 minutes and 40 cycles of amplification comprising 95°C for 15 seconds and 60°C for 1 minute. The PCR assay was performed with a sequence detector (Prism 7700; ABI). MCT primers and probes (Table 2) were selected from GenBank (<http://www.ncbi.nlm.nih.gov/genbank>); provided in the public domain by the National Center for Biotechnology

TABLE 2. Effect of Experimental Diabetes on the Total Retinal Levels of MCT1, -2, and -4 mRNAs by Real-Time PCR Relative to Control Rats

Treatment	MCT1	MCT2	MCT4
Control	1.00 (0.93–1.08)	1.00 (0.81–1.23)	1.00 (0.88–1.14)
Diabetic	0.90 (0.83–0.98)	1.04 (0.89–1.22)	1.00 (0.88–1.13)

All values are normalized to the level expressed in control rats. Student's unpaired *t*-test showed no significant differences. *n* = 20–21.

Information, Bethesda, MD) and designed with primer design software (Primer Express; Applied Biosystems), whereas GAPDH primers and probe were obtained from rodent GAPDH control reagents (TaqMan; ABI). Amplification of GAPDH mRNA was performed as the internal control gene, to account for variations in RNA levels between different samples. To allow a comparison to be made between the levels of expression of the different MCT isoforms in the retinas of control and diabetic rats, results obtained from the real-time PCR experiments were quantified by using the comparative threshold ( $2^{-\Delta\Delta C_T}$ ) method.<sup>33</sup> This method essentially comprises two steps: First, the amount of target gene (MCT) in the retina is normalized to the levels of an endogenous housekeeping gene (GAPDH), and second, the normalized amount of target gene in diabetic retinas is expressed relative to the normalized amount of target gene in the control retinas. The threshold cycles ( $C_T$ ) were calculated on computer (Prism 7000 SDS Software; ABI).

### Immunohistochemistry

Retinas were taken from rats and fixed in 4% paraformaldehyde for 30 minutes and cryopreserved in 10- $\mu$ m-thick frozen sections. Sections were incubated at 4°C overnight with primary antibody against MCT1 (polyclonal rabbit, 1:1000), MCT2 (polyclonal chicken, 1:200), and MCT 4 (polyclonal goat, 1:100). Protein was visualized with the sensitive avidin-biotin-peroxidase complex kit, according to the manufacturer's documentation, with the appropriate secondary antibody, 3,3'-diaminobenzidine, and 0.1% hydrogen peroxide used as substrates.

### Assessment of DNA Breakdown by the TUNEL Method

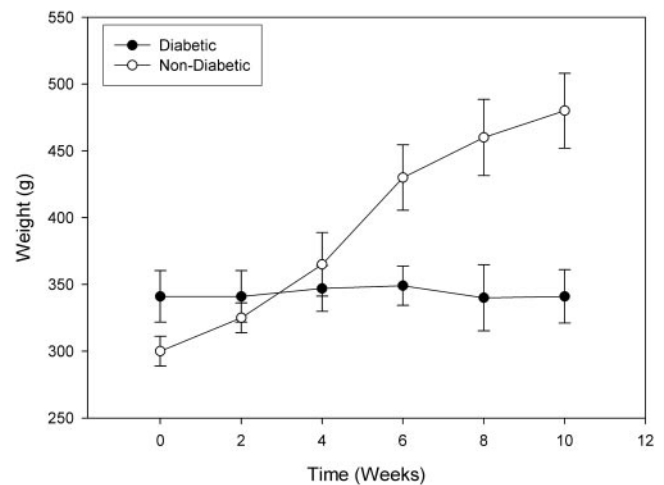
TUNEL was performed as described previously.<sup>34</sup> Briefly, free DNA ends were labeled in retinal sections by incubating in buffer (30-mM Tris-HCl [pH 7.2], containing 140-mM sodium cacodylate and 1-mM cobalt chloride) along with 0.25 U/ $\mu$ L of terminal deoxynucleotidyl transferase (Promega, Southampton, UK) and 10  $\mu$ M biotin-16-dUTP (Roche Diagnostics, Lewes, UK). The reaction was stopped by two 15-minute washes in saline sodium citrate buffer (30 mM sodium citrate with 300 mM sodium chloride). After an additional 15-minute wash in 1% bovine serum albumin in phosphate-buffered saline solution, stained nuclei were visualized with CY-3 labeled streptavidin (Fluorolink; GE Healthcare). Stained nuclei were quantified and averaged for each retinal section for normal and diabetic groups.

### ERG Methodology

Maximum amplitude responses from Wistar rats were recorded after dark adaptation for at least 2 hours and preparation under dim red illumination. Anesthesia was achieved with a dose of 0.063 mg/kg fentanyl, 2 mg/kg fluanisone, and 1 mg/kg diazepam, and the pupils were dilated with cyclopentolate (1%). After instillation of a single drop of 0.4% benoxinate, the eyelids were separated by placing a cotton thread loosely around the equator of the globe, and the rat was placed on a stereotactic frame facing the stimulus at a distance of 50 cm. A reference electrode was placed through the ear, a grounding electrode was attached to the scruff of the neck, and a platinum electrode was placed in contact with the central cornea. The cornea was intermittently irrigated with physiologic saline (BSS; Alcon, Fort Worth, TX), to maintain the baseline recording and to prevent exposure keratopathy. All recordings were made in near darkness, and the rats were kept warm during and after the procedure.

After a single flash, responses to four subsequent flashes (10  $\mu$ s, 0.1 Hz) from a photic stimulator (Grass PS33-plus; Grass-Telefactor, Instrument Division, West Warwick, RI) set at maximum brightness (setting 16,  $\sim$ 17.5 cd-s/m<sup>2</sup>) were amplified (gain set at 300), filtered (100-Hz low-pass filter, DC high-pass filter, and 50-Hz filter notch activated) and averaged (1902 Signal Conditioner/1401 Laboratory Interface; CED, Cambridge, UK) to give the maximum ERG response. The b-wave amplitude was measured from the trough of the a-wave to the peak of

### Weight Gain of Experimental Rats



**FIGURE 1.** Serial weight measurements in the diabetic and control groups. Rats were weighed fortnightly from the time diabetes was confirmed, with random blood glucose measurement. Error bars are  $\pm$  SEM. Statistically significant differences in the weights of the groups relative to baseline measurements were evident after 4 weeks of diabetes.  $P < 0.05$  by Student's *t*-test with 14 animals in each group.

the b-wave, and the a-wave was measured as the difference in amplitude between the recording at onset and the trough of the negative deflection. Oscillatory potentials were obtained by treating recordings with a digital band-pass filter between 60 and 200 Hz and total oscillatory potentials measured from trough to adjacent peak of the four largest potentials. Recordings were taken 9 weeks after the administration of streptozotocin, 1 week before sample collection.

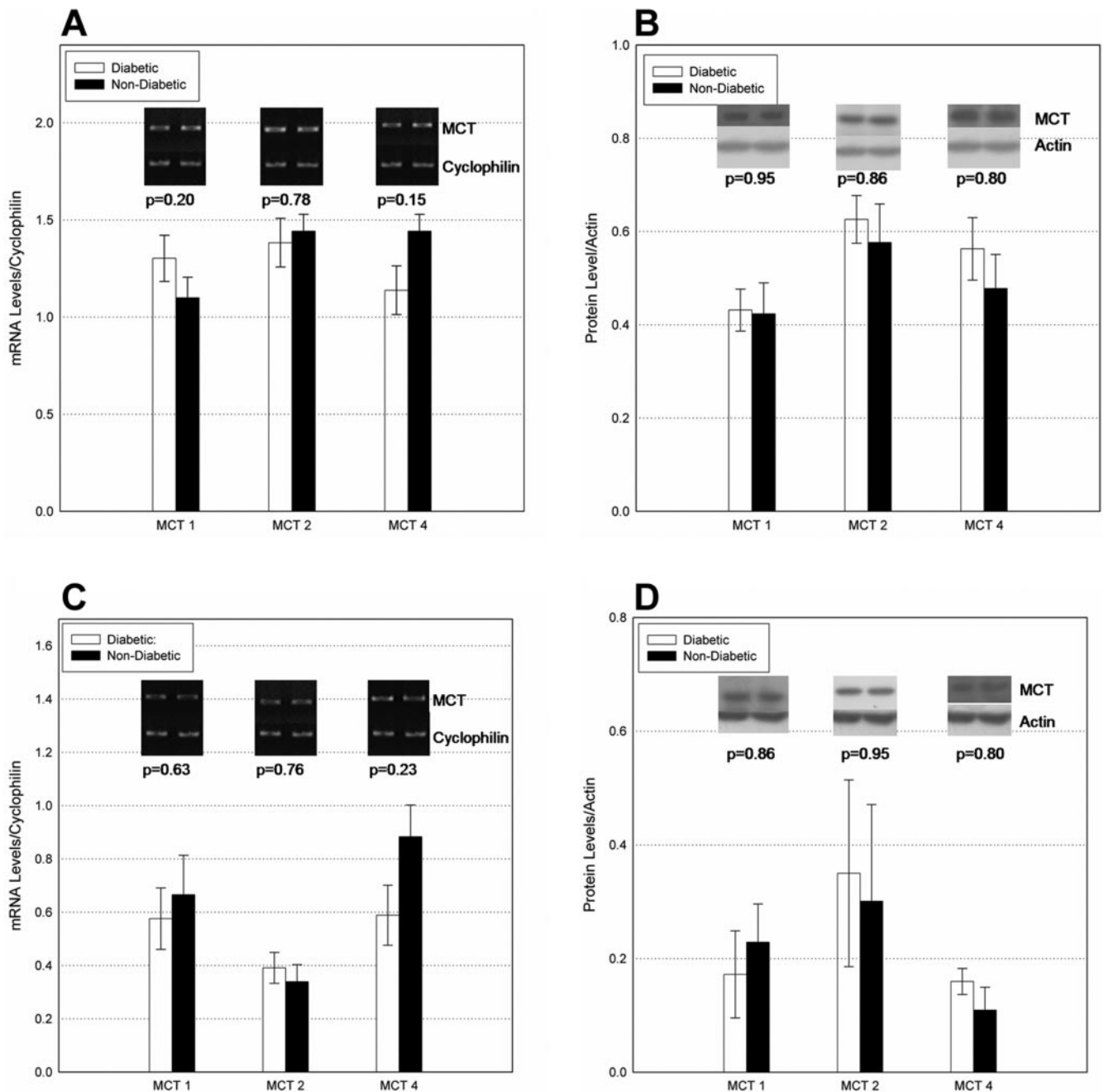
### Statistical Analysis

Analysis was performed on computer (SPSS statistical package, ver. 11; SPSS Science, Chicago, IL). Animal weights and blood glucose levels were compared by using Student's unpaired *t*-test and the Mann-Whitney test. Both produced similar results. mRNA and protein expression in the retina and hippocampus was analyzed with the aid of the Oxford University Department of Statistics, using both samples from each animal and the repeated-measures function of the analysis software (SPSS) to allow for intra- and interanimal variability. All results are given as the mean  $\pm$  SEM.  $P < 0.05$  was considered significant.

## RESULTS

### Confirmation of Experimental Diabetes

Before induction of diabetes, there was no significant difference between control and diabetic animals' weights or random blood glucose levels. No rats had obvious cataract at the beginning of the experiment. Ten weeks after injection of streptozotocin, the random blood glucose concentration in the diabetic and control group was  $31.4 \pm 0.9$  and  $8.2 \pm 0.3$  mM, respectively ( $P < 0.001$ ). Ten of the 14 diabetic rats were noted to have cataracts, and the water intake of diabetic groups was markedly increased. Although slightly heavier at the beginning of the experiment due to the randomization process, the diabetic group did not gain weight at any time during the experimental period (Fig. 1), with final weights of  $340.0 \pm 24.6$  g compared with  $482 \pm 28.5$  g in the control group ( $P < 0.001$ ).



**FIGURE 2.** MCT expression in the retina. (A) mRNA levels in the retina by semiquantitative PCR. (B) Protein levels in the retina. (C) mRNA levels in the hippocampus by semiquantitative PCR. (D) Protein levels in the hippocampus. Probabilities reflect significance differences, allowing for intraclass correlation between the eyes, with 20 eyes in each group.

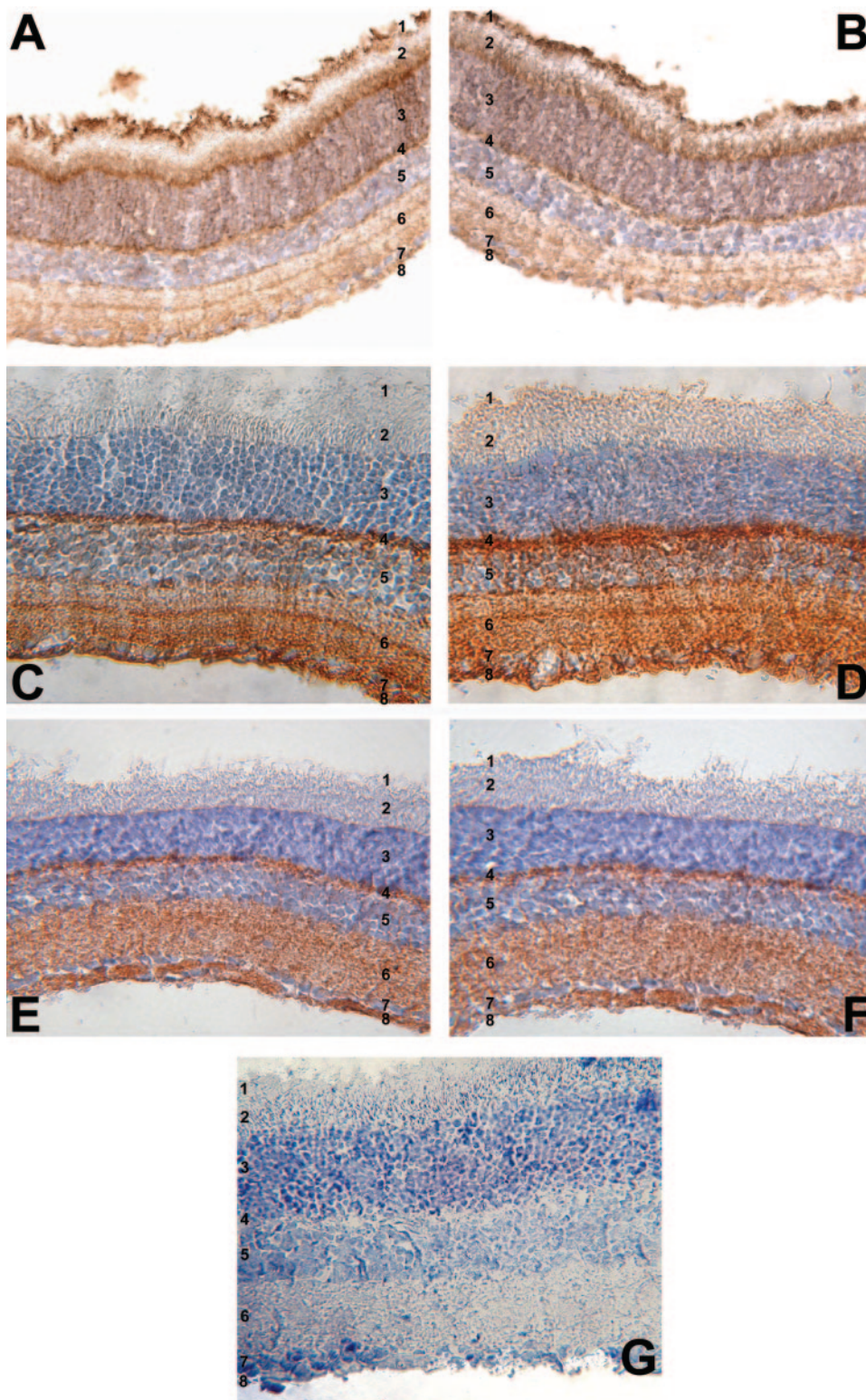
### Changes in MCT Expression in Experimental Diabetes

Figure 2 shows that levels of MCT mRNA and protein were not significantly changed in the diabetic retina. Hippocampal samples also showed no change in the levels of these transporters in diabetes. These results were supported by the real-time PCR technique, which showed no changes in mRNA expression of MCTs (Table 2). The close correlation between the methods supports results of previous work showing that semiquantitative PCR has a precision similar to

that of real-time PCR in the analysis of whole retinal lysates.<sup>35</sup> Immunohistochemistry supports this finding (Fig. 3). No obvious change in the intensity or distribution of MCT1, -2, or -4 was evident in sections of control or diabetic retina taken at similar eccentricities.

### Markers of Retinopathy and Neuropathy

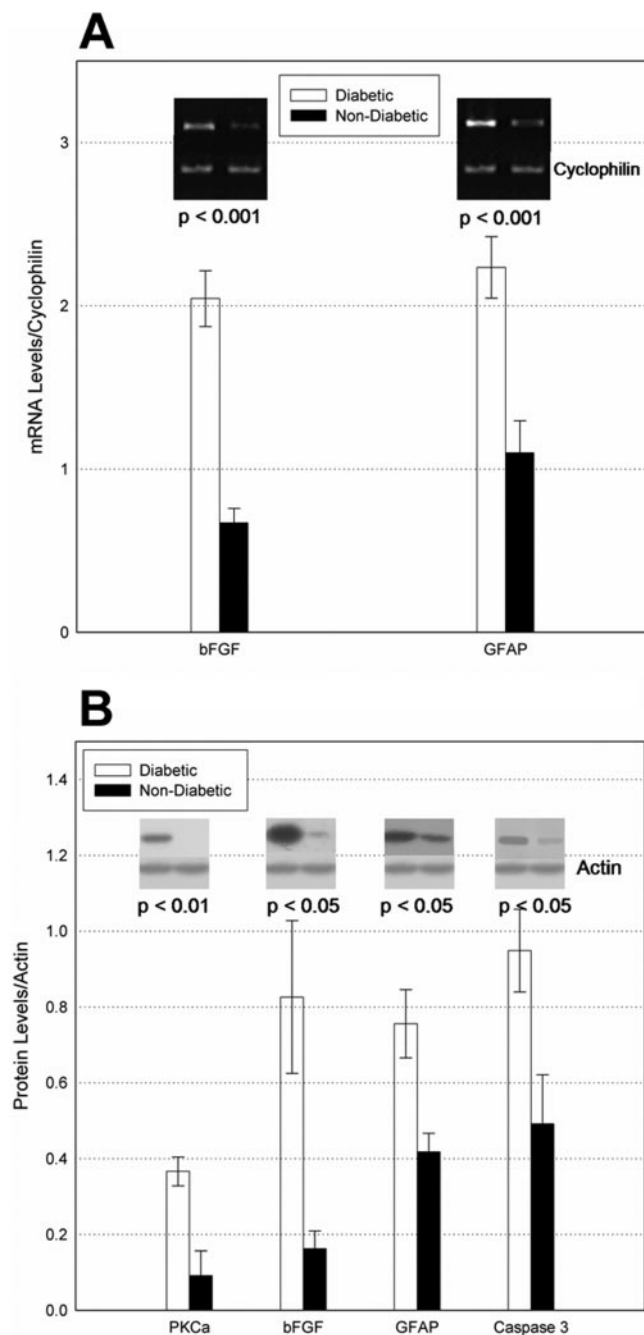
Protein and mRNA levels of both bFGF and GFAP (Fig. 4) were elevated in the diabetic groups compared with the control animals. Protein levels of PKC $\alpha$  increased significantly in the



**FIGURE 3.** Immunohistochemistry showing MCT1, -2, and -4 distribution in the retina in control and diabetic rats. (A) MCT1 retinal distribution in a control eye. (B) MCT1 distribution in diabetes. In both retinas, MCT1 was localized in the RPE, inner segments of the photoreceptors, and the outer nuclear layer. Some staining was noted in the inner plexiform layer. (C) MCT2 retinal distribution in the control eye. (D) MCT2 retinal distribution in the diabetic eye. In both retinas, MCT2 was localized in the plexiform layers and the inner limiting membrane. (E) MCT4 expression in the control eye. (F) MCT4 expression in the diabetic eye. In both retinas, MCT4 was localized in the plexiform layers and the nerve fiber layer. (G) Retinal section incubated with no primary antibody and polyclonal rabbit, chicken, and goat. No background staining was noted. 1, retinal pigment epithelium; 2, photoreceptor layer; 3, outer nuclear layer; 4, outer plexiform layer; 5, inner nuclear layer; 6, inner plexiform layer; 7, ganglion cell layer; 8, nerve fiber layer/inner limiting membrane. Magnification,  $\times 180$ .

experimental groups, and activated caspase-3 levels were also elevated in diabetes (Fig. 4), but levels of both substances were unchanged in the hippocampus (results not shown). Cells staining prominently for TUNEL were very few in number, with the most evident TUNEL staining in the photoreceptor layer (Fig. 5). Some very weakly stained TUNEL cells appeared also in the inner retinal layers of diabetic animals (Fig. 5). In

addition, ERG measurements showed significant reduction in oscillatory potentials, a measure of inner retinal function, in the diabetic rats ( $P < 0.001$ ). The b-wave amplitude was also reduced, probably representing a loss of the oscillatory potentials at the peak of the b-wave, but the change was not significant ( $P = 0.1$ ), and the a-wave amplitude was unchanged, indicating that cataract did not reduce the amount of light



**FIGURE 4.** Markers of pathologic processes in the diabetic retina. (A) mRNA markers of diabetic processes in the eye. (B) Protein markers of diabetic processes in the eye. Probabilities represent significance differences after accounting for intra- and interclass correlation within animals and within groups, with 28 eyes in each group.

reaching the retina sufficiently to produce a submaximum response (Fig. 6).

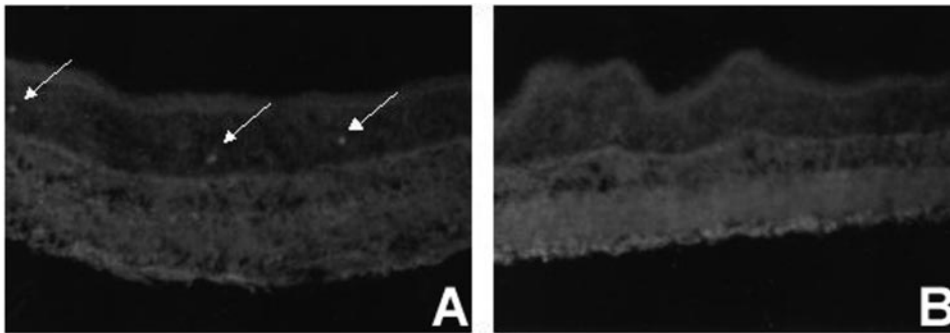
## DISCUSSION

In this investigation, neither MCT proteins or mRNA levels in the retina were significantly affected in the diabetic group. This was somewhat surprising, because MCT1 has been shown to be altered in the brain within hours by ischemia<sup>26</sup> and after 6 weeks in diet induced-ketosis,<sup>27</sup> both of which conditions

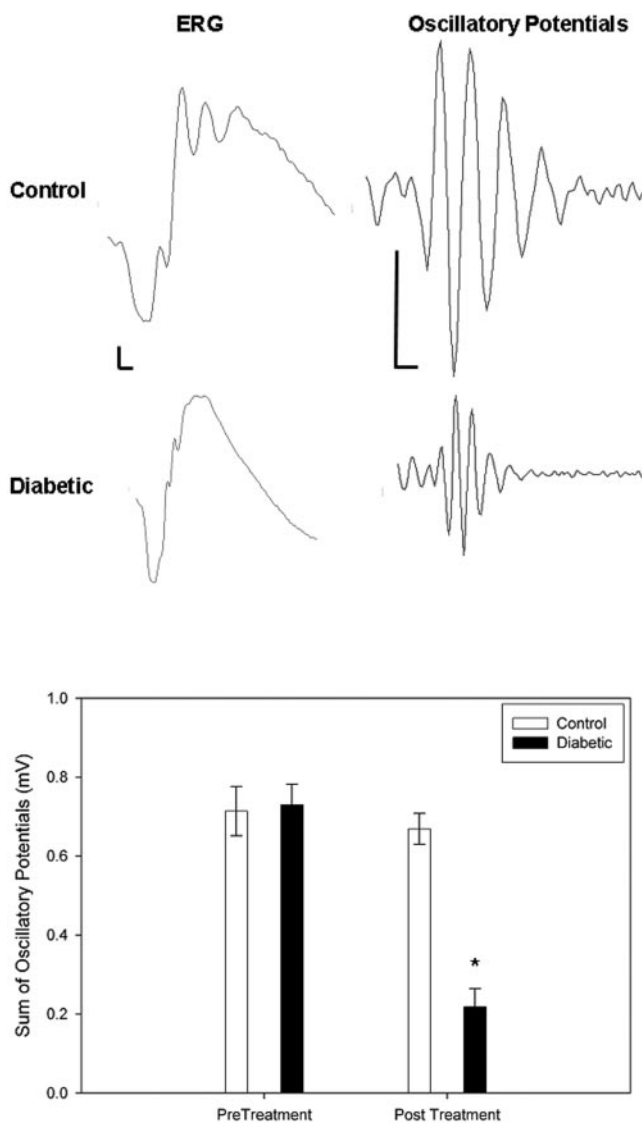
reflect some aspects of the diabetic process in the retina. In addition, it was also recently reported that lactate levels are decreased in the diabetic retina (Ambrosio AF, et al. *IOVS* 2004;45:ARVO E-Abstract 3222). It is possible that changes caused by these factors were altered by other manifestations associated with diabetes but absent in models of ischemia or ketosis (for example, elevated glucose) which may produce counterregulatory influences on the transcription and translation of the MCTs. In this study, rats were kept diabetic for 10 weeks to allow the development of diabetic retinal neuropathy described by other investigators,<sup>31</sup> and it is possible that MCTs were altered earlier in this model and were normalized by the 10-week endpoint. However the lack of change in MCT expression in the retina in diabetes reflects findings from previous work in diabetic muscle.<sup>36</sup> These findings do not rule out the possibility that a retinal failure in MCT regulation contributes to retinal damage. However, this possibility is less likely, given the findings in the hippocampal sections, where similar expression profiles were found.

The rats in the diabetic group displayed the classic signs of polyuria, polydipsia, and cataract at the end of the experiment. Blood glucose elevations were significant, and consistently elevated blood glucose concentrations were noted over the 10 weeks. In addition, the rats showed elevations in bFGF, PKC $\alpha$ , and GFAP in the retina. bFGF, an important factor associated with CNS injury and angiogenesis, is thought to be a causative factor in the progression of proliferative diabetic retinopathy,<sup>37</sup> but it also exhibits potent neuroprotective properties.<sup>38</sup> It has been suggested that its release in diabetes is a response to diabetic retinal insult,<sup>1</sup> and work in our laboratory supports this finding (Layton CJ, unpublished data, 2004). PKC $\alpha$  is a substance involved in second-messenger signal transduction and is heavily expressed in rod bipolar cells and characteristically upregulated in the diabetic retina. In diabetes, it both induces and is induced by angiogenic substances such as vascular endothelial growth factor (VEGF),<sup>39</sup> and the changes shown in the current study may therefore indicate a change in rod bipolar function in diabetes. GFAP is a marker of glial cell activation that appears between 2 and 6 months after the onset of diabetes and has been used by other investigators as an endpoint in evaluating treatments of diabetic retinopathy.<sup>40</sup> Together, these three findings indicate that the diabetic process was well established in the retina at a point when the MCT levels seemed stable.

The evidence presented herein also shows that previously reported signs of diabetic retinal neuropathy were evident in the experimental group after 10 weeks of the disease. ERG recordings demonstrated a significant decrease in oscillatory potentials, traditionally interpreted as a functional impairment in the inner retina. The slight but clear increase in the number of TUNEL-stained cells in our retinal sections, consistent with results of the work of other investigators, showed most of the apoptotic cells in the photoreceptor layer, as has also been reported by Park et al.<sup>41</sup> Clear staining of TUNEL-positive cells in the inner retina or blood vessels as previously reported in other investigations involving human tissue<sup>7</sup> was not confirmed. Although the apoptotic activity in the retina noted in this study was subtle, together these results show both inner and outer retina to be affected at this stage of diabetes. Unfortunately, TUNEL staining indicates early apoptotic changes in localized cells and does not represent conclusive proof of retina-wide neural disease. Therefore, the demonstration of elevated levels of activated caspase 3 in whole retinal samples is perhaps more significant. Caspase 3 is an "executioner" caspase, activated only late in the apoptotic cascade, and the observed elevations show that cell death processes are advanced after 10 weeks of experimental diabetes. This demon-



**FIGURE 5.** TUNEL-stained retinas in (A) diabetic and (B) nondiabetic groups. Diabetic retinas displayed  $6.2 \pm 1.1$  TUNEL-positive cells in the photoreceptor layer per section, compared with  $2.1 \pm 1.2$  in the control retinas.  $P < 0.01$  by Student's *t*-test, with eight independent eyes in each group and 20 sections of each retina. Magnification,  $\times 180$ .



**FIGURE 6.** ERG oscillatory potentials in diabetic and nondiabetic groups. *Top:* a representative ERG (*left*) and isolated oscillatory potential (*right*). Both scale bars represent 0.1 mV and 10 ms. Neither a ( $P < 0.6$ ) or b-wave ( $P = 0.1$ ) amplitude was significantly altered in the diabetic group. *Bottom:* quantitation of the total of four oscillatory potentials, measured from trough to peak, demonstrating that total oscillatory potentials were reduced after 9 weeks of experimental diabetes. \* $P < 0.01$  after accounting for intra- and interclass variation between eyes and within groups, with 28 eyes in each group.

strates an earlier increase in caspase-3 than shown previously by other workers, who have found that, although caspase 2 and 6 were elevated after 8 weeks of diabetes, activated caspase 3 did not increase in diabetic retinas until after 6 months.<sup>5</sup> This discrepancy may be due to the larger sample size in the present study, but is more likely a reflection of the different dose of streptozotocin used in this study and the subsequently accelerated diabetic response.

Diabetes is a chronic condition in humans, and perhaps MCT levels are altered later in the disease. However, in this study, retinal disease is well established before any effect on MCT levels can be discerned. This does not rule out the possibility that a lack of compensatory MCT changes in the retina in response to an important diabetic stressor contributes to diabetic retinopathy, but this possibility is reduced by the finding that MCT levels are also unaffected in the diabetic hippocampus, where signs of retinopathy do not occur. These results therefore suggest it is unlikely that regulation of these energy transporters is critical in the pathogenesis of diabetic changes in the retina. However, it should be noted that although there was no sign of altered distribution of MCTs in the immunocytochemistry presented in the present study, subcellular expression of MCTs may be altered in diabetes. Basigen (CD147) colocalizes with MCTs and has been shown to be vital in targeting the transporters to the cell surface<sup>42</sup>; however, recent work by the same group showed that MCT expression in the RPE depends on MCT gene expression and was not modulated by CD147.<sup>43</sup> Our results do not exclude the possibility that diabetes could regulate MCT function through this protein, altering the subcellular distribution of the transporters and causing subtle changes that are not detectable by the methodology used in this study. It is also possible that other, unknown lactate transporters may be present in the retina and contribute to diabetic changes or that an earlier change in MCT levels, such as that reported after 6 weeks of ketosis in the brain<sup>27</sup> is a central part of the process.

The difference between streptozotocin-induced diabetes and type-1 diabetes also should be mentioned. It is probable that the induction of diabetes is not the sole action of streptozotocin and that the agent is likely to have other effects. Because streptozotocin obtains access to the cell via the GLUT 2 transporter, it may be toxic to other cells that express this transporter. In the mature rat retina, only Müller cells express GLUT 2<sup>44</sup>; and, for this reason, a wide range of diabetic endpoints, produced by a variety of retinal cell types, were used in this investigation to confirm the development of significant disease.

In summary, this study has shown that early retinal changes in diabetes, including retinal neuropathy, are readily demonstrated after 10 weeks of streptozotocin-induced diabetes in the rat. Despite these changes, MCT levels and distribution appear to remain constant during this period. Therefore, it is

unlikely that changes in MCT expression explain the recently noted phenomenon of diabetic retinal neuropathy.

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### References

- Gardner TW, Antonetti DA, Barber AJ, LaNoue KF, Levison SW. Diabetic retinopathy: more than meets the eye. *Surv Ophthalmol*. 2002;47(suppl 2):S253-S262.
- Stavrou EP, Wood JM. Letter contrast sensitivity changes in early diabetic retinopathy. *Clin Exp Optom*. 2003;86:152-156.
- Cho NC, Poulsen GL, Ver Hoeve JN, Nork TM. Selective loss of S-cones in diabetic retinopathy. *Arch Ophthalmol*. 2000;118:1393-1400.
- Tzekov R, Arden GB. The electroretinogram in diabetic retinopathy. *Surv Ophthalmol*. 1999;44:53-60.
- Mohr S, Xi X, Tang J, Kern TS. Caspase activation in retinas of diabetic and galactosemic mice and diabetic patients. *Diabetes*. 2002;51:1172-1179.
- Kowluru RA, Koppolu P. Diabetes-induced activation of caspase-3 in retina: effect of antioxidant therapy. *Free Radic Res*. 2002;36:993-999.
- Barber AJ, Lieth E, Khin SA, et al. Neural apoptosis in the retina during experimental and human diabetes: early onset and effect of insulin. *J Clin Invest*. 1998;102:783-791.
- Barber AJ. A new view of diabetic retinopathy: a neurodegenerative disease of the eye. *Prog Neuropsychopharmacol Biol Psychiatry*. 2003;27:283-290.
- Salceda R, Vilchis C, Coffe V, Hernandez-Munoz R. Changes in the redox state in the retina and brain during the onset of diabetes in rats. *Neurochem Res*. 1998;23:893-897.
- Van den Enden MK, Nyengaard JR, Ostrow E, Burgan JH, Williamson JR. Elevated glucose levels increase retinal glycolysis and sorbitol pathway metabolism: implications for diabetic retinopathy. *Invest Ophthalmol Vis Sci*. 1995;36:1675-1685.
- Vicario C, Arizmendi C, Malloch G, Clark JB, Medina JM. Lactate utilization by isolated cells from early neonatal rat brain. *J Neurochem*. 1991;57:1700-1707.
- Schurr A, West CA, Rigor BM. Lactate-supported synaptic function in the rat hippocampal slice preparation. *Science*. 1988;240:1326-1328.
- Schurr A, Payne RS, Miller JJ, Rigor BM. Brain lactate, not glucose, fuels the recovery of synaptic function from hypoxia upon reoxygenation: an in vitro study. *Brain Res*. 1997;744:105-111.
- Walz W, Mukerji S. Lactate release from cultured astrocytes and neurons: a comparison. *Glia*. 1988;1:366-370.
- Korf J. Intracerebral trafficking of lactate in vivo during stress, exercise, electroconvulsive shock and ischemia as studied with microdialysis. *Dev Neurosci*. 1996;18:405-414.
- Pellerin L, Magistretti PJ. Glutamate uptake into astrocytes stimulates aerobic glycolysis: a mechanism coupling neuronal activity to glucose utilization. *Proc Natl Acad Sci USA*. 1994;91:10625-10629.
- Pellerin L, Pellegrini G, Bittar PG, et al. Evidence supporting the existence of an activity-dependent astrocyte-neuron lactate shuttle. *Dev Neurosci*. 1998;20:291-299.
- Winkler BS, Sauer MW, Starnes CA. Modulation of the Pasteur effect in retinal cells: implications for understanding compensatory metabolic mechanisms. *Exp Eye Res*. 2003;76:715-723.
- Winkler BS. Glycolytic and oxidative metabolism in relation to retinal function. *J Gen Physiol*. 1981;77:667-692.
- Tsacopoulos M, Poitry-Yamate CL, MacLeish PR, Poitry S. Trafficking of molecules and metabolic signals in the retina. *Prog Retin Eye Res*. 1998;17:429-442.
- Tsacopoulos M, Magistretti PJ. Metabolic coupling between glia and neurons. *J Neurosci*. 1996;16:877-885.
- Poitry-Yamate CL, Poitry S, Tsacopoulos M. Lactate released by Muller glial cells is metabolized by photoreceptors from mammalian retina. *J Neurosci*. 1995;15:5179-5191.
- Zeevalk GD, Nicklas WJ. Lactate prevents the alterations in tissue amino acids, decline in ATP, and cell damage due to glycemias in retina. *J Neurochem*. 2000;75:1027-1034.
- Ransom BR, Fern R. Does astrocytic glycogen benefit axon function and survival in CNS white matter during glucose deprivation? *Glia*. 1997;21:134-141.
- Bergersen L, Rafiki A, Ottersen OP. Immunogold cytochemistry identifies specialized membrane domains for monocarboxylate transport in the central nervous system. *Neurochem Res*. 2002;27:89-96.
- Tseng MT, Chan SA, Schurr A. Ischemia-induced changes in monocarboxylate transporter 1 reactive cells in rat hippocampus. *Neurosci Res*. 2003;25:83-86.
- Leino RL, Gerhart DZ, Duelli R, Enerson BE, Drewes LR. Diet-induced ketosis increases monocarboxylate transporter (MCT1) levels in rat brain. *Neurochem Int*. 2001;38:519-527.
- Foster DW. Diabetes mellitus. In: Fauci AS, Braunwald E, Isselbacher KJ, Wilson JD, Martin JB, eds. *Harrison's Principles of Internal Medicine*. 14th ed. New York: McGraw-Hill; 1998:2060-2081.
- Bui BV, Vingrys AJ, Wellard JW, Kalloniatis M. Monocarboxylate transport inhibition alters retinal function and cellular amino acid levels. *Eur J Neurosci*. 2004;20:1525-1537.
- Barber AJ, Antonetti DA, Gardner TW. Altered expression of retinal occludin and glial fibrillary acidic protein in experimental diabetes. The Penn State Retina Research Group. *Invest Ophthalmol Vis Sci*. 2000;41:3561-3568.
- Rungger-Brandl E, Dosso AA, Leuenberger PM. Glial reactivity, an early feature of diabetic retinopathy. *Invest Ophthalmol Vis Sci*. 2000;41:1971-1980.
- Nash MS, Osborne NN. Assessment of Thy-1 mRNA levels as an index of retinal ganglion cell damage. *Invest Ophthalmol Vis Sci*. 1999;40:1293-1298.
- Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. *Methods*. 2001;25:402-408.
- Wood JP, Osborne NN. Induction of apoptosis in cultured human retinal pigmented epithelial cells: the effect of protein kinase C activation and inhibition. *Neurochem Int*. 1997;31:261-273.
- Chidlow G, Osborne NN. Rat retinal ganglion cell loss caused by kainate, NMDA and ischemia correlates with a reduction in mRNA and protein of Thy-1 and neurofilament light. *Brain Res*. 2003;963:298-306.
- Py G, Lambert K, Milhavel O, et al. Effects of streptozotocin-induced diabetes on markers of skeletal muscle metabolism and monocarboxylate transporter 1 to monocarboxylate transporter 4 transporters. *Metabolism*. 2002;51:807-813.
- Cai J, Boulton M. The pathogenesis of diabetic retinopathy: old concepts and new questions. *Eye*. 2002;16:242-260.
- Li Q, Stephenson D. Postischemic administration of basic fibroblast growth factor improves sensorimotor function and reduces infarct size following permanent focal cerebral ischemia in the rat. *Exp Neurol*. 2002;177:531-537.
- Mamputu JC, Renier G. Advanced glycation end products increase, through a protein kinase C-dependent pathway, vascular endothelial growth factor expression in retinal endothelial cells: inhibitory effect of glizalide. *J Diabetes Complications*. 2002;16:284-293.
- Asnagli V, Gerhardinger C, Hoehn T, Adeboje A, Lorenzi M. A role for the polyol pathway in the early neuroretinal apoptosis and glial changes induced by diabetes in the rat. *Diabetes*. 2003;52:506-511.
- Park SH, Park JW, Park SJ, et al. Apoptotic death of photoreceptors in the streptozotocin-induced diabetic rat retina. *Diabetologia*. 2003;46:1260-1268.
- Philp NJ, Ochrietor JD, Rudoy C, Muramatsu T, Linser PJ. Loss of MCT1, MCT3, and MCT4 expression in the retinal pigment epithelium and neural retina of the 5A11/basigin-null mouse. *Invest Ophthalmol Vis Sci*. 2003;44:1305-1311.
- Philp NJ. *Properties and Regulation of Monocarboxylate Transporter in the Retinal Pigment Epithelium*. International Conference of Eye Research, Sydney, Australia, 2004.
- Watanabe T, Mio Y, Hoshino FB, et al. GLUT2 expression in the rat retina: localization at the apical ends of Muller cells. *Brain Res*. 1994;655:128-134.