DIABETES-INDUCED DYSFUNCTION OF RETINAL MÜLLER CELLS

BY *Donald G. Puro, MD, PhD*

ABSTRACT

Purpose: This study tested the hypothesis that the function of the glutamate transporter in retinal Müller cells is compromised early in the course of diabetes by a mechanism involving oxidation. Dysfunction of this transporter, which removes glutamate from the extracellular space, may play a critical role in the disruption of glutamate homeostasis that occurs in the diabetic retina. Because glutamate is toxic to retinal neurons and is likely to exacerbate oxidative stress, elucidation of the mechanisms by which diabetes elevates the concentration of this amino acid may help to better understand the pathogenesis of diabetic retinopathy.

Methods: Müller cells were freshly isolated from normal rats and those made diabetic by streptozotocin injection. The activity of the Müller cell glutamate transporter, which is electrogenic, was monitored via the perforated-patch configuration of the patch-clamp technique.

Results: Four weeks after the onset of hyperglycemia, dysfunction of the Müller cell glutamate transporter was detected ($P = .005$). After 13 weeks of streptozotocin-induced diabetes, the activity of this transporter was decreased by 67% ($P = .001$). Consistent with oxidation causing this dysfunction, exposure to a disulfide-reducing agent rapidly restored the activity of this transporter in Müller cells from diabetic retinas.

Conclusions: Soon after the onset of experimental diabetes, the function of the glutamate transporter in Müller cells is decreased by a mechanism that is likely to involve oxidation. The demonstration that the activity of this transporter can be rapidly restored raises the possibility that targeting this molecule for therapeutic intervention may restore glutamate homeostasis and, thereby, ameliorate sight-threatening complications of diabetic retinopathy.

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INTRODUCTION

The focus of this thesis is on diabetes-induced changes in the function of retinal Müller cells. These glia, which are critically positioned between the vasculature and the neurons of the retina, are of particular interest because reports in the literature suggest that their role in regulating the molecular composition of the retinal microenvironment may be compromised early in the course of diabetic retinopathy.

The objective of this study was to test the hypothesis that the ability of Müller cells to remove glutamate from the extracellular space is diminished soon after the onset of experimental diabetes. Since extracellular glutamate is neurotoxic, dysfunction of the mechanisms regulating the concentration of this amino acid could contribute to nerve cell damage in the diabetic retina. In the long term, identifying early events in diabetic retinopathy may provide therapeutic targets for preventing sight-threatening complications of this disorder.

From the Departments of Ophthalmology and Visual Sciences and Physiology, University of Michigan, Ann Arbor. This project was supported in part by grants EY07003 from the National Institutes of Health; Senior Scientist Awards from Research to Prevent Blindness, Inc; and the Mid-West Eye Bank and Transplantation Center.

DIABETIC RETINOPATHY: MORE THAN A VASCULAR DISORDER

Although vascular changes are the classic hallmark of this disorder, a number of observations suggest that microangiopathy is only one aspect of a more widespread retinal dysfunction.[1-3] The concept that neurons as well as capillaries are affected by diabetes is not new. In the early 1960s, Wolter[4] and Bloodworth[5] documented that diabetic retinopathy is associated with degeneration of neurons, especially those located in the inner retina. In fact, diabetes-induced changes in retinal neurons and glia may precede the onset of clinically evident vascular injury.[3-7] For example, loss of color[8-10] and contrast sensitivity[11-14] and abnormalities in the electroretinogram[15-24] have been documented in patients before detection of the vascular changes that are traditionally used to diagnose diabetic retinopathy.

Early neuronal and glial alterations are also evident in rats with chemically induced diabetes. These changes include decreases in components of the electroretino-

gram[25] and increased apoptosis of retinal neurons.[26,27] In addition, early in the course of diabetic retinopathy, Müller cells markedly upregulate their expression of glial fibrillary acidic protein,[28-31] which is a nonspecific response to pathophysiological conditions.[32] Thus, a comprehensive understanding of diabetic retinopathy requires elucidation of the mechanisms by which diabetes affects nonvascular, as well as vascular, cells of the retina.

MÜLLER CELLS: CRITICAL ELEMENTS IN A HEALTHY RETINA

Müller cells are the principal glia of the retina. Although the physiology of these cells was previously thought to be rather simple, investigations during the past 2 decades have revealed that Müller cells express a diversity of ion channels and transporters, release a variety of cytokines and survival factors, and have receptors for numerous neurotransmitters and growth factors.[33,34] As a result, it is now evident that Müller cells play an active, dynamic role in the retina.[33]

A major physiological function of these cells is to regulate the ionic and molecular composition of the extracellular space (Table I). Consistent with a homeostatic role, Müller cells are well positioned to interact with the retinal microenvironment.[35] These glial cells are radially oriented and span the depth of the retina from the vitreal border to the interphotoreceptor matrix of the subretinal space. Their processes are in close apposition to neuronal cell bodies, neurites, and synapses as well as the blood vessels.

TABLE I: PUTATIVE FUNCTIONS OF MÜLLER CELLS

PHYSIOLOGICAL
Homeostatic
Maintain a low concentration of glutamate in the microenvironment
Redistribute extracellular potassium
Regulate pH
Nutritive
Store glycogen
Provide lactate for neuronal nutrition
Trophic:
Release photoreceptor and neuronal survival factors
Vascular regulation
Facilitate blood-retinal barrier development
Influence blood flow
Modulation of retinal function
Recycle glutamate/glutamine
Communicate with neurons
PATHOBIOLOGICAL
Protective
Decrease neurotoxic levels of glutamate at sites of blood-retinal barrier breakdown and neuronal injury
Release molecules that enhance neuronal and photoreceptor survival
Phagocytose retinal debris
Serve as an antigen-presenting cell
Detrimental
Migration from the retina
Proliferation

An intensively studied function of Müller cells is their uptake of synaptically released glutamate,[33,34,36] which is a neurotransmitter at more than 90% of the synapses in the retina.[37] Not only is this amino acid released by the photoreceptors, but many retinal neurons also use glutamate as a neurotransmitter. By removing extracellular glutamate, Müller cells help to terminate transmission at glutamatergic synapses.[38,39] After uptake via a glutamate transporter, glutamate is rapidly converted in Müller cells to glutamine, which is subsequently recycled to neurons, where it is converted back into glutamate for release at synapses.[34,40] Consistent with Müller cells having a vital role in regulating retinal glutamate levels, Vorwerk and colleagues[41] reported that treatment of rats with antisense oligonucleotides directed against the Müller cell glutamate transporter caused more than a threefold increase in the vitreal concentration of glutamate.

In addition to playing a role in terminating glutamatergic transmission, prompt removal of synaptically released glutamate is necessary, since prolonged activation of certain glutamate receptors, for example, the calcium-permeable *N*-methyl-*D*-aspartate (NMDA) receptors, can cause an excessive influx of calcium, which can kill retinal neurons.[42-44] Thus, mechanisms to efficiently remove synaptically released glutamate are necessary for the maintenance of a healthy retina.

GLUTAMATE HOMEOSTASIS: DISRUPTION IN THE DIABETIC RETINA

Despite intensive study of the mechanism by which Müller cells remove glutamate under normal conditions, much less is known about this vital function in the diabetic retina. This gap in knowledge is likely to be significant, because the role of Müller cells in maintaining a low extracellular concentration of glutamate may be particularly critical in diabetes. As in the normal retina, synaptically released glutamate must be removed. However, in addition, neurons in the diabetic retina must be protected from glutamate leaking into the retina because the blood-retinal barrier is compromised early in diabetes.[45,46] Since plasma contains 100 to 300 μM of this amino acid[47] and as little as 5 μM of glutamate can be lethal to retinal neurons,[43,48] it seems apparent that a breakdown in the blood-retinal barrier could have dire consequences for retinal function and neuronal survival. Thus, the transport of glutamate into Müller cells may be essential in order to prevent toxic levels of this amino acid from reaching neurons located near defects in the blood-retinal barrier.

Glial cells, whose processes completely ensheath the retinal vasculature,[49] are well positioned to remove glutamate at sites of a breakdown in the blood-retinal barrier. In diabetes, however, the ability of Müller cells to regulate the extracellular concentration of glutamate may be

compromised. Support for this possibility is that levels of this amino acid are elevated in the retinas of diabetic rats,[28,50] even though glutamate synthesis is unaffected.[51] Also, the increased concentration of glutamate in the vitreous of patients with diabetic retinopathy is likely to reflect the presence of high concentrations of this amino acid in the retina.[52] These various observations suggest that the regulatory mechanisms to control glutamate are dysfunctional in the diabetic retina.

GLUTAMATE UPTAKE BY MÜLLER CELLS: A VULNERABLE STEP

An essential step in the regulation of extracellular glutamate is the transport of this amino acid into Müller cells via a high-affinity glutamate transporter, which is named GLAST (the human analog is named EAAT1). GLAST is the only glutamate transporter detected in Müller cells, and it is not found in other types of retinal cells.[53] A potentially critical feature of GLAST is the presence of redox-sensing elements, which regulate this transporter via thiol-disulfide redox interconversion.[54,55] Consistent with the presence of redox-sensitive sites, Trotti and colleagues[54] demonstrated that chemical oxidation or reduction altered the activity of cloned GLAST molecules, which had been placed in artificial liposomes. These investigators found that oxidizing agents decreased GLAST function. In contrast, exposure to a chemical reductant restored the activity of this transporter.[54] However, although evidence is good that cloned GLAST expressed in liposomes possesses redox sensitivity, this has not been demonstrated for native GLAST molecules located in cells, such as Müller cells, which exclusively express this type of glutamate transporter.

A central premise of this thesis is that the redox sites on the GLAST molecules in Müller cells render this transporter vulnerable to diabetes-induced dysfunction (Figure 1). This idea is based, in part, on the observation that oxidative stress occurs in the retina[50,56-58] and other tissues[59] early in the course of diabetes. These observations, plus evidence that glutamate homeostasis is disrupted in the diabetic retina,[28,51,52] are the basis for the hypothesis that oxidation of the glutamate transporter is one mechanism by which diabetes compromises the ability of Müller cells to regulate glutamate concentration.

EXPERIMENTAL STRATEGY

The aim of this thesis is to test the hypothesis that early in the course of diabetic retinopathy, the function of the glutamate transporter in retinal Müller cells is compromised by a mechanism involving oxidation. To achieve this aim, this study was designed to quantify GLAST activity in Müller cells that were freshly isolated from normal rats and those made diabetic by injection of streptozotocin.

Electrophysiological Assay of GLAST Activity

A key feature of the glutamate transporter in Müller cells is that it is electrogenic.[60-62] Briefly stated, a net inward current is generated during the transport of glutamate as two sodium ions enter, but only one potassium ion exits; the influx of the negatively charged amino acid is balanced with an entering proton (Figure 2). In addition, there is an associated chloride conductance. However, this conductance is not coupled to the transport of glutamate.[62]

As a result of the movement of ions and glutamate during the uptake of this amino acid, the GLAST transporter generates a net influx of a positive charge as glutamate enters the cell. In standard electrophysiological terminology, a positive charge moving into a cell is classified as being an inward, negative current. Thus, GLAST activity generates an influx of positive ions that can be detected electrophysiologically as an inward (negative) current.

Because the glutamate transporter of a Müller cell is electrogenic, electrophysiological techniques can be used to detect the negative, inward current generated as glutamate is transported into the cell. The ability to use elec-

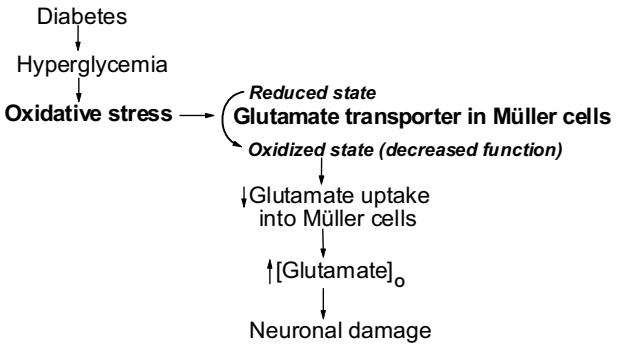

FIGURE 1

Hypothesized mechanism by which diabetes causes dysfunction of the glutamate transporter in Müller cells. $[Glutamate]_o$, extracellular glutamate concentration.

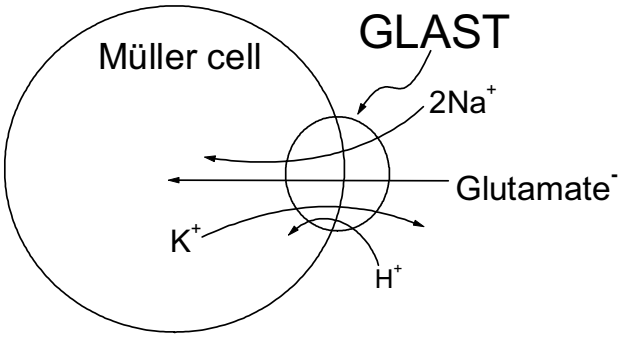

FIGURE 2

Kinetic scheme for the Müller cell glutamate transporter, GLAST. With transport of a glutamate molecule via GLAST, there is a net influx of a positive ion. Since a positive current is by definition the movement of a positive ion out of a cell, then the net influx of a positive charge, as occurs with transport of glutamate by GLAST, is an inward, negative current.

trophysiological methods to quantify in "real-time" the transporter-induced current in freshly isolated Müller cells has resulted in a detailed understanding of the physiology of this glutamate transporter.[63] However, despite extensive knowledge of the normal function of GLAST in Müller cells, information concerning the activity of this transporter in the diabetic retina is extremely limited.

This project appears to be the first electrophysiological assessment of the effect of diabetes on Müller cell function. In the experiments presented in this study, the currents of isolated rat Müller cells were monitored using the perforated-patch configuration of the patch-clamp technique.[64,65] A major advantage of the perforated-patch configuration, as compared with the standard whole-cell recording method, is that it minimally disrupts the cytoplasm of the sampled cell and, consequently, minimizes the loss of intracellular regulatory molecules, which may affect GLAST activity. Use of this recording method permitted quantification of glutamate transporter activity before, during, and after the sampled Müller cell was exposed to a perfusate supplemented with glutamate or l-trans-pyrrolidine-2, 4-dicarboxylate (PDC), which is a specific ligand for this transporter.[66,67]

Streptozotocin Model of Diabetes

To test the hypothesis that the function of the Müller cell glutamate transporter is decreased in the diabetic retina, Müller cells from rats with streptozotocin-induced diabetes were studied. Injection of streptozotocin creates an experimental model of type 1 diabetes.[68] Streptozotocin, which is essentially a glucose molecule linked to a reactive nitrosourea moiety, is internalized into cells via glucose transporters. Once inside a cell, the nitrosourea moiety is released and kills the cell by cross-linking vital structures. Because the beta cells of the pancreas are more active than other cells in taking up glucose, they are also more sensitive to streptozotocin toxicity than other cells. As a result, at an appropriate dose, streptozotocin preferentially kills beta cells and thereby causes insulin levels to plummet and blood glucose levels to rise.

The streptozotocin model of diabetes differs from the usual clinical situation in that blood glucose levels are not controlled by treatment with insulin. In contrast, patients diagnosed as having type 1 diabetes are promptly placed on insulin therapy. However, despite differing from the typical clinical course, use of this experimental model in numerous studies has provided useful insights into the effects of hyperglycemia and insulin deficiency. For this reason, a vast literature now exists on the effects of streptozotocin-induced diabetes on the rat retina. This information is useful, since new observations from this thesis project can be correlated with previously identified retinal changes that occur in this experimental model of diabetes.

Isolated Müller Cells

Because technical challenges precluded the use of an electrophysiological assay of transporter activity in Müller cells in vivo, or in the intact retina, the experiments performed in this study used Müller cells that were freshly isolated from the retina. Over the past 15 years, electrophysiological studies of freshly isolated Müller cells have been very fruitful. There is now a detailed understanding of the mechanisms by which GLAST molecules transport glutamate from the extracellular space into a Müller cell (Figure 2),[62,63] although the function of the glutamate transporter in Müller cells of diabetic retinas has not been assessed prior to this project.

A caution in the interpretation of experimental results obtained from isolated Müller cells is that any effects of diabetes on the glutamate transporter of these cells must ultimately be confirmed in the retina in vivo. However, despite not assaying the transporter acitivity in Müller cells of the intact retina, the use of the patch-clamp technique to monitor freshly isolated cells provides a powerful experimental approach. The ability to perform real-time quantification of glutamate transporter activity permits the testing of hypotheses concerning the mechanisms by which diabetes affects Müller cell function.

The results presented in this thesis revealed that the activity of the glutamate transporter in rat Müller cells decreased significantly within 4 weeks after the onset of streptozotocin-induced diabetes. With exposure of diabetic Müller cells to a chemical reductant, glutamate transporter activity was fully restored. Taken together, the experimental findings of this study support the hypothesis that early in the course of diabetic retinopathy, the function of the glutamate transporter in Müller cells is decreased by a mechanism involving oxidation.

METHODS

MODEL OF DIABETES IN THE RAT

This study conformed to the guidelines of the Association for Research in Vision and Ophthalmology and the University of Michigan University Committee on the Use and Care of Animals. After an overnight fast, 5- to 6-week-old Long-Evans rats (Harlan Sprague Dawley, Inc, Indianapolis, Indiana) received an intraperitoneal injection of streptozotocin (75 mg/kg) diluted in 0.8 mL of 0.03 M citrate buffer (pH 4.7). Subsequently, the animals received food and water ad libitum. The vivarium was maintained on a 12-hour alternating light-dark cycle. Three days after streptozotocin injection, diabetes was confirmed by assaying the glucose concentration (One Touch Basic, LifeScan, Milpitas, California) in blood obtained from the tail vein. Rats having glucose levels of greater than 250 mg/dL were classified as being diabetic.

Age-matched rats served as controls. Immediately prior to the harvesting of retinal Müller cells, the blood glucose level was 378 ± 6 mg/dL in the 23 diabetic rats used in this study.

FRESH MÜLLER CELLS

Freshly dissociated Müller cells were prepared from rats that were euthanized with carbon dioxide. Immediately after death, the retinas were rapidly removed and incubated in 2.5 mL Earle's balanced salt solution (Invitrogen, San Diego, California), which was supplemented with 0.5 mM EDTA, 1.5 mM $CaCl_2$, 1 mM $MgSO_4$, 20 mM glucose, 26 mM sodium bicarbonate, 2 mM cysteine, 0.04% DNase, and 15 units of papain (Worthington Biochemical Corp, Freehold, New Jersey), for 40 minutes at 30°C while 95% oxygen–5% carbon dioxide was bubbled through to maintain pH and oxygenation. After transfer to a solution containing 140 mM NaCl, 3 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgCl_2$, 10 mM Na-Hepes, 15 mM mannitol, and 5 mM glucose at pH 7.4 with osmolarity adjusted to 310 mOsm L^{-1}, the retinas were gently triturated, and a suspension of cells was placed on a glass coverslip (diameter, 15 mm; Warner Instrument Corp, Hamden, Connecticut) that was positioned in a recording chamber mounted on the stage of an inverted microscope. Müller cells were identified by their characteristic morphology (Figure 3).

ELECTROPHYSIOLOGY

Recordings from fresh Müller cells were made at room temperature (22° to 24°C) within 3 hours of cell isolation. A gravity-fed system with multiple reservoirs was used to continuously perfuse (~2 mL/min^{-1}) the recording chamber (0.5 mL volume) with various solutions. Whole-cell currents were monitored using the perforated-patch configuration of the patch-clamp technique. Unless noted otherwise, the bathing solution (solution A) consisted of 140 mM NaCl, 3 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgCl_2$, 3 mM $BaCl_2$, 10 mM Na-Hepes, and 5 mM glucose at pH 7.4 with the osmolarity adjusted by less than 5% to 310 mOsm L^{-1}. Detection of currents generated by the glutamate transporter was facilitated by using barium to block the large ionic currents generated by the inwardly rectifying potassium channels, which are the predominant ion channels of these glia.[69-71]

Using a multistage programmable puller (Sutter Instruments, San Rafael, California), patch pipettes were pulled from Corning No. 7052 glass tubing (Gardner Glass Co, Claremont, California) and heat-polished to tip diameters of 2 to 3 µm. The pipette solution consisted of 50 mM KCl, 65 mM K_2SO_4, 6 mM $MgCl_2$ and 10 mM K-Hepes, 240 µg mL^{-1} amphotericin B, and 240 µg mL^{-1} nystatin at pH 7.4 with the osmolarity adjusted to 280 mOsm L^{-1}. The resistances of the pipettes used were approximately 5 MΩ when tested in the bathing solution.

The pipettes were mounted in the holder of a Dagan 3900 patch-clamp amplifier (Dagan Corp, Minneapolis, Minnesota) and sealed to the cell bodies of Müller cells (Figure 4). Seals generally formed over a period of 1 to 30 seconds and reached resistances of greater than 1GΩ. As amphotericin-nystatin perforated the patch, the access resistance to the cell usually decreased to less than 20 MΩ within 10 minutes for the Müller cells analyzed. Recordings were used after the ratio of cell membrane to series resistance was greater than 10. This ratio was monitored periodically; if the ratio decreased to below 10, the analysis of the cell was terminated. Series resistance was not corrected, but the error due to the voltage drop across the patch pipette was always less than 10% of the applied voltage. Cell membrane capacitance was estimated using circuits of the Dagan 3910 expander module (Dagan Corp). Adjustment for the calculated[72] liquid junction potential was made after data collection.

Currents were evoked by a voltage ramp protocol, which was controlled by pClamp 8 software (Axon Instruments, Inc, Foster, California). During a voltage ramp, the applied voltage changed from negative to positive membrane potentials at the rate of 66 mV s^{-1}. The recorded currents were filtered at 1 kHz with a four-pole Bessel filter, digitally sampled at 1-msec intervals using a Digidata 1200B acquisition system (Axon Instruments) and stored by a Pentium class computer that was equipped with pClamp 8 and Origin (Version 6,

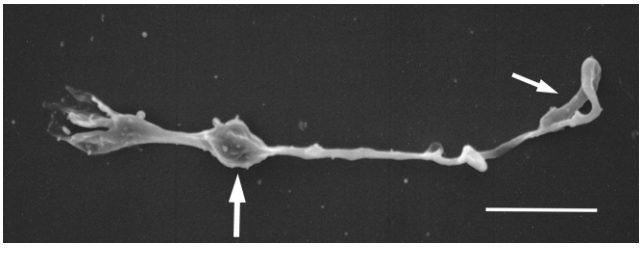

FIGURE 3

Scanning electron photomicrograph of a Müller cell freshly isolated from the rat retina. Larger arrow points to cell soma; smaller arrow points to Müller cell endfoot. Bar shows 10 µm.

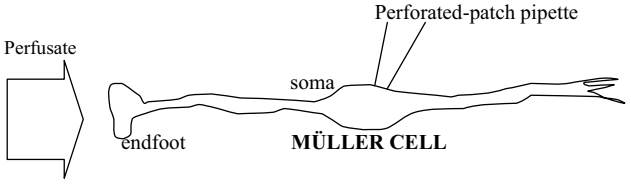

FIGURE 4

Schematic drawing of a perforated-patch recording from the soma of an isolated Müller cell.

OriginLab Corp, Northampton, Massachusetts) software for data analysis and graphics display.

The inward currents induced by glutamate or PDC were measured 1.5 to 2 minutes after the onset of exposure to the ligand. The amplitudes of the induced currents were measured at –120 mV. The chord conductance for the inwardly rectifying potassium current was calculated by measuring the amplitude of the barium-sensitive current at –120 mV.

SCANNING ELECTRON MICROSCOPY

Standard techniques, as described previously,[73] were used to prepare glutaraldehyde-fixed isolated Müller cells for scanning electron microscopy, which was performed by the University of Michigan Anatomy and Cell Biology Core Facility.

CHEMICALS

Chemicals were from Sigma (St Louis, Missouri) unless otherwise noted.

STATISTICS

Data are given as means ± SEM. Probability was evaluated by the Student's *t* test.

RESULTS

The perforated-patch configuration of the patch-clamp technique was used to monitor the currents of isolated rat Müller cells. Because the glutamate transporter of Müller cells is electrogenic, its function can be quantitatively assessed by an electrophysiological method. An advantage of the perforated-patch configuration is that it minimizes the disruption of the recorded cell's cytoplasm and, consequently, the loss of intracellular regulatory molecules that may influence the functioning of the glutamate transporter.

Since a recording pipette detects currents generated by the activity of ion channels, as well as the electrogenic transporters such as GLAST, it was preferable in many experiments to use a ligand that is selective for the glutamate transporter. For this reason, PDC, which is more selective for GLAST than glutamate,[66,67] was often used in this study. The selectivity of PDC results in the activation of the glutamate transporter, without the confounding effects by also activating glutamate receptors,[66,67,74] which modulate the activity of ion channels in Müller cells.[75,76] Because PDC has not been used previously to monitor glutamate transporter function in mammalian Müller cells, however, it was necessary to establish that the PDC-induced current in rat Müller cells had characteristics consistent with GLAST activity.

THE PDC-INDUCED CURRENT IN MÜLLER CELLS

Figure 5 shows the effect of PDC on the inward current monitored in a Müller cell freshly isolated from a control rat. Consistent with activation of GLAST, there was an increase in an inward (negative) current during exposure of the Müller cell to PDC. The PDC-induced current reversed rapidly when the perfusate was switched to one lacking PDC. Similar observations were made in 18 Müller cells isolated from normal retinas.

Because GLAST activity is known to be dependent on the concentration of the transported ligand,[60,61,67] the concentration dependence of the PDC-induced inward current in isolated rat Müller cells was determined. As shown in Figure 6, the half-maximally effective concentration of PDC was approximately 10 μM. This concentration is very similar to the value determined by Sarantis and coworkers[67] for the activation by PDC of currents in salamander Müller cells. In addition, the half-maximally effective concentration for PDC is similar to that for glutamate when tested on Müller cells from the salamander,[61] rabbit[62] and rat (this thesis, Figure 12).

Similar to the electrogenic glutamate transporter observed in Müller cells from other species,[61,74] the PDC current induced in rat Müller cells was dependent on the voltage across the cell membrane. As illustrated in Figure 7, the PDC-induced current increased in amplitude with membrane hyperpolarization. Also, there was no sign of an induced outward current (Figure 7B), as can occur when glutamate-gated ion channels are activated.[76] Similar observations were made in 18 sampled Müller cells.

As reviewed (Figure 2), the transport of glutamate

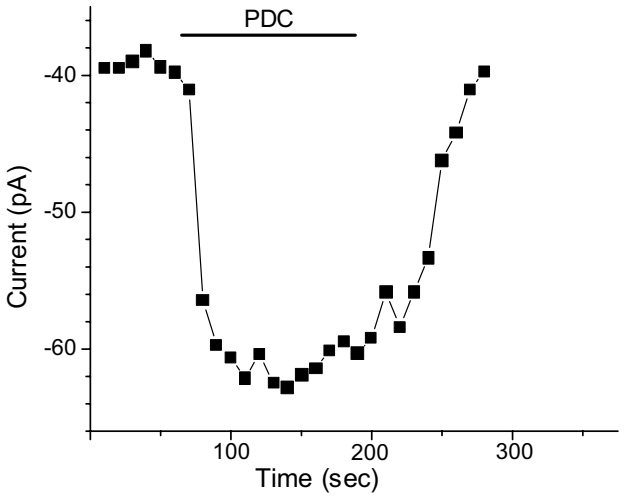

FIGURE 5

Time course for the effect of PDC on the current of a freshly isolated Müller cell monitored via a perforated-patch pipette. Amplitude of current was measured at 10-second intervals while membrane potential of Müller cell was voltage-clamped to -120 mV. Bar shows time at which perfusate was supplemented with 100 μM PDC. Induction of an inward, negative current is consistent with activation of GLAST, which causes a net movement of positive charge into a cell as this transporter moves its ligand (in this case, PDC) from extracellular space into cytoplasm.

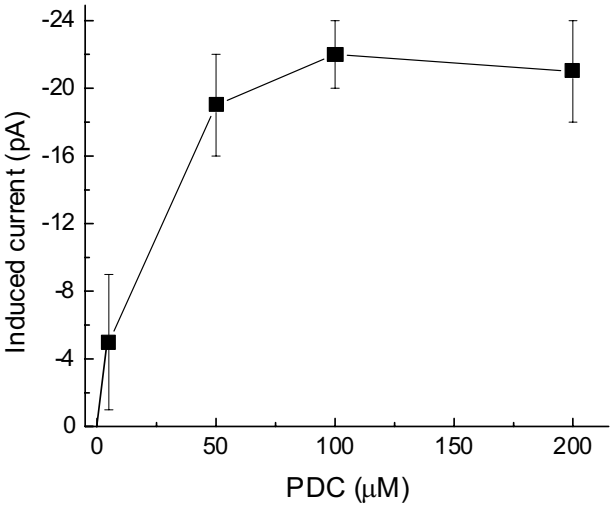

FIGURE 6

Dose response for current induced in Müller cells during exposure to PDC. Note that vertical axis indicates an increase in inward, negative current; greater transporter activity causes a more negative, inward current. A mean of 7 ± 4 cells was sampled per data point. The amplitude of PDC-induced inward current in Müller cells is concentration-dependent.

into a cell via GLAST is dependent on external sodium. Consistent with a sodium dependence of the PDC-induced current, the experiment illustrated in Figure 8 demonstrated that perfusion with a sodium-free solution eliminated the PDC-induced current in a rat Müller cell. Similar observations were made in four Müller cells isolated from rat retinas. Taken together, the results of these experiments are consistent with PDC activating GLAST in Müller cells freshly isolated from the rat retina.

GLAST CURRENT IN MÜLLER CELLS OF DIABETIC RATS

The working hypothesis of this study was that diabetes causes dysfunction of the Müller cell glutamate transporter. To test this hypothesis, the PDC current was quantified in Müller cells isolated from control and diabetic rats (Figure 9). Four weeks after administration of streptozotocin, the amplitude of the current induced by 100 μM PDC decreased significantly (P = .005). At 13 weeks after streptozotocin injection, which was the maximum duration of diabetes studied in this project, the amplitude of the PDC-induced current was decreased by 67% (P = .001) as compared with the control value.

Although there are distinct advantages to using PDC to selectively activate the GLAST, it seemed reasonable to also quantify the current induced in normal and diabetic Müller cells during exposure to 100 μM glutamate, which is the in vivo ligand for this transporter. As illustrated in Figure 10, the glutamate-induced current in Müller cells from retinas of diabetic rats was significantly (P = .004) diminished. This finding with glutamate is consistent with the observed decrease in the PDC-induced currents in

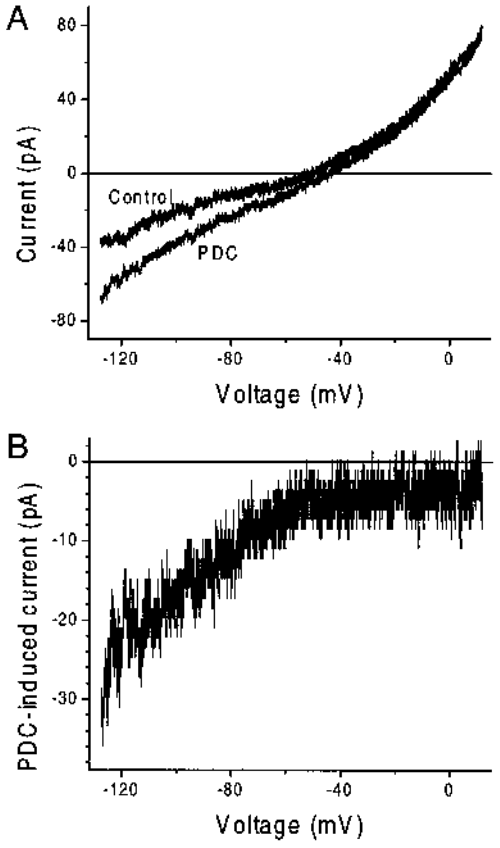

FIGURE 7

The effect PDC on the currents of a freshly isolated Müller cell. A, Effect of 100 μM PDC on current-voltage (I-V) relationship of same Müller cell as studied in Figure 5. B, I-V plot of PDC-induced current obtained by subtracting curves in A. PDC activated a voltage-dependent inward current in Müller cells.

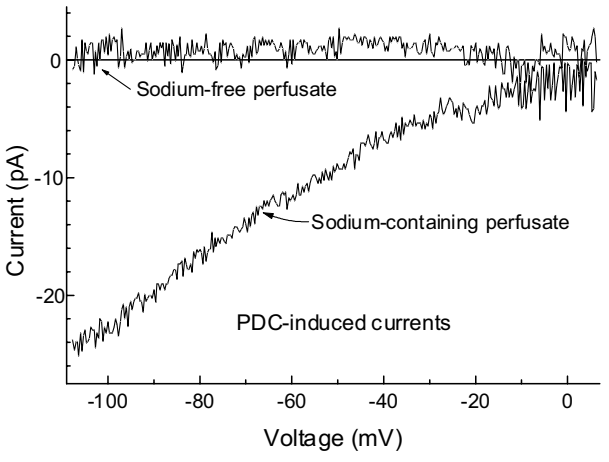

FIGURE 8

Effect of external sodium on PDC-activated current in an isolated rat Müller cell. I-V relationships of the current induced by 100 μM PDC in a rat Müller cell perfused with either solution A, which contained 150 mM Na^+, or a modification of solution A, in which NaCl was replaced with choline chloride and *N*-methyl-*D*-glucamine-Hepes was substituted for Na-Hepes. Consistent with PDC activating GLAST, the PDC-induced current in Müller cells was dependent on external sodium.

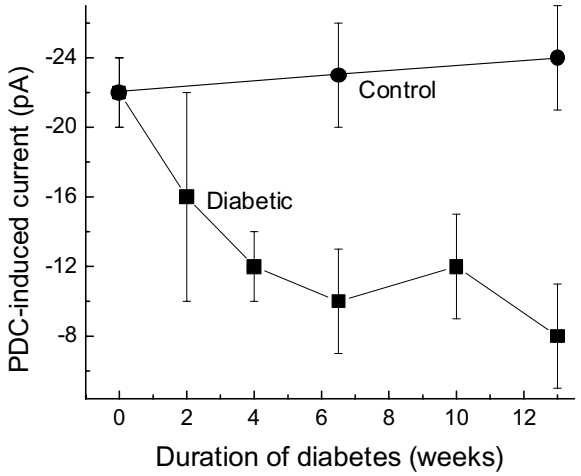

FIGURE 9

Effect of diabetes on the amplitude of the PDC-induced current in Müller cells. Inward (negative) currents induced by PDC were measured in Müller cells isolated from control (●) and diabetic (■) rats. Note that the more negative the current, the greater the PDC-induced transporter activity. Over 13-week course of streptozotocin-induced diabetes, blood glucose levels prior to sacrifice were not significantly ($P > .1$) different; mean level of blood glucose was 378 ± 6 mg/dL^{-1} in the diabetic rats. A mean of 11 ± 2 Müller cells was sampled for each time point. The amplitude of the PDC-induced inward current decreases soon after the onset of experimental diabetes.

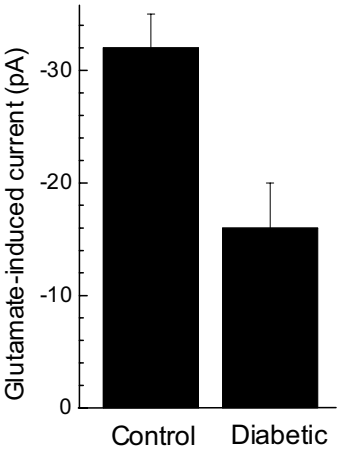

FIGURE 10

Effect of diabetes on glutamate-induced current in Müller cells. Mean induced currents in a series of 11 Müller cells from control rat retinas and 8 cells isolated from rats having diabetes for 9 ± 1 weeks. Note that the greater the glutamate-induced activation of the transporter, the more negative the current. During experimental diabetes, amplitude of Müller cell current induced by glutamate decreases.

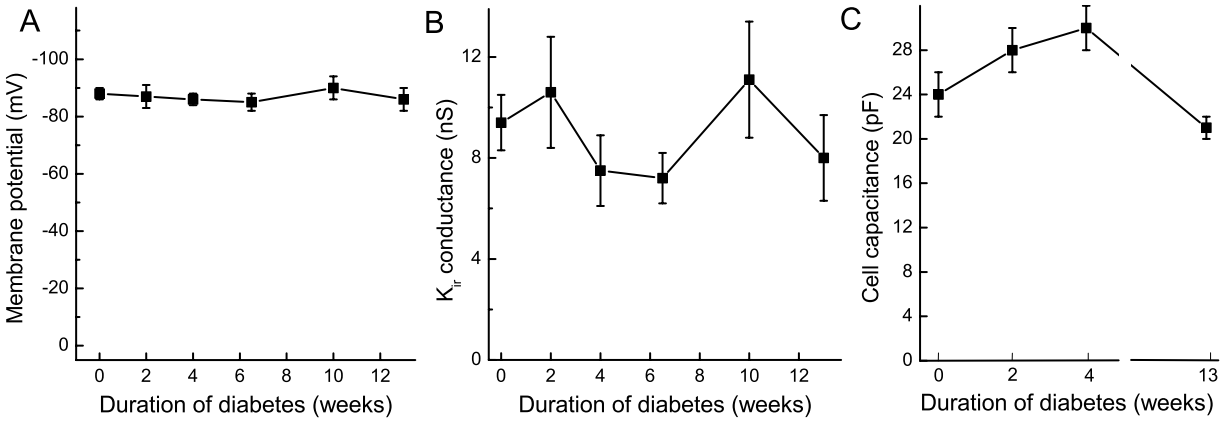

FIGURE 11

Physiological parameters of Müller cells at various times after streptozotocin injection. A, Membrane potential. B, Conductance of inwardly rectifying potassium (Kir) current. C, Cell membrane capacitance. For each panel, a mean of 8 ± 1 Müller cells were sampled for each point. No significant changes occurred in these parameters of Müller cell physiology during 13 weeks of streptozotocin-induced diabetes.

Müller cells from diabetic retinas (Figure 9). Thus, it appears that early in the course of diabetes, these glia have a diminished capability to remove glutamate from the extracellular space.

One possible explanation for observation that glutamate transporter activity decreases in Müller cells from diabetic rats is that there is a generalized disturbance in physiology of these glia. However, this seems unlikely, since the membrane potentials of the sampled cells were not significantly ($P > .3$) different in the control and diabetic groups (Figure 11A). In addition, the amplitude of the inwardly rectifying potassium current, which is the predominant ionic current of Müller cells,[70,71] was not significantly ($P > .16$) different in the Müller cells isolated from diabetic and control rats (Figure 11B).

An alternative possibility to account for the reduction in the amplitude of the current generated by the glutamate transporter is that diabetes causes Müller cells to

become smaller. With a smaller surface area, there may be fewer GLAST molecules per cell and, thereby, a smaller current generated as glutamate is transported across the membrane. However, measurements of cell membrane capacitance, which is an indicator of cell size, did not reveal a significant ($P > .05$) change from control values during 13 weeks of streptozotocin-induced diabetes (Figure 11C).

The stability of the resting membrane potential, potassium conductance, and cell membrane capacitance during the initial 13 weeks of experimental diabetes suggests that the observed decrease in the activity of the glutamate transporter was not part of a generalized physiological deterioration of Müller cells. Rather, it appears likely that early in diabetic retinopathy, there is a selective vulnerability of this transporter molecule.

OXIDATION AND GLAST FUNCTION IN DIABETIC MÜLLER CELLS

This study also addressed the issue of the mechanism by which diabetes causes dysfunction of the glutamate transporter in Müller cells. Experiments were performed to help test the hypothesis that oxidative mechanisms play a role. This seems to be a reasonable idea because diabetes is associated with oxidative stress[59] and the cloned GLAST molecule possesses redox-regulatory elements.[54]

Because studies on cloned GLAST demonstrated that oxidation diminished transporter function without altering the half-maximally effective concentration of glutamate,[54] the dose-response relationship for the glutamate-induced current was assessed in Müller cells from control and diabetic rats. As shown in Figure 12, the half-maximally effective concentration of glutamate was approximately 8 μM in both the control and experimental groups. Of further interest, this value is almost identical to the half-maximally effective concentration reported for the glutamate transporters of Müller cells from salamanders and rabbits, the only other species studied previously.[60,61] Thus, diabetes is associated with a significant decrease in the activity of the Müller cell glutamate transporter (Figure 9) but does not alter the half-maximally effective concentration of glutamate, at least during the initial 13 weeks of experimental diabetes. Since oxidation of GLAST is also reported to decrease the maximal activity of this transporter, the results presented here are consistent with, although not proof of, the possibility that an oxidative mechanism causes GLAST dysfunction in Müller cells of the diabetic retina.

To more directly test the hypothesis that GLAST dysfunction in diabetic Müller cells may be due to oxidation, the effect of a chemical reductant was tested. If a chemical reducing agent reversed the dysfunction of this transporter, then this would be support for the idea that the Müller cell glutamate transporter in the diabetic retina was in an oxidized state. On the basis of this reasoning, the effect of the disulfide-reducing agent, disulfide dithiothreitol (DTT), was assessed (Figure 13).

As illustrated in Figure 13, exposure of a diabetic Müller cell to DTT promptly and markedly increased the amplitude of the inward current induced by PDC. Subsequent exposure of this Müller cell to PDC alone (without DTT) resulted in a response that was similar in amplitude to the current that was previously induced during exposure to PDC plus DTT. The persistent restoration of transporter activity is consistent with DTT having chemically modified the glutamate transporter (ie, changed this transporter molecule from being in an oxidated state to being in a reduced state).

In a series of experiments, the effect of DTT was tested on control Müller cells and those isolated from rats that were diabetic for various durations (Figure 14). Exposure to this disulfide-reducing agent completely reversed the diabetes-associated decrease in the amplitude of the PDC-induced current. This effect was not due to DTT itself inducing a current that was independent of PDC, since the basal currents of Müller cells did not change significantly ($P > .05$, n = 5) when the perfusate contained DTT without PDC.

The DTT-mediated recovery of glutamate transporter activity in diabetic Müller cells is consistent with this transporter molecule being oxidized in the diabetic retina. Taken together, the experimental results presented in this study support the hypothesis that early in the course of diabetic retinopathy, the function of the gluta-

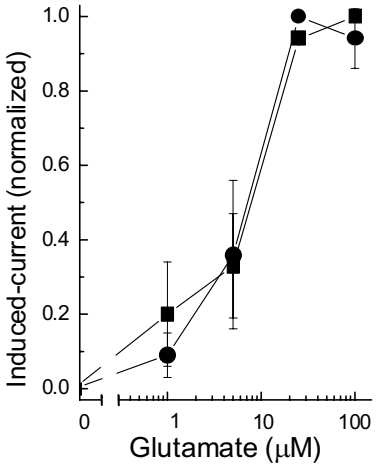

FIGURE 12

Dose-response relationship for glutamate-induced inward current in control Müller cells (n = 4) and in Müller cells from rats that were diabetic for 9 weeks (n = 4). Since glutamate transporter activity in diabetic Müller cells was decreased relative to controls (Figure 10), currents for each group were normalized. Concentration of glutamate that evokes a half-maximal current is similar in diabetic and control Müller cells. ■= Diabetic, ●= Control.

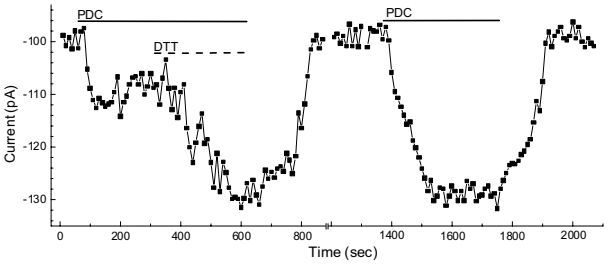

FIGURE 13

Effect of reducing agent, DTT, on current induced by PDC in a Müller cell from a diabetic retina. Current amplitudes were measured at 10-second intervals in a Müller cell isolated from a rat made diabetic by streptozotocin injection 6 weeks earlier. Bars show times during which perfusate contained 100 μM PDC (solid lines) and 3 mM DTT (dashed line).

mate transporter in retinal Müller cells is decreased by a mechanism involving oxidation.

DISCUSSION

The results of this study show that the function of the glutamate transporter in rat Müller cells decreases by 67% during the initial 13 weeks of streptozotocin-induced diabetes. This conclusion is based on experiments that used an electrophysiological assay, which permitted real-time quantitative monitoring of the activity of this transporter in Müller cells freshly isolated from the rat retina. Consistent with the dysfunction of this glutamate transporter being caused by oxidation, exposure of diabetic Müller cells to a disulfide-reducing agent rapidly and fully restored the activity of this transporter. Thus, the experimental findings of this study support the hypothesis that early in the course of diabetic retinopathy, the function of the glutamate transporter in retinal Müller cells is decreased by a mechanism involving oxidation.

DIABETIC RETINOPATHY AND THE GLUTAMATE TRANSPORTER

The finding that the activity of the Müller cell glutamate transporter is decreased in experimental diabetes provides new insight into putative mechanisms that may account for observations made in previous studies of the diabetic retina. For example, a decrease in the ability of Müller cells to transport glutamate from the extracellular space would likely cause levels of this amino acid to increase in the microenvironment. In agreement with this prediction, Lieth and colleagues[28] and Kowluru and colleagues[50] reported that the concentration of retinal glutamate is elevated early in the course of experimental diabetes. Also, the finding by Ambati and colleagues[52] of raised glutamate levels in the vitreous of patients with diabetic retinopathy suggests that diabetes is associated with an elevated concentration of this amino acid in the human retina.

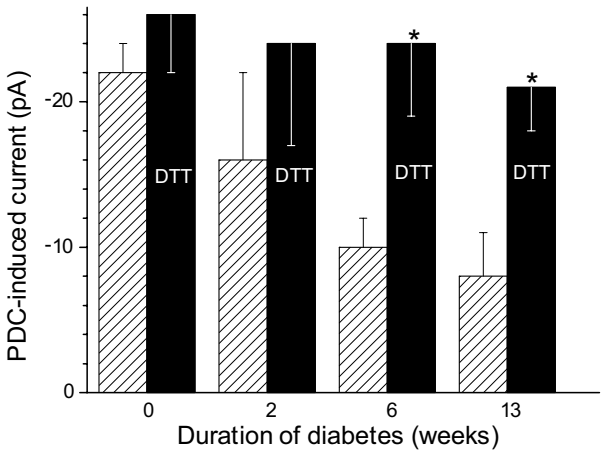

FIGURE 14

Effect of reducing agent, DTT, on current induced by PDC in control and diabetic Müller cells. Striped bars show current induced by 100 μM PDC in absence of DTT. Solid bars show induced current in presence of 3mM DTT. For each value, a mean of 9 ± 2 cells were sampled. Asterisk indicates a significant ($P < .001$) increase in PDC-induced current during exposure to DTT. This reducing agent restores glutamate transporter activity in Müller cells isolated from diabetic rats.

Recent observations suggest that the mechanism by which glutamate levels are elevated in the diabetic retina may involve oxidative stress. For example, Kowluru and colleagues[50] documented that diabetic rat retinas with increased glutamate levels also had a 100% increase in thiobarbituric acid–reactive substances (TBARS), which are indicators of oxidative stress. Further support for a link between oxidative stress and elevated glutamate levels in the diabetic retina is that treatment of diabetic rats with a mixture of antioxidants blocked the increase in TBARS and in the concentration of glutamate in the retina. Taken together, the findings of Kowluru and colleagues[50] suggest that oxidative stress causes a rise in glutamate levels in the diabetic retina.

How are oxidative stress and glutamate homeostasis linked? The findings presented in this thesis indicate that one mechanism by which oxidative stress may increase the levels of glutamate in the diabetic retina involves an inhibition of the Müller cell glutamate transporter. Inhibition of this transporter compromises the ability of Müller cells to remove glutamate from the extracellular space. As a result, the levels of this amino acid in the retinal microenvironment would increase. Thus, on the basis of recent reports[28,50,52] and the experimental findings of this study, a likely scenario is that an oxidation-induced inhibition of GLAST molecules in Müller cells contributes significantly to the disruption of glutamate homeostasis in the diabetic retina.

Dysfunction of the glutamate transporter in Müller cells may augment the level of oxidative stress in the diabetic retina. Support for this idea is that glutamate

enhances the generation in the retina of oxidative stress, as indicated by an increase in TBARS.[50] Pharmacologic experiments indicate that the glutamate-induced increase in retinal oxidants involves the activation of *N*-methyl-*D*-aspartate receptors and the production of nitric oxide (NO).[50] In an environment of excess reactive oxygen species and oxidative stress, NO is readily converted into potent oxidants,[77] which can cause dysfunction of GLAST molecules.[78] These observations suggest that in the retina there is a positive feedback loop involving glutamate and oxidative stress. By failing to maintain glutamate homeostasis in the diabetic retina, Müller cells may play a key role in augmenting oxidative stress.

A positive-feedback loop involving oxidative stress and dysfunction of GLAST molecules in Müller cells may be important in the progression of diabetic retinopathy (Figure 15). This idea is based on the emerging concept[59] that oxidative stress initiates each of the four main molecular mechanisms implicated in the pathogenesis of diabetic complications: increased polyol synthesis, formation of advanced glycation end products, activation of protein kinase C, and enhanced flux through the hexosamine pathway. Thus, by augmenting oxidative stress in the retina, the inhibition of the Müller cell glutamate transporter may be an important step in the development of sight-threatening complications of diabetic retinopathy.

EXPERIMENTAL LIMITATIONS

As with any study, there are limitations in the interpretation of the experimental results. For example, although streptozotocin-injected rats provide an intensively studied model of diabetes, they are not identical to the clinical situation. One reason for this is that uncontrolled hyperglycemia is not typical in patients. Also, of course, rats may respond differently to hyperglycemia and hypoinsulinemia than humans. Additionally, a chemically induced loss of beta cell function is very rare in humans. However, the possibility of a direct toxic effect of streptozotocin on Müller cells seems unlikely in this study, since the membrane potential, predominant ionic conductance, and cell size were not significantly changed in the sampled cells during 13 weeks of experimental diabetes. In addition, the rapid and complete recovery of glutamate transporter activity during exposure of Müller cells to a chemical reducing agent suggests a diabetes-induced oxidative effect rather than nonspecific damage caused by streptozotocin. Thus, despite some limitations, the observations made in this study of Müller cells isolated from rats with streptozotocin-induced diabetes provide a framework for future experimental work on Müller cells from human diabetic donors.

An additional experimental limitation is the use of Müller cells isolated from the retina. Because an in vivo application of the electrophysiologic technique used in this study seems impractical at present, it remains to be demonstrated that oxidation compromises the function of the glutamate transporter in Müller cells in the diabetic retina in vivo. However, although there clearly is a need for caution in extrapolating results from isolated cells to the in vivo situation, use of freshly isolated Müller cells provides some important experimental advantages. For example, the composition of the extracellular solution can be controlled without secondary effects of chemicals that may be released by other retinal cells. In addition, the use of isolated Müller cells in this study, which is the first electrophysiologic analysis of glia from the diabetic retina, allowed comparison with the substantial body of knowledge derived from studies of Müller cells isolated from normal retinas. Overall, it seems reasonable to predict that when electrophysiologic methods are perfected to assay Müller cells in the diabetic retina in vivo, the results of this thesis will help in the design of experiments to assess the functional effects of diabetes on these glia.

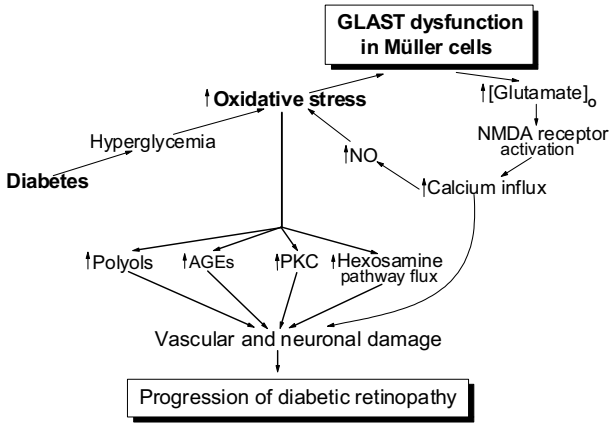

FIGURE 15

Model of putative mechanisms by which Müller cells may play a role in progression of diabetic retinopathy. Oxidative-induced dysfunction of Müller cell glutamate transporter (GLAST) and resulting elevation of glutamate levels may create a positive feedback loop that further increases oxidative stress and, thereby, progression of diabetic complications in retina. Although almost certainly overly simplistic, this model should help in formulation of future studies to assess the role of Müller cells in the pathogenesis of diabetic retinopathy. NMDA, *N*-methyl-*D*-aspartate; NO, nitric oxide; NOS, nitric oxide synthase; AGEs, advanced glycation end products; PKC, protein kinase C; $[glutamate]_o$, extracellular glutamate concentration.

CONCLUDING COMMENTS

A review of the literature led to the formulation of the hypothesis that the ability of retinal Müller cells to remove glutamate from the extracellular space is compromised in the diabetic retina. This hypothesis was extended to also propose that, by a mechanism involving oxidation, diabetes causes dysfunction of the glutamate transporter in Müller cells. This seemed to be a reasonable hypothesis

because oxidative stress is a hallmark of diabetes and recent work indicates that the type of glutamate transporter expressed by Müller cells contains redox-sensitive elements. Experiments to test this hypothesis demonstrated that the function of the glutamate transporter in Müller cells freshly isolated from the rat retina was significantly decreased within 4 weeks after the onset of streptozotocin-induced diabetes. Furthermore, the rapid and complete recovery of transporter function during exposure of diabetic Müller cells to a chemical reducing agent supports the idea that oxidation plays a role in decreasing the function of this glutamate transporter.

Dysfunction of the Müller cell glutamate transporter is one of the earliest reported diabetes-induced changes in these glia. This change precedes the decrease in the activity of glutamine synthetase, the rate-limiting enzyme in Müller cells for the conversion of glutamate to glutamine. This enzyme's activity is not affected until after 8 weeks of experimental diabetes.[51] Likewise, it is not until 6 to 8 weeks of experimental diabetes that there is an up-regulation in the expression of glial fibrillary acidic protein,[28-31] which is an intermediate filament of uncertain function that is expressed by Müller cells in response to a multitude of retinal perturbations. Thus, the glutamate transporter of Müller cells appears to be a molecule that is particularly vulnerable early in the course of diabetes.

The demonstration in this study that the diabetes-induced dysfunction of the Müller cell glutamate transporter can be rapidly reversed, at least early in the course of diabetic retinopathy, renders GLAST as a potential target for pharmacologic intervention. In the future, enhancing the ability of Müller cells to regulate glutamate levels in the diabetic retina may prevent or diminish subsequent molecular events that lead to sight-threatening complications.

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