Diabetes Downregulates GLUT1 Expression in the Retina and Its Microvessels but Not in the Cerebral Cortex or Its Microvessels

Gamal A. Badr, Jie Tang, Faramarz Ismail-Beigi, and Timothy S. Kern

Capillaries in the retina are more susceptible to develop microvascular lesions in diabetes than capillaries in the embryologically similar cerebral cortex. Because available evidence implicates hyperglycemia in the pathogenesis of diabetic retinopathy, differences in glucose transport into the retina and brain might contribute to this observed tissue difference in susceptibility to diabetes-induced microvascular disease. Thus, we compared levels of GLUT1 and GLUT3 expression in the retina, cerebrum, and their respective microvessels by Western blot analysis. In nondiabetic animals, the content of GLUT1 protein in retina and its microvessels was multifold greater than that of cerebral cortex gray matter and its microvessels. Streptozotocin-induced diabetes of a 2-week or 2-month duration reduced GLUT1 expression in the retina and its microvasculature by ~50%, but it resulted in no reduction in GLUT1 expression in cerebrum or its microvessels. The density of capillaries in retinas of diabetic animals did not change from normal, and so the observed decrease in GLUT1 expression in the retina and retinal capillaries of diabetic animals cannot be attributed to fewer vessels. Despite the diabetes-induced reduction of GLUT1 expression in retina, neural retina of diabetic rats still possessed more GLUT1 than the cerebrum. Retinal pigment epithelium (RPE) possessed more GLUT1 than neural retina or its microvessels, and expression of the transporter in the RPE was not affected by diabetes. GLUT3 levels were greater in cerebral gray matter than in retina, and they were unaffected by diabetes in either tissue. The effect of diabetes on GLUT1 expression differs between retina and cerebral cortex, suggesting that glucose transport is regulated differently in these embryologically similar tissues. Because diabetes results in downregulation of GLUT1 expression in retinal microvessels, but not in RPE, the fraction of the glucose entering the retina in diabetes is likely to be greater across the RPE than across the retinal vasculature. *Diabetes* 49:1016–1021, 2000

Hyperglycemia is sufficient to initiate the development of diabetic retinopathy. This fact has been made especially clear by the development of diabetic-like retinopathy in normal nondiabetic dogs and rodents that were experimentally made hyperglycemic by feeding them a diet enriched with galactose (1–5). Studies demonstrating that intensive therapy sufficient to minimize hyperglycemia inhibits the development of retinopathy (6–8) are consistent with the evidence that hyperglycemia plays a critical role in the pathogenesis of retinal disease. However, the mechanism by which hyperglycemia causes diabetic retinopathy remains unclear. Excessive transport or concentration of glucose within cells of the retina is a common thread underlying most of the biochemical mechanisms that have been postulated to play a role in the pathogenesis of diabetic retinopathy.

Retina and cerebral cortex are embryologically similar, yet, we and others (9–11) have found them to differ in their susceptibility to develop microvascular lesions of diabetes. Unlike the extensive microvascular pathology that develops in retinal capillaries in diabetes, cerebral capillaries show no increase in the number of acellular capillaries, microaneurysms, or pericyte ghosts (11). Because both vascular beds are exposed to similar concentrations of blood hexose, these observations suggest that factors in addition to systemic blood glucose concentration influence the development of retinal vascular disease in diabetes.

Transport of glucose into cells can vary among cell types, and such differences might be important in determining which cells are adversely affected by hyperglycemia. Transport of glucose into retina and brain occurs across the blood-retinal and blood-brain barriers via the GLUT1 transporter. Effects of diabetes on GLUT1 expression in cerebral cortex and its microvessels have been examined by several investigators, but the results have been controversial (12–16). In contrast, effects of diabetes on GLUT1 expression in retinal microvessels have not been studied as thoroughly (17). In the present study, retina and cerebral cortex, their respective microvessels, and retinal pigment epithelium (RPE) have been directly compared with respect to the effects of diabetes on GLUT1 expression.

RESEARCH DESIGN AND METHODS

Male Sprague-Dawley rats weighing 225–250 g were used in this study. Rats were randomly assigned to become diabetic or to remain nondiabetic controls. Diabetes was induced by the injection of a freshly prepared solution of streptozotocin in citrate buffer (pH 4.5) at a dose of 60 mg/kg body wt in the tail vein.

From the Departments of Medicine, Ophthalmology, and Physiology and Biophysics, and the Center for Diabetes Research, Case Western Reserve University, Cleveland, Ohio.

Address correspondence and reprint requests to Timothy S. Kern, PhD, Clinical and Molecular Endocrinology, Case Western Reserve University, Cleveland, OH 44106-4951. E-mail: tsk@po.cwru.edu.

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G.A.B. and J.T. contributed equally to this work.

RPE, retinal pigment epithelium; TBS, Tris-buffered saline; TBST, Tris-buffered saline containing Tween.

After 1 week, a sample of blood was obtained from the tail vein for measurement of the serum glucose concentration to verify the presence of hyperglycemia. Glycated hemoglobin levels were measured at 2 months by affinity chromatography (Glyc-Affin; Pierce, Rockford, IL) after an overnight fast. Insulin levels were measured in representative animals by a double-antibody radioimmunoassay (18). Our treatment of the animals conformed to the resolution of the Association for Research in Vision and Ophthalmology on the treatment of animals in research.

Rats were killed at the indicated times by CO$_2$ inhalation anesthesia followed by decapitation. For the 2-week study, cerebral cortexes and retinas were isolated, frozen in dry ice, and stored at –80°C until used. For the 8-week study, control and diabetic rats were divided into replicate groups (2–3 rats per replicate). Retinal and cerebral microvessels were isolated as later described for Western blot analysis of GLUT1. After removal of pia, a slice of gray matter (based on gross appearance of thin slices of cerebrum that did not encompass more than the outer 5 mm of cerebrum from the frontal lobe) and whole retina were used for quantitation of whole-tissue GLUT1 by Western blot. Segments of frontal lobe, hippocampus, and retina were saved from each replicate group for quantitation of vascular density. After removal of neural retina, RPE was isolated by the method described by Cingle et al. (19), in which 1 mmol/l EDTA was added to the eyecup for 20 min, followed by gentle brushing to dislodge the RPE cells.

Samples of brain, retina, and RPE were disrupted using a Potter-Elveheim homogenizer (1:15 dilution) in a solution containing 0.25 mol/l sucrose, 10 mmol/l Tris, and 0.5 mmol/l EGTA, pH 7.4. Homogenates were centrifuged at 2,000*g* for 5 min, and the resulting postnuclear supernatants were used in Western blot analysis. It is possible that some intact vessel fragments were pelleted by this procedure, but in other samples, lower speed (500*g*) or sonication to totally disrupt all cells did not alter results or conclusions. For the cerebrum, up to 50 and 20 µg protein were loaded per lane in blots to be reacted with anti-GLUT1 and anti-GLUT3 antibodies, respectively. For the retina, up to 30 and 75 µg protein were loaded per lane in blots for analyses of GLUT1 and GLUT3, respectively. RPE was loaded at a concentration of 2 µg for analysis of GLUT1. Samples were solubilized in SDS and subjected to SDS-PAGE (12% acrylamide gels). After SDS-PAGE and the transfer to a nitrocellulose membrane (BAS-85; Schleicher and Schuell, Keene, NH), blots were incubated in Tris-buffered saline (TBS) (20 mmol/l Tris and 137 mmol/l NaCl, pH 7.6) containing 0.1% Tween 20 (TBST) and 5% nonfat milk. After 3 washes of each for 10 min in TBST, the blots were incubated with 1:300 dilution of affinity-purified rabbit anti-GLUT1 or 1:10,000 dilution of anti-GLUT3 IgG in TBST for 1 h. After 3 washes with TBST, blots were reacted for 1 h with 1:3,000 dilution of peroxidase-labeled goat anti-rabbit IgG (Gibco BRL, Gaithersburg, MD) in TBST containing 0.15% nonfat milk and were then washed 3 times with TBS devoid of the detergent and were developed using an enhanced chemiluminescence kit (Amersham Life Science, Buckinghamshire, U.K.). Films were digitized, and the intensity of the bands was quantitated using OS-Scan Image Analysis software (USB State Biochemical, Cleveland, OH). Tests that used either purified erythrocyte GLUT1 or erythrocyte cell membrane indicated that the methods used were linear in the range of interest. Antibodies against the glucose transporters were obtained from Charles River Pharmaservices (Southbridge, MA).

Several steps were taken to ensure that comparable amounts of protein had been loaded onto each lane of the gels. After staining with anti-GLUT1, membranes were destained and then restained with Ponceau S staining solution (Sigma, St. Louis, MO). The intensities of the representative bands were visually similar across the lanes. Results were not regarded as reliable unless the intensities of multiple bands were judged to be comparable among the lanes. To further assess the validity of the conclusions derived from this method, other membranes were stripped after staining with anti-GLUT1 antibody and were then restained with antibody against Na,K-ATPase (α1 subunit) (obtained from the University of Iowa Hybridoma facility), GADPH (Chemicon, Temecula, CA), or actin (Sigma). Bands were quantitated by scanning densitometry. Calculating GLUT1 expression relative to intrinsic proteins, such as Na,K-ATPase, GADPH, and actin, was found to be useful in assessing the effect of diabetes on GLUT1 within a tissue; however, it was not as useful for making comparisons among tissues, because the different tissues were found to have different amounts of each of the intrinsic proteins. Of the intrinsic proteins examined, GAPDH expression was found to differ least between retina and cerebral cortex.

Isolation of microvessels. Microvessels from cerebral cortex gray matter were isolated using previously published methods (20,21) with some modifications. Briefly, brains from 3 rats were rapidly removed and immersed in ice-cold Earl's-HEPES buffer containing Earl's salts, 20 mmol/l HEPES (pH 7.4), and 1% bovine serum albumin. The brain stem and cerebellum were dissected away and discarded, and the pia and other large superficial blood vessels were removed using fine forceps. The gray matter was isolated using a sharp razor blade and minced with fine scissors. The small pieces of gray matter were homogenized by hand (3–4 strokes) in 30 ml of the previously described buffer by using a Dounce homogenizer (Fisher, Pittsburgh, PA) with a loosely fitting pestle. The homogenate was sieved through 210 µm nylon mesh, and the material collected on the mesh was homogenized again in 20 ml of the buffer using 2–3 strokes. The homogenate was sieved through 105 µm nylon mesh, and microvessels collected on the mesh were washed gently with 10 ml of the buffer and were then collected. The material was layered over 5 ml of 1.3 mol/l sucrose containing 10 mmol/l Tris (pH 7.4) and was centrifuged at 10,000*g* for 10 min at 4°C. The pellet containing purified microvessels was washed twice with ice-cold phosphate-buffered saline. The purity of each microvessel preparation was monitored by light microscopy and was similar in control and diabetic groups. We estimated that contaminants (mostly naked nuclei) did not exceed 5% of the nuclei in the preparation and that the yield of microvessels from both experimental groups was equal (~60 µg protein).

The recovery of microvessels from pooled retinas from 3 rats by using the sieving method previously described was poor. Thus, retinal vasculature was isolated from whole retina by an osmotic method that we have used previously (22). Briefly, freshly isolated retina were incubated in distilled water for 1 h, followed by a 5-min incubation with DNase I (2 mg/ml). The retinal microvasculature was isolated under microscopy by repetitive inspiration and ejection through Pasteur pipettes with sequentially narrower tips. Retinal microvessels isolated by this method showed a normal complement of nuclei and were devoid of all nonvascular materials.

The microvessels were prepared for Western blots, as previously described, except they were sonicated briefly in SDS sample buffer to assist in solubilization. Protein was measured by Micro BCA reagent (Pierce), and Western blots were prepared using 5 µg protein/sample as previously described.

The density of microvessels in the retina and the gray matter of the frontal lobe was quantitated in paraffin sections using point-counting methods (23). Hematoxylin and eosin-stained sections were examined at ×250 magnification, and the fraction of grid points (10 × 10 grid) intersecting with capillary cross-sections was counted. For frontal lobes, the density of capillary profiles was calculated by averaging 4 different regions per tissue section (total area of 0.67 mm^2). The density of capillaries in retinal cross-sections was estimated as the number of capillary profiles observed per 40 mm linear length of retina. Because the number of microvessels counted in retinal cross-sections was modest, the density of retinal microvessels was also assessed in Periodic acid-Schiff-stained flat preparations of isolated retinal vasculature. The 2 methods used for measurement of retinal vascular density yielded similar conclusions.

Results are expressed as means ± SE. Student's unpaired 2-tailed t test was used, and a $P < 0.05$ was considered significant.

RESULTS

Diabetic rats were hyperglycemic and insulinopenic, and they failed to gain weight at a normal rate. Body weight at 8 weeks averaged 387 and 256 g for normal and diabetic rats, respectively. Serum glucose levels (4.4 ± 0.7 and 21.7 ± 4.3 mmol/l for normal and diabetic groups, respectively) and glycosylated hemoglobin concentrations (4.7 ± 0.2 and 12.2 ± 0.6%) were greater than normal in diabetic rats. Serum insulin levels in diabetic rats were low (0.4 ± 0.1 ng/ml) compared with those in controls (2.5 ± 0.2 ng/ml).

The content of GLUT1 protein in retina of normal rats was greater than that in cerebral cortex gray matter; retina pos-

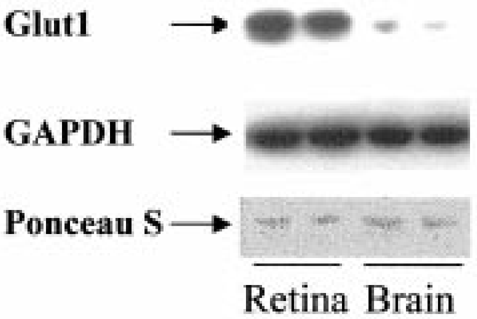

FIG. 1. GLUT1 expression in retina of nondiabetic rats is greater than that in cerebral gray matter. GAPDH expression and Ponceau S staining of protein on the same gel are included to demonstrate comparable loading of lanes.

sessed 6 times more of the transporter than cortex (Fig. 1). In homogenates of retina and cerebrum, the transporter was about 45 kDa, and retina showed a wider band of staining than cerebrum. Diabetes of a 2-week (data not shown) or 8-week (Fig. 2) duration significantly reduced GLUT1 content of retina by ~33 and 50%, respectively. In contrast, GLUT1 in cerebral cortex gray matter was not reduced by diabetes at either time point (Fig. 2).

Microvessels from both retina and cerebrum had the 55-kDa isoform of GLUT1 (Fig. 3). Consistent with findings in whole tissues, the content of GLUT1 in microvessels isolated from retinas of nondiabetic rats was multifold greater than that in cerebral microvessels. Diabetes reduced GLUT1 levels in retinal microvessels by >60% compared with nondiabetic rats, whereas expression in cerebral microvessels did not decrease. Diabetes of a 2-month duration did not significantly alter the microvascular density in either retina (normal 7.9 ± 0.5 capillaries/40 mm retina, diabetes 7.9 ± 0.7 capillaries/40 mm retina) or the brain (normal 5.0 ± 0.5 capillaries/mm^2, diabetes 5.9 ± 0.4 capillaries/mm^2), indicating that the observed loss of retinal GLUT1 in diabetes was not due to a change in vascular density. RPE had a multifold greater expression of GLUT1 than neural retina and cerebrum, and expression of the transporter in RPE was not affected by diabetes (Fig. 4).

The content of GLUT3 was ~12 times greater in cerebral cortex gray matter than in retina (Fig. 5). In contrast to GLUT1, the content of GLUT3 was unaffected by diabetes in either tissue.

DISCUSSION

Elevation of systemic levels of glucose or galactose in diabetes or experimental galactosemia can induce a variety of abnormalities of metabolism of the retina and its microvasculature, some of which, presumably, contribute to the development of retinopathy. To date, however, the mechanism by which hyperglycemia causes retinopathy remains unclear. Abnormalities that have attracted interest as potential causes of retinopathy include excessive polyol pathway activity, nonenzymatic glycation, oxidative stress, and stimulation of reti-

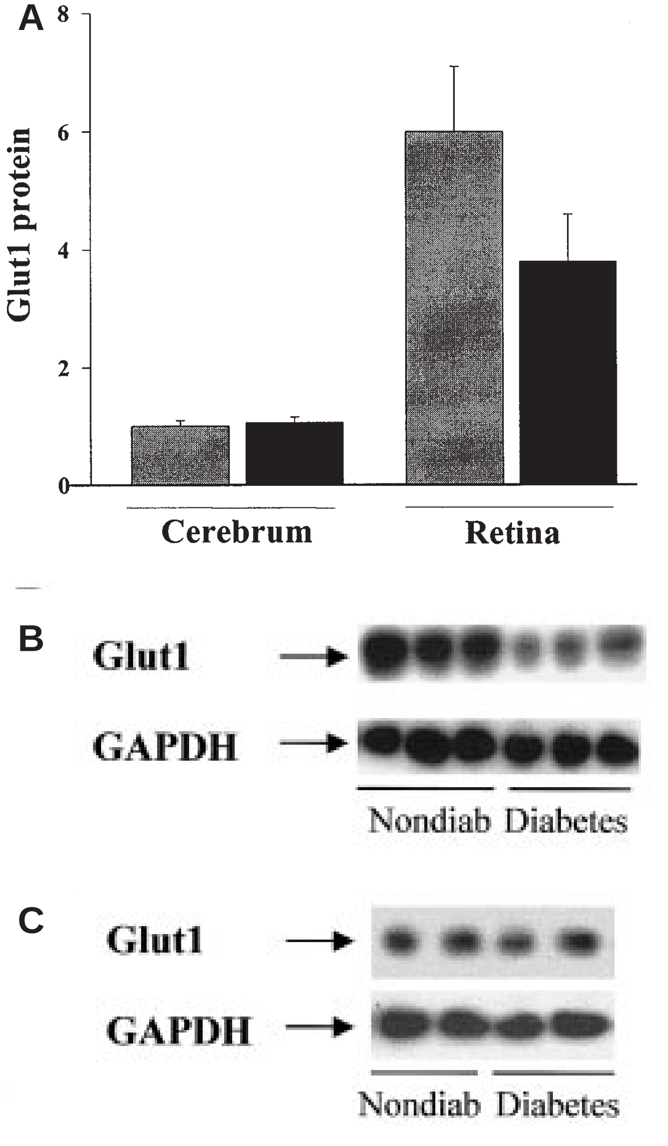

FIG. 2. Diabetes of an 8-week duration results in subnormal expression of the glucose transporter in retina, whether expressed as relative to the protein loaded (*A*) or the GAPDH expression (*B*). Diabetes did not decrease GLUT1 in cerebral gray matter (*A* and *C*). The *y*-axis in *A* is *n*-fold the value of nondiabetic cerebral gray matter. Data are means ± SE (*n* >8 per group). ▨, Nondiabetic rats; ■, diabetic rats. Nondiab, nondiabetic.

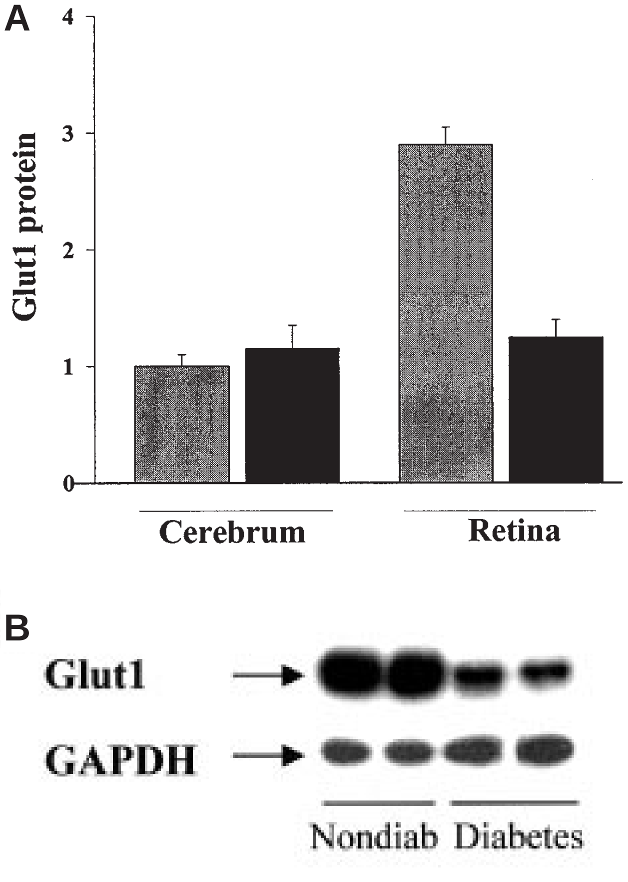

FIG. 3. GLUT1 expression in microvessels isolated from retina, but not in microvessels from cerebral tissue of the same animals, is decreased by diabetes of an 8-week duration. Graphical summary (*A*) and raw Western blots (*B*) are provided. The *y*-axis in *A* is *n*-fold the value of nondiabetic cerebral microvessels. Data are means ± SE (*n* = 6 per group). ▨, Nondiabetic rats; ■, diabetic rats. Nondiab, nondiabetic.

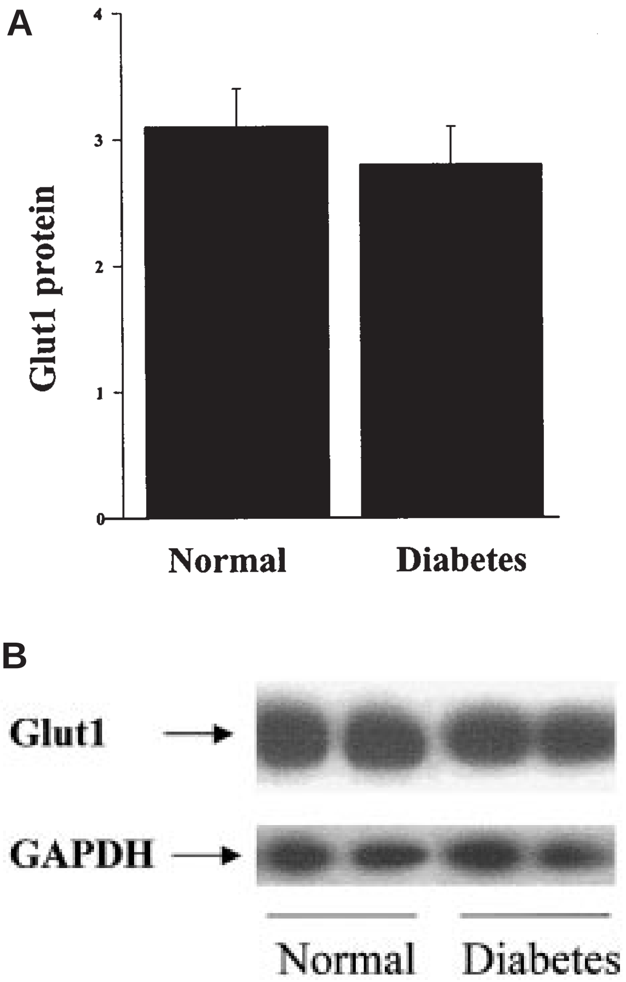

FIG. 4. Diabetes of an 8-week duration does not alter GLUT1 expression in RPE, whether expressed as relative to the microgram of protein loaded (*A*) or relative to GAPDH expression (*B*).

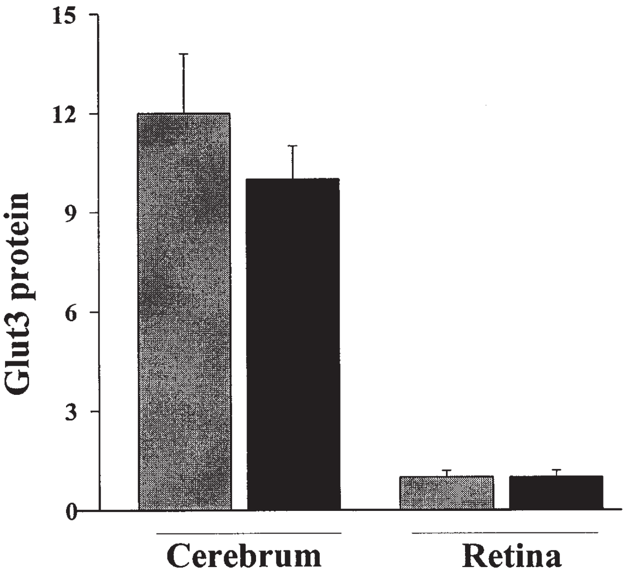

FIG. 5. GLUT3 expression in retina and cerebral cortex is unaffected by diabetes. The duration of diabetes is 2 weeks. The *y*-axis is *n*-fold the value of nondiabetic retina. Data are means ± SE (*n* = 8 per group). ▨, Nondiabetic rats; ■, diabetic rats.

nal protein kinase C activity (22,24–32). Most of these metabolic abnormalities are dependent on excessive transport of hexose into retinal cells or increased concentration of hexose within those cells.

Diabetic retinopathy is generally regarded to primarily affect the microvasculature; therefore, metabolic abnormalities of microvascular cells have been of special interest. Endothelial cells apparently differ from many other cell types in that transport of glucose into the cells is more rapid than metabolism of the glucose (i.e., transport is not rate-limiting) (33–36). Thus, glucose can reach high concentrations intracellularly, especially during periods of hyperglycemia.

Transport of glucose and several other hexoses across plasma membranes is mediated mainly by a gene family of Na^+-independent glucose transporters; of this family, there are at least 7 different forms (37). GLUT1 is a widely expressed isoform that is also expressed at sites of barrier function, such as the blood-retinal and blood-brain barriers (38). Glucose reaches individual cells of the retina and brain normally via GLUT1-mediated transfer of the sugar across the microvascular endothelial cells from the blood or across the RPE. Transport of glucose into some nonvascular cells of the retina and brain is also mediated by GLUT1, with the GLUT1 transporter having been identified immunohistochemically in glial cells, ganglion cells, photoreceptors, and pigment epithelial cells (39–41). Low levels of GLUT3 also are detectable within retina. GLUT3 is highly expressed in cerebral neurons (16,38,42–44), suggesting that this isoform mediates glucose transport into neuronal cells.

Retina and cerebral cortex are embryologically similar, yet we and others (9–11) have shown them to differ in their susceptibility to develop microvascular lesions in diabetes. Cerebral cortical vessels of dogs with either diabetes or experimental galactosemia of a 5-year duration were found to possess none of the microaneurysms, acellular capillaries, and pericyte ghosts that occurred in retinal vessels of the very same animals (11). Differences in tissue architecture or in activity of particular enzymes might account for the tissue difference in susceptibility to developing diabetic microvascular disease, but differences in the amount of glucose transport into the tissues might also be a factor.

There have been several reports on the effects of diabetes on GLUT1 expression in cerebral cortex and cerebral microvessels, but the results of these reports remain controversial. Investigators have reported a decrease (12,13,15,16) or no changes (14,45) in GLUT1 expression in cerebral microvessels of diabetic rats. Likewise, the density of cytochalasin B–binding sites, which reflect GLUT1 concentration in the vascular endothelium (because GLUT1 is the major glucose transporter present in those cells), has yielded contradictory results; glucose-displaceable cytochalasin B–binding has been reported to be decreased, increased, and unchanged in cerebral microvessels of diabetic rats (14). Some investigators have reported increased levels of GLUT1 mRNA in cerebral microvessels in diabetes despite normal GLUT1 protein concentrations (45), suggesting a diabetes-induced defect in translation of the transporter.

Effects of diabetes on the in vivo expression of GLUT1 in retina and its microvessels has been reported by only a single laboratory (17,39). These authors initially reported that diabetes did not alter the distribution of GLUT1 in the retina, but they were unable to determine if the amount of the transporter was altered (39). The same investigators later quantitated the effect of diabetes on GLUT1 expression in retinal microvessels, although the analysis was based on only 3 patients (17). In those 3 patients, diabetes was associated with an increase or no change in the expression of GLUT1 in the retinal endothelium. Other studies from the same laboratory (using the same immunochemical methods) reported that diabetes decreased expression of GLUT1 in endothelial cells of the cerebral cortex (13), thus offering a possible mechanism for the different susceptibilities of retinal and cerebral microvessels to hyperglycemia-induced microvascular disease.

Our studies, however, do not support the aforementioned conclusions. Our findings, based on Western blots of whole tissue and isolated microvessels from each tissue, indicate that diabetes for up to a 2-month duration decreased expression of GLUT1 in neural retina, whereas expression of the transporter in cerebral cortex remained largely unaffected. The microvascular density at 2 months of diabetes was not abnormal; thus, the observed decrease in GLUT1 content of the retina was not secondary to diabetes-induced changes in vascular density. The different conclusions between our study and that of Kumagai et al. (17) might be due to differences in species, durations of diabetes, and methods of GLUT1 quantitation. Quantitation of GLUT1 in the studies of Kumagai et al. (17) and Pardridge et al. (13) was very precise, because it involved ultrastructural localization and quantitation of GLUT1 sites, but their analyses included only a small number of capillaries from each tissue. In contrast, our studies involved essentially the entire vasculature of the retina, but the method we used lacked the ability to determine the cellular and subcellular distribution of the transporters. More work must be accomplished to resolve the different conclusions of the studies and to determine their significance.

The RPE constitutes the second site of the blood retinal barrier. It is estimated that a significant amount of all glucose enters the retina via the RPE, and these estimates are consistent with the presence of appreciable amounts of GLUT1 in that cell type. In our studies, diabetes of a 2-month duration had no effect on the expression of GLUT1 in the RPE, despite significantly inhibiting expression of the transporter in the retinal vasculature. Regulation of GLUT1 expression apparently is under different control in different cell types. In diabetes, the combination of increased systemic glucose concentration and unchanged expression of GLUT1 in RPE will likely result in a considerable increase in total glucose transport into the retina across the RPE. Moreover, a greater fraction of the glucose entering the retina in diabetes is likely to occur via the RPE due to the decrease in GLUT1 expression in retinal microvessels.

The present comparisons suggest that the retina and its vasculature express more GLUT1 than the cerebrum and its vasculature. Thus, even though GLUT1 expression was downregulated in retinal vessels in diabetes, it is probable that glucose transport across retinal vessels remained at least as great as that across cerebral vessels of diabetic rats. Rates of glucose transport and metabolism in diabetes need to be compared in retina and cerebral cortex to more fully understand the difference in susceptibility of retina and cerebrum to diabetic microvascular disease. In diabetes, downregulation of GLUT1 in retinal microvessels likely minimizes excess transport of glucose across the vasculature, but unchanged expression of the transporter in RPE probably diminishes that effect by allowing a greater fraction of glucose entry into the retina across the RPE. Ultimately, the downregulation of GLUT1 in retinal vessels in diabetes is not sufficient to prevent diabetes-induced retinopathy.

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