Inner Blood–Retinal Barrier GLUT1 in Long-Term Diabetic Rats: An Immunogold Electron Microscopic Study

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PURPOSE. The GLUT1 glucose transporter mediates glucose entry into the endothelial cells of the inner blood–retinal barrier (BRB). In many cell types, exposure to high glucose concentrations or diabetes downregulates GLUT1. To determine whether long-standing diabetes alters the expression and distribution of inner BRB GLUT1, changes in immunoreactive retinal endothelial cell GLUT1 were studied in Goto-Kakizaki (GK) rats, an animal model of type 2 diabetes.

METHODS. Immunogold staining for GLUT1 was performed on ultrathin sections of retinal specimens obtained from 1-year-old GK rats and age-matched Wistar controls. Retinal capillary endothelial cells were visualized by transmission electron microscopy, and GLUT1 immunogold was quantified on the luminal and abluminal membranes of endothelial cells from digital microphotographs of individual vessels by computer.

RESULTS. Forty-one microvessels from six diabetic rats and 43 microvessels from six nondiabetic Wistar control rats were analyzed. Densitometric quantification revealed an asymmetry of GLUT1 distribution between luminal and abluminal membranes of both diabetic and nondiabetic rats, with a lumino-to-abluminal ratio of approximately 1 to 3. The distribution pattern and density of retinal endothelial GLUT1 immunoreactivity were not significantly different between the diabetic and control rats.

CONCLUSIONS. As determined by GLUT1 immunogold distribution, there is no compensatory downregulation of GLUT1 on the inner BRB in an animal model of long-standing diabetes. (Invest Ophthalmol Vis Sci. 2003;44:3150–3154) DOI: 10.1167/iovs.02-1284

Glucose is an essential metabolic substrate for retinal metabolism, and its transport from blood to retina is tightly coupled with retinal glucose utilization.1 Glucose entry into the retina occurs at two major anatomic interfaces: the retinal capillary endothelial cells and the retinal pigment epithelium.2 These structures serve specialized barrier functions, in that, similar to the brain capillaries of the blood–brain barrier (BBB), the presence of intercellular tight junctions prevents the passive diffusion of glucose and other nutrients across these barriers from blood to the neuroretina.3 Glucose entry into the endothelial cells of the so-called inner BRB is mediated by a saturable, facilitated transport process involving GLUT1, a member of a family of sodium-independent glucose transporter proteins.2,4

The transport of glucose into the retinal endothelial cells occurs in excess of its phosphorylation by intracellular hexokinase,5 and therefore intracellular glucose may be detected in both isolated retinal capillaries6 and in the living retina.6 Glucose transport in retinal endothelial cells is mediated by a number of different factors, including hypoxia and cytokines, through a variety of different signaling pathways.7–10 In situations that involve increased concentrations of intracellular glucose, glucose may be available for a variety of different metabolic fates, including the generation of oxygen free radicals and oxidative stress,11 increased production of diacylglycerol and protein kinase C activity,12 and increased intracellular glycation.13

To protect the intercellular environment from excessive glucose flux, most tissues downregulate glucose transport in response to elevated extracellular glucose concentrations and/or diabetes.13–15 In endothelial cells of the retinal microvasculature, however, the situation is not as clear. Primary cultures of bovine retinal endothelial cells (BRECs) do not demonstrate a change in the uptake of the glucose analog, 3-O-methylglucose, after exposure for 8 days to 20 mM glucose, compared with retinal pericytes, which exhibit a 30% decrease in transport rates under similar conditions.16 In a streptozotocin-diabetic rat model, Badr et al.17 recently reported a decrease of approximately 50% in total retinal GLUT1 and retinal microvascular GLUT1 after 8 weeks of diabetes. In contrast, we previously used GLUT1 immunogold electron microscopy to demonstrate a localized increase in GLUT1 on the retinal endothelial cells of the inner BRB in postmortem retina specimens from patients with long-standing diabetes (>17 years), compared with nondiabetic control specimens.18

To investigate changes in inner BRB GLUT1 in an animal model of long-standing diabetes, we performed GLUT1 immunogold electron microscopy on retina specimens from 1-year-old Goto-Kakizaki (GK) diabetic rats, a rodent model of spontaneous, nonobese type 2 diabetes. The GK rat, developed from a Wistar background, begins to develop chronic hyperglycemia at 4 to 6 weeks of age.19 Chronic hyperglycemia is associated with hyperinsulinemia and glucose intolerance in this rat model,19,20 and diabetic GK rats also demonstrate metabolic and anatomic changes similar to those in human diabetic retinopathy, including increased endothelial cell-pericyte ratios,21 abnormalities of retinal circulation,22 decreased retinal glutathione concentrations,23 and increased intravitreal...
vascular endothelial growth factor (VEGF) levels, compared with Wistar control animals.

The objectives of the present study were twofold: first, to determine the subcellular distribution of GLUT1 in the endothelial cells of the rat inner BRB, and, second, to investigate changes in inner BRB expression of GLUT1 in long-standing diabetes.

**Materials and Methods**

**Animals**

GK and control Wistar rats were obtained from a local breeding colony maintained at the Faculty of Medicine, University of Coimbra. After 6 weeks of age, the GK rats showed persistent hyperglycemia. Five 1-year-old diabetic GK rats and four age-matched nondiabetic Wistar control rats were used in these experiments. The rats were fed normal rat chow ad libitum and maintained in temperature-controlled facilities with 12-hour light–dark cycles. Glucose concentrations were measured on tail blood samples using the glucose monitor (Gluco Touch; Lifescan, Milpitas, CA). All animals were handled in accordance with ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

**Materials**

Paraformaldehyde, glutaraldehyde, an embedding resin kit (LR Gold), fish gelatin, osmium tetroxide, and anti-rabbit IgG conjugated to 10 nM colloidal gold (Auroprobe EM-GAR10) were purchased from Ted Pella (Redding, CA). Filters (Millex-GV; pore size, 0.22 µm) were from Millipore (Bedford, MA). Ultrapure bovine serum albumin (BSA) and all other reagents were obtained from Sigma-Aldrich (St. Louis, MO).

**Preparation of Specimens**

The GK and Wistar control rats were killed, and their eyes were immediately removed and briefly rinsed in ice-cold phosphate-buffered saline (PBS, pH 7.4). The eyes were bisected 1 to 2 mm posterior to the limbus, and the anterior chamber and the lens and vitreous were carefully removed. Fixation was performed as described previously with minor modifications. Briefly, the eyes were immersed in 4% paraformaldehyde in 0.05 M sodium phosphate (pH 7.4) containing 8.5% sucrose, 0.1% glutaraldehyde, and 1 mM CaCl₂. After 30 minutes, the eyes were cut in half along the axis of the optic nerve and placed back in the same fixative for an additional 30 minutes at room temperature. The eyes were rinsed briefly with cold PBS and placed overnight in 4% paraformaldehyde in 0.2 M NaHCO₃ buffer (pH 10.4) containing 1 mM CaCl₂. The next day, the eyes were rinsed with ice-cold 50 mM PBS (pH 7.4) with 1 mM CaCl₂, and 0.5 to 1.0-mm segments were cut under a dissecting microscope and dehydrated by immersion in an ethanol series (90-minute periods in 50%, 70%, 95%, and 100% ethanol) under a dissecting microscope and dehydrated by immersion in an ethanol series (90-minute periods in 50%, 70%, 95%, and 100% ethanol) at 4°C. All specimens were embedded in gold resin (LR Gold), and after blocking in gelatin capsules, the resin was polymerized overnight at 30°C, according to the manufacturer’s instructions. Semithin sections were cut and stained with trypsin blue to enhance the presence of retinal capillaries. These sections were used to guide trimming of ultrathin sections. Two to five blocks of retinas per rat were sectioned.

**Immunohistochemistry**

**Anti-GLUT1 Antiserum.** A rabbit polyclonal antibody raised against purified human erythrocyte glucose transporter (GLUT1) was a kind gift from Christin Carter-Su. This antibody is specific for GLUT1 and has been characterized previously.

**Immunogold Staining Procedures.** Immunogold staining was performed as previously described. Briefly, the sections were hydrated in buffer A (0.01 mM sodium phosphate [pH 7.4], 0.15 M NaCl, 0.05% Tween-20, 0.1% fish gelatin, and 1% BSA) for 10 minutes at room temperature. After hydration, the grids were transferred to blocking solution (buffer A containing 2.5% BSA and 2.5% normal goat serum) and incubated for 30 minutes at room temperature. Grids were then incubated overnight at 4°C on drops of anti-GLUT1 antiserum or normal rabbit serum at a dilution of 1:800. All the solutions, antisera or sera were filtered (GV; Millipore) through filters before use. The grids were then washed for 10-minute periods on drops of filtered buffer A, followed by incubation for 2 hours at room temperature in a 1:40 dilution of 10-nm gold-particle-conjugated goat anti-rabbit immunogoldulin antiserum (Auroprobe EM-GAR10) diluted in buffer A, followed by washing for six 10-minute periods on drops of 0.1 sodium phosphate buffer (pH 7.4). The grids were then fixed for 5 minutes in 1% glutaraldehyde in 0.1 M Na₂HPO₄ (pH 7.4), rinsed with distilled water, stained with 2% aqueous osmium tetroxide for 10 minutes, rinsed with filtered water, stained with 2% (wt/vol) aqueous uranyl acetate for 30 minutes, rinsed with distilled water, and air-dried. Grids were examined in a transmission electron microscope (CM-100; Philipps, Eindhoven, The Netherlands).

**Quantification of Immunogold.** Retinal microvessels were visualized at 19,000× to 34,000×, and digital photographs were taken of sections of capillary profiles. The digital image of each complete vessel cross section was a reconstructed montage of the photomicrographs of the corresponding partial microvessel profiles using an image-processing program (Jasc Paint Shop Pro; Minneapolis, MN; Fig. 1).

The immunogold particles, represented by discrete black spots on the photomicrographs (Fig. 2), were counted on the luminal and abluminal membranes of the endothelial cells, and the lengths of the luminal and abluminal membranes of each vessel were measured with a customized software program (MetaMorph; Universal Imaging Corp., Downingtown, PA). For purposes of quantification, immunogold particles that demonstrated clear evidence of aggregation and lengths of membrane...
The average blood glucose concentrations and body weight for the 1-year-old diabetic GK rats and age-matched Wistar control rats are provided in Table 1. The average blood glucose concentrations for diabetic animals were significantly higher ($P < 0.01$) than that of control animals, and hyperglycemia persisted throughout the experiment in the diabetic rats.

**Statistical Analysis**

Samples were taken from five diabetic GK and four control Wistar rats. Between 2 and 12 vessels per block and at least 2 to 4 blocks per rat were studied. For comparative analysis, the mean immunogold density per micrometer of luminal and abluminal membranes from all samples from each rat were quantified and averaged over each group (diabetic and nondiabetic), and comparisons were made of the mean values between the GK diabetic and Wistar control groups. All data are expressed as the mean ± SD. Comparisons between groups were made with an unpaired two-tailed Student’s $t$-test. $P < 0.05$ was considered statistically significant.

**RESULTS**

The average blood glucose concentrations and body weight for the 1-year-old diabetic GK rats and age-matched Wistar control rats are provided in Table 1. The average blood glucose concentrations for diabetic animals were significantly higher ($P < 0.01$) than that of control animals, and hyperglycemia persisted throughout the experiment in the diabetic rats.

**TABLE 1.** Body Weight and Plasma Glucose Levels for Nondiabetic Wistar and Diabetic GK Rats.

<table>
<thead>
<tr>
<th></th>
<th>Wistar Rats (n=4)</th>
<th>GK Rats (n=5)</th>
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<tbody>
<tr>
<td>Body weight (g)</td>
<td>$858 \pm 48^*$</td>
<td>$424 \pm 39^*$</td>
</tr>
<tr>
<td>Serum glucose (mg/dL)</td>
<td>$108 \pm 7^*$</td>
<td>$303 \pm 46^*$</td>
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Values are averages ± SE. Data gathered at 12 months. $^* P < 0.01$ compared to Wistar controls.

The retinal microvessels showed good ultrastructural preservation and allowed for the identification of many features of the endothelial cells, including the plasma membrane and basal lamina (Figs. 1, 2) and for the quantification of GLUT1 immunogold particles. Specific immunogold staining was confirmed in all specimens by negligible staining with preimmune serum (data not shown). A total of 41 retinal microvessel profiles from the five diabetic rats and 43 profiles from the four nondiabetic control rats were analyzed. For both groups, an asymmetrical distribution of GLUT1 immunogold was seen on the luminal and abluminal membranes of the retinal microvascular endothelial cells. Quantitative analysis of the GLUT1 immunolabeling in the sections showed an approximate threefold enrichment of GLUT1 on the abluminal membrane compared with the luminal membrane (Fig. 3). Luminal-to-abluminal ratios of approximately 1 to 3.0 ($1.15 \pm 0.17$ vs. $3.18 \pm 0.37$ gold [Au]/µm) and 1 to 3.2 ($1.19 \pm 0.12$ vs. $3.45 \pm 0.22$ Au/µm) were observed for the nondiabetic control rats and the GK diabetic rats, respectively ($P < 0.003$).

As seen in Figure 4, densitometric analysis revealed that the distribution and density of GLUT1 immunoreactivity in the retinal capillary endothelial cells were virtually identical between the nondiabetic and diabetic rat retinas. In the luminal membranes, the density of GLUT1 immunogold was $1.19 \pm 0.28$ Au/µm in the microvessels from the diabetic retinas and $1.15 \pm 0.35$ Au/µm in the nondiabetic retinas. In the abluminal

**FIGURE 2.** GLUT1 immunogold staining of rat retina. Representative partial cross-sectional profiles of retinal microvessels from nondiabetic (A) and diabetic (B) rats stained with a 1:800 dilution of anti-GLUT1 antiserum, followed by incubation with a secondary antibody coupled to 10-nm colloidal-gold. Counterstained with uranyl acetate. Immunogold labeling is represented by discrete black spots along luminal or abluminal membranes or within cytoplasm of the microvascular endothelial cell (arrows). L, lumen of capillary. Original magnification of individual photomicrographs, $\times 34,000$.

**FIGURE 3.** Subcellular distribution of GLUT1 immunogold. Gold particles were quantified per micrometer luminal (Lum) or abluminal (Abl) membrane length of endothelial cells from composite photomicrographs of retinal capillaries of GK diabetic (GK) or nondiabetic Wistar control rats (Control). Results are expressed as the ratio of GLUT1 in abluminal (Abl) to luminal (Lum) membranes.

**FIGURE 4.** Inner BRB GLUT1 immunogold labeling from 1-year-old diabetic and nondiabetic rats. GLUT1 immunogold particles (Au) were quantified per micrometer length of luminal (Lum) or abluminal (Ablum) endothelial cell membrane length from composite photomicrographs of retinal capillaries of GK diabetic (Diabetic) or nondiabetic Wistar control (Control) rats. Results are expressed as density of GLUT1 immunogold in abluminal and luminal membranes. $^* P < 0.003$ compared with luminal membrane.
membrane, the density of GLUT1 was $3.46 \pm 0.50$ and $3.18 \pm 0.74 \text{Au/\mu m}$ in the microvessels from the diabetic and nondiabetic retinas, respectively. Among the diabetic retina microvessel profiles analyzed, no statistically significant differences were found in GLUT1 between the normal and diabetic rats on either luminal or abluminal membranes.

**DISCUSSION**

The present study used immunogold electron microscopy to investigate GLUT1 expression in the nondiabetic and diabetic inner BRB of the rat. This technique differs from Western blot analysis of the luminal and abluminal membranes,20 isolated capillaries, or whole retina samples,17 in that it allows for simultaneous subcellular localization of GLUT1 in the same specimens in which quantitative analysis is performed. It also allows for quantification of GLUT1 in situ—that is, free of possible changes due to isolation procedures. The present study demonstrates an asymmetrical distribution of GLUT1 between the luminal and abluminal membranes of the normal rat inner BRB, with an approximately threefold higher density of GLUT1 on the abluminal than in the luminal membranes (Fig. 3). This asymmetrical distribution was similar to that of our previous report of an approximate 2-to-1 ratio of GLUT1 in the abluminal and luminal membranes of the endothelia of the human inner BRB18 and is also similar to the 4-to-1 ratio of GLUT1 observed in the structurally similar blood–brain barrier of the rat.22 Asymmetrical distribution of transporter proteins is characteristic of polarized endothelial cells, in that intercellular tight junctions allow for division of the cells into luminal and abluminal surfaces and for the nonuniform distribution of transport proteins within the cell.20,29 With regard to glucose, the higher density of GLUT1 on the abluminal membrane of the retinal endothelial cells suggests that glucose transport is limited at the blood–luminal, rather than the abluminal–interstitial, interface.

The present study also demonstrates that GLUT1 expression in the inner BRB of the rat does not change with long-standing diabetes (Fig. 4). These findings suggest that unlike other cell types, retinal endothelial cells do not downregulate GLUT1 as a compensatory mechanism in the presence of chronic hyperglycemia.13–15 These data also support observations in retinal endothelial cell culture models of unchanged glucose transport rates after 8 days of elevated glucose concentrations.16 The latter model, however, may not be entirely applicable to human diabetic retinopathy, because the risk and severity of retinopathy are dependent on long-term exposure to hyperglycemia.30 Recently, Badr et al.17 reported a 50% decrease in retinal microvascular GLUT1 after 2 or 8 weeks of streptozotocin-induced diabetes. The results in Badr et al. differ from those in the present experiments because of the different approach used to quantify GLUT1 (Western blot versus immunogold electron microscopy), as well as the shorter duration of diabetes (8 weeks versus 12 months). It is possible that changes that occur relatively early in the development of microvascular disease are not sustained in diabetes of longer duration; however, differences in the methods or experimental models of diabetes used (streptozotocin versus spontaneous diabetes in the GK rats) cannot be ruled out.

Several factors associated with the development and progression of diabetic retinopathy, such as hypoxia,7 VEGF,8 and insulin-like growth factor (IGF)-110 have been demonstrated to increase GLUT1 expression and/or glucose transport in primary retinal endothelial cell cultures. Busik et al.31 have recently reported an increase in nonzero transglucose uptake in human retinal endothelial cells without a change in endothelial cell GLUT1. They attribute this increased uptake to the activation of GLUT1 by elevated glucose concentrations.31

We have reported a localized increase in GLUT1 expression in the endothelial cells of the human BRB in retina specimens from individuals with diabetes of greater than 17 years’ duration and no clinical evidence of diabetic retinopathy.19 The findings of the present study differ from those in our previous report, in that no change in inner BRB GLUT1 density was observed in GK diabetic rats after approximately 1 year of diabetes (Fig. 4). The general method—immunogold electron microscopy—was the same for both studies; however, in the present study, cytosolic GLUT1 was not compared. Although cytosolic GLUT1 was clearly detectable in the rat retinal endothelial cells (data not shown), the present study concentrated on quantification of the abundance and distribution of immunoactive GLUT1 on the luminal and abluminal membranes to study changes in functional glucose transporters on the rat inner BRB. Because GLUT1 is a transmembrane transporter, it is active only when present on the plasma membrane surface. Therefore, changes in membrane-bound GLUT1 on the luminal and abluminal membranes of the endothelial cells are of the greatest relevance in determining whether changes in GLUT1 expression in diabetes correlate with changes in glucose transport into and through the inner BRB. The differences between changes in membrane-bound GLUT1 of the inner BRB of humans and GK rats with chronic hyperglycemia may be explained either in terms of duration of diabetes or species differences. Although the GK rat exhibits some changes similar to those in human diabetic retinopathy,21–24 it is also possible that there are differences between the GK rat model of diabetic retinopathy and the actual disease process in humans. Nonetheless, there is an absence of compensatory downregulation of GLUT1 expression in both the human18 and the GK rat inner BRB (this study), and the absence of this protective mechanism may expose the internal environment of the retinal endothelial cells to elevated free glucose concentrations and may therefore provide an explanation for the exquisite vulnerability of this cell type to chronic hyperglycemia.

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