Effect of High Glucose on Permeability of Retinal Capillary Endothelium In Vitro

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Purpose. To study the effect of high glucose on the permeability of bovine retinal capillary endothelial cell (BRCEC) monolayers.

Methods. The paracellular permeability of second-passage BRCEC cultured on millipore filters in two chamber transwell inserts was assayed by measuring the peak trans-monolayer electrical resistance and percent equilibration of 14C-inulin 48 hours after it had been added to the luminal chamber.

Results. High glucose increased the paracellular permeability of BRCEC monolayers independently of its hypertonic action (5 mM glucose: 154.2 ± 21.2 and 19.5 ± 2.4; 30 mM glucose: 134.2 ± 5.1 [P = 0.01] and 23.5 ± 2.1 [P = 0.01]; 5 mM glucose + 25 mM mannitol: 168.7 ± 13.7 ohm·cm² [P = 0.04] and 19.3% ± 1.2% 48-hour equilibration of inulin [P = 0.008]).

In a separate series of experiments, the authors were unable to show that either aminoguanidine or ponalrestat prevented the effect of high glucose on permeability (30 mM glucose 95.1 ± 16.7 and 45.4 ± 5.6; 5 mM glucose: 122.9 ± 14.2 [P = 0.02] and 75.3 ± 14.9 [P = 0.06]; 30 mM glucose + aminoguanidine 87.9 ± 17.5 [P = 0.4] and 75.3 ± 14.9 [P = 0.6]; 30 mM glucose + ponalrestat 79.9 ± 12.7 ohm·cm² [P = 0.1] and 48.2 ± 2.5% 48-hour equilibration of inulin [P = 0.15]). Ponalrestat did not abrogate the effect of high glucose despite its ability to reduce a high glucose-induced increase in BRCEC intracellular sorbitol levels.

Conclusions. The data are consistent with a role for increased paracellular permeability in breakdown of the blood–retinal barrier in diabetic retinopathy, which appears to be independent of both nonenzymatic glycosylation and the polyol pathway. Invest Ophthalmol Vis Sci. 1997; 38:635–642.
ments, including alterations in lipid metabolism as well as increased production of superoxide, and, possibly, nitric oxide.\textsuperscript{10} The same rodent model also has been used to show the ability of aminoguanidine to prevent the same diabetic retinal vascular leakage. Whether this effect is because of the prevention of the formation of advanced glycosylation end products, which are thought to have a number of detrimental effects, nitric oxide, or both, is uncertain.\textsuperscript{11,12}

In this article, we describe the effect of high glucose on the paracellular permeability of retinal capillary endothelial cell monolayers in vitro and the influence on this of inhibitors of both aldose reductase and the formation of advanced glycosylation end products. We have shown previously that this model, which comprises bovine retinal capillary endothelial cells (BRCECs) grown on polycarbonate filters, forms a barrier that is an order of magnitude "tighter" than that formed by cells derived from the peripheral circulation,\textsuperscript{13} and that it is responsive to cytokines and other mediators.\textsuperscript{14} The advantages of such a model are, first, that it allows examination of the endothelial cell in isolation and, second, that the mechanism of changes in permeability can be studied more easily and more precisely than is possible in in vivo studies. Increased paracellular permeability, which is the result of the formation of gaps between cells and is the subject of this article, is reflected by a reduction of the transmonolayer electrical resistance and increased permeability of inulin, which does not cross cell membranes.\textsuperscript{15}

\textbf{METHODS}

\textbf{Cell Culture}

The BRCEC were isolated as described previously for human tissue.\textsuperscript{16} Procurement of tissue and all experiments were conducted according to the ARVO statement for the use of animals in ophthalmic and vision research. Cells were grown on 60-mm tissue culture dishes coated with 100 \textmu g fibronectin (Trace Biosciences, Sydney, Australia) and 50-\textmu g collagen type IV (Collaborative Research Incorporated, Bedford, MA). The culture medium consisted of Dulbecco’s modified Eagle’s medium (Gibco, Grand island, NY) with 5.5 mM glucose supplemented with 15% pooled human platelet poor serum, 20 \textmu l/ml bovine retinal extract, 90 \textmu g/ml heparin, 0.2 \textmu g/ml insulin, 2.5 \textmu g/ml transferrin, 5 \textmu g/ml ascorbic acid, 100 IU/ml penicillin, and 100 \textmu g/ml streptomycin. Second-passage cells were used for all experiments.

\textbf{Preparation of Cell Monolayers}

Second-passage cells were cultured on 0.33-cm\textsuperscript{2} transwell polycarbonate filters (Costar, Cambridge, MA) with 3.0-\textmu m pores as described previously.\textsuperscript{13} Transwells were precoated with 35 \textmu l/well of 0.1% gelatin and then coated with 1 \textmu g of collagen type IV, 1 \textmu g laminin, and 1.5 \textmu g fibronectin at room temperature for 3 hours. Cells were seeded at approximately 3 \times 10\textsuperscript{4} cells per transwell or 1 \times 10\textsuperscript{5} cells per cm\textsuperscript{2} and cultured for 2 weeks. The culture medium was replaced after the first 24 hours, then every second day, and 150 \textmu l was added to the luminal chamber and 700 \textmu l to the abluminal chamber.

\textbf{Exposure to Factors}

In the first experiment where the effect of glucose was compared to that of mannitol, cells were introduced to high-glucose medium after 5 days in culture in the wells. In the second series of experiments, cells exposed to high glucose received it from the first day in culture, and ponalrestat (statil) or aminoguanidine also was added on day 1.

\textbf{Electrical Resistance}

Electrical resistance of cell monolayers in the transwells was measured with a Millicell Electrical Resistance System electrical resistance meter (Millipore, Bedford, MA) and calculated as described previously.\textsuperscript{17} The results were expressed as ohm \cdot cm\textsuperscript{2}. The average resistance of coated filters (without cells) was subtracted from the resistance of filters with cells. Although the electrical resistances of the different groups might have been compared at any point during the experiment, it was decided prospectively that the differences between groups would be analyzed when the cells grown in 5 mM glucose had attained their "peak resistance," because this would represent the closest approximation of the blood–retinal barrier in vivo. In studies in which serial measurements are made on respective groups, it may be more appropriate to analyze the maximum values achieved rather than to compare groups at timepoints that are determined retrospectively after the graphs have been plotted.\textsuperscript{17}

At the end of some experiments, cells were removed from the filters by exposure to 0.02 N ammonium hydroxide for 30 minutes followed by washing five times with distilled water. This allowed the determination of the electrical resistance of basement membrane material, which had been secreted by the cells during their 2 weeks in culture. The small increase in resistance found in wells in which cells had been grown in high glucose only was approximately 1% of the total resistance and did not affect the overall significance of the results.

\textbf{Permeability of 14C-inulin}

\textsuperscript{14}C-inulin (Du Pont, Wilmington, DE), 0.05 \textmu Ci/well, was added to the luminal chamber of each well after cell monolayers had reached what was judged to be...
High Glucose and Retinal Capillary Permeability In Vitro

was calculated as:

\[
\% \text{ equilibrium} = \frac{\text{dpm of sample}}{\text{dpm of total equilibrium of each well}} \times 100\%
\]

Lactate and Pyruvate Measurement

Cells were cultured in six-well tissue culture dishes and treated with high glucose for 5 days, after which the various factors were added for 2 days while exposure to high glucose continued. The culture medium was collected and stored at -70°C. The cell number of each well was counted with a hemocytometer after the medium was collected. The lactate and pyruvate levels of the medium were measured by an enzymatic assay (Behring Diagnostics, Marburg, Germany) using a Cobas Bio analyzer (Roche Diagnostics, Basel, Switzerland).

Sorbitol and Fructose Measurement

Cells were grown in 75-cm² flasks because they could not be harvested from the wells in sufficient numbers for meaningful analysis. They were grown using the same medium with high glucose for 5 days, after which 10-μM ponalrestat was added for 2 days. The cell number in each flask was determined, and the cells were washed with cold phosphate-buffered saline three times, then stored at -70°C. Immediately before the assay, the cells were thawed and homogenized in 2 ml of cold water. Xylitol was added as an internal standard to each sample. The proteins were precipitated with ethanol (70% of final volume) and eliminated by centrifugation at 13,000 G for 15 minutes. The supernatants then were lyophilized. To obtain derivatives of sugars and polyols, 100 μl of pyridine and 100 μl of N-trimethylsilylimidazole (Alltech, Sydney, Australia) were added to the lyophilized sample and incubated at 75°C for 15 minutes. The trimethylsilylimidazole derivatives were analyzed by gas liquid chromatography mass spectrometry.

Statistical Analysis

Data are presented as mean ± standard deviation. Each experiment included at least four, and usually six, wells per group. The principal findings were confirmed in at least two duplicate experiments, except for the determination of intracellular polyol levels, which represent pooled results from duplicate experiments, the results of which were similar.

For comparisons between groups, analysis of variance was first performed. Student’s t-test then was used to make those comparisons specified a priori by the experimental design, using the error mean square and its degrees of freedom from the analysis of variance. In all cases, this meant the comparison of all groups with only one other, either the 5-mM glucose group (Fig. 1) or the 30-mM glucose group (Fig. 2, Tables 1 and 2). No allowance of multiple comparisons was made because all experiments were interpreted together using the overall pattern of results.

RESULTS

High Glucose Reduced Electrical Resistance and Increased Inulin Permeability Independently of its Hypertonic Action

High glucose consistently reduced the electrical resistance of BRCEC cell monolayers. In the first experiment, the effect of varying concentrations of high glucose on electrical resistance and inulin permeability was compared with that of high mannitol, which was used to control for the hyperosmolar action of high glucose. Monolayers exposed to 15-, 30-, and 40-mM glucose all exhibited a similar reduction in their electrical resistance when compared to those exposed to 5-mM glucose (Fig. 1A). Cells grown in 40-mM glucose were affected earlier than were cells exposed to 30- and 15-mM glucose, but all three groups developed less electrical resistance compared with cells grown in 5-mM glucose. After 13 days in culture, which is when the cells grown in 5-mM glucose reached their peak resistance, analysis of variance showed there was a significant difference between the mean resistance for the four different doses of glucose (F3,8 = 6.89, P = 0.01), and inspection of the means showed that this was because of the mean for the cells grown in 5-mM glucose being significantly greater than that of the three higher dose groups, which were similar. Equivalent concentrations of mannitol, however, had only a small effect on the electrical resistance of BRCEC initially (Fig. 1B) and were not significantly different at day 13 (F3,8 = 1.12; P = 0.4). The results of the 14C-inulin permeability studies complemented the electrical resistance data (Fig. 1C). Cells grown in high glucose displayed significantly higher permeability than did cells grown in normal glucose (P = 0.01), whereas high mannitol had no effect (P = 0.8).

Effect of High Glucose and Ponalrestat on Activity of the Polyol Pathway

Fructose was not detected in cells grown in either 5- or 30-mM glucose. A small amount of sorbitol was detected in cells grown in 5-mM glucose, which was at the limit of detection of the assay despite the use of 2 × 10⁷ cells per group. A greater amount of sorbi-
FIGURE 1. Paracellular permeability of bovine retinal capillary endothelial cell (BRCEC) grown in varying concentrations of high glucose and high mannitol. (A) Electrical resistance versus days after seeding onto filters of BRCEC exposed to varying concentrations of glucose added as indicated; open circles = 5 mM; open squares = 15 mM; open triangles = 30 mM; closed circles = 40 mM glucose. (B) Electrical resistance of BRCEC exposed to 5 mM glucose plus varying concentrations of mannitol; open circles = 0 mM; open squares = 10 mM; open triangles = 25 mM; closed circles = 35 mM mannitol. (C) Forty-eight-hour inulin equilibration of BRCEC exposed to N (5 mM) glucose, high (30 mM) glucose, and high (25 mM) mannitol. For the sake of clarity, error bars (± standard deviation) in B are included only for cells grown in 5 mM glucose, which displayed the greatest variation.

tol was found in cells grown in 30-mM glucose, and this increase was reduced by treatment with ponalrestat (10 μM) (Table 1). Growth of the cells in either 30-mM glucose, ponalrestat, or both, did not have any significant effect on lactate-pyruvate ratios in BRCEC supernatants (Table 2). High glucose-induced increased paracellular permeability was not reversed by either ponalrestat or aminoguanidine.

Aminoguanidine (0.1, 0.5, and 5 mM) and ponalrestat (10 and 100 μM) were both consistently unable to prevent the high glucose-induced reduction in electrical resistance (Fig. 2A). In the experiment shown, cells exposed to 5-mM glucose had developed significantly greater electrical resistance 5 days after seeding onto filters than those grown in 30-mM glucose, but there was no significant difference when cells grown in 30-mM glucose were exposed to either aminoguanidine or ponalrestat (30-mM glucose 95.1 ± 16.7; 5-mM glucose: 122.9 ± 14.2 [P = 0.02]; 30-mM glucose + aminoguanidine 87.9 ± 17.5 [P = 0.4]; 30-mM glucose + ponalrestat 79.9 ± 12.7 ohm cm² [P = 0.1]). Studies of the permeability of ¹⁴C-inulin again reinforced the electrical resistance data (Fig. 2B). High glucose caused a significant increase in inulin permeability in untreated cells (P = 0.001), but adding either aminoguanidine (P = 0.6) or ponalrestat (P = 0.15) had no significant effect. Higher doses of aminoguanidine seemed, if anything, to aggravate the effect of high glucose on monolayer permeability.

DISCUSSION

The reduction in electrical resistance and increased inulin permeability of BRCEC monolayers induced by high glucose in this study suggests that breakdown of the capillary blood–retinal barrier in diabetes may be accomplished at least partly through the paracellular route. This effect of high glucose appears to be independent of nonenzymatic glycosylation of proteins or increased activity of the sorbitol pathway, despite ele-
High Glucose and Retinal Capillary Permeability In Vitro

FIGURE 2. Paracellular permeability of bovine retinal capillary endothelial cell monolayers grown in high glucose with aminoguanidine (0.5 mM) or ponalrestat (10 μM). Cells were exposed to high glucose and factors 24 hours after seeding. (A) Electrical resistance versus days after seeding onto filters of bovine retinal capillary endothelial cell; open circles, 5-mM glucose; closed circles, 30-mM glucose; open squares, 30-mM glucose plus aminoguanidine; open triangles, 30-mM glucose plus ponalrestat. (B) Forty-eight hour inulin equilibration of bovine retinal capillary endothelial cell monolayers exposed to 5-mM glucose (solid) or 30-mM glucose (plain).

vated sorbitol levels in BRCEC associated with high glucose, which were reversed largely by treatment with ponalrestat. Exposure to high glucose appeared to result in changes to the endothelial basement membrane that were prevented by treatment with aminoguanidine.

The increase in paracellular permeability associated with high-glucose treatment strongly suggests damage to interendothelial tight junctions. Electrical resistance, which reflects the permeability of a barrier to electrolytes, has been correlated with the amount and complexity of tight junctions between epithelial cells20,21 and is accepted as a sensitive indication of the barrier function of brain capillary endothelial cells in vitro.15 The gradual development of increased electrical resistance over several days by cell monolayers in vitro after they have reached confluence, which was seen in this study, has been observed previously and is thought to reflect the maturation of intercellular junctional complexes.20 Peripheral vascular endothelium in vitro or in vivo does not develop transmonolayer electrical resistances any where near the level found in this study for BRCEC13 and would not constitute a valid model with which to study glucose-induced breakdown of the blood–retinal barrier.

The study of the permeability of retinal capillary endothelial cell monolayers in vitro may yield new insights into diabetic retinopathy as long as its limitations are understood. Despite some variation in the time and magnitude of the peak electrical resistance achieved by cells, as well as the permeability of inulin, from one experiment to the next, all groups acted similarly in each experiment and permeability was re-

TABLE 1. Intracellular Fructose and Sorbitol of BRCEC

<table>
<thead>
<tr>
<th>Culture Conditions</th>
<th>Fructose</th>
<th>Sorbitol</th>
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<tbody>
<tr>
<td>5 mM glucose</td>
<td>ND</td>
<td>2.2 ± 1.9*</td>
</tr>
<tr>
<td>5 mM glucose + ponalrestat</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>30 mM glucose</td>
<td>ND</td>
<td>21.2 ± 2.0†</td>
</tr>
<tr>
<td>30 mM glucose + ponalrestat</td>
<td>ND</td>
<td>5.4 ± 1.3†</td>
</tr>
</tbody>
</table>

ND = not detected.
* P = 0.001.
† P = 0.001.

Units are nmol/10⁷ cells.
BRCEC = bovine retinal capillary endothelial cells.

TABLE 2. Lactate/Pyruvate in BRCEC Culture Medium (X10⁻³)

<table>
<thead>
<tr>
<th>Culture Conditions</th>
<th>Lactate</th>
<th>Pyruvate</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 mM Glucose</td>
<td>9.1 ± 1.2*</td>
<td></td>
</tr>
<tr>
<td>5 mM Glucose + ponalrestat</td>
<td>8.1 ± 0.3</td>
<td></td>
</tr>
<tr>
<td>30 mM Glucose</td>
<td>7.9 ± 0.1†</td>
<td></td>
</tr>
<tr>
<td>30 mM Glucose + ponalrestat</td>
<td>7.6 ± 0.2†</td>
<td></td>
</tr>
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</table>

* P = 0.1.
† P = 0.7.

BRCEC = bovine retinal capillary endothelial cells.
duced consistently by high glucose. Although factors of undoubted importance, such as abnormal blood rheology and the influence of neighboring cell types, are excluded by this model, this also may be an advantage if one wants to examine the contribution of the endothelium alone. The potential exists for the model to be expanded in a controlled fashion to include conditioned medium and to direct coculture with other cells to explore these processes in more detail than could be achieved with in vivo studies.

The clear and consistent increase in the paracellular permeability of BRCEC monolayers caused by high glucose was not affected by aminoguanidine or ponalrestat in this study. Because these drugs would be expected at least to attenuate the detrimental effects of advanced glycosylation end products and to increase activity of the sorbitol pathway, this suggests that increased paracellular permeability across the capillary blood–retinal barrier may be because of an effect of high glucose, which is independent of these two mechanisms.

Our data show that there are small but significant levels of sorbitol in BRCEC. Despite earlier work, where aldose reductase activity was not detected in retinal capillary endothelium using immunohistochemical techniques, sorbitol has been detected previously in BRCEC using a fluorescent enzymatic assay. The levels we detected in both 5- and 30-mM glucose are similar to those recorded in aortic smooth muscle cells and substantially less than those reported for pericytes in vitro. The attenuation of the increased intracellular sorbitol caused by high glucose by treatment with ponalrestat strongly suggests that aldose reductase is present in BRCEC. The supernatants of these cells in high glucose did not, however, show increased lactate–pyruvate ratios, which would be expected with significant activity of the polyol pathway, as would the intracellular accumulation of fructose, which we did not detect in significant quantities.

It is possible that a detrimental effect of the polyol pathway, or nonenzymatic glycosylation, is exerted through mechanisms that are excluded by our model. These include increased transcellular transport, an affect on neighboring cell types, such as pericytes, which do express much higher aldose reductase activity, or rheologic factors, such as the increased clotting tendency found in patients with diabetes. Although the supernatants of RPE cells exposed to high glucose in vitro did not exhibit increased lactate–pyruvate ratios either, elevated ratios have been detected in retinal explants, and these could be normalized by an aldose reductase inhibitor. The influence of an external factor does not explain, however, the results of the current study in which a detrimental effect of high glucose has been shown on BRCEC alone. The rodent model used by Williamson, Tilton, and their colleagues, which has been used to study high glucose-induced retinal extravasation of albumin in vivo, does not identify how the leak occurs, that is, what cells are affected or what route the leak takes (transcellular or paracellular, inner or outer blood–retinal barrier). Moreover, it reflects clearance across the vascular endothelium rather than permeability alone, the former being influenced by blood flow. Although there is strong evidence from some of the field’s foremost investigators that aldose reductase inhibitors inhibit high glucose-induced leaks from retinal vessels in rodent retinae, this was not confirmed by another group, and the issue of whether these drugs can prevent diabetic retinopathy in animal models or humans remains controversial.

We can only speculate on how high glucose has increased the paracellular permeability of our system. Ultimately, there must be changes in intercellular tight junctions, which are the anatomic correlate of the blood–retinal barrier, resulting in the formation of gaps between cells or, possibly, in reduced complexity of the junctions without gap formation. Intracellular mechanisms that might control this process and that could be affected by high glucose include changes in intracellular cyclic adenosine monophosphate as well as phosphorylation of adherens junction-associated proteins. High glucose augments autacoid-induced stimulation of Ca entry into vascular endothelial cells, associated with increased formation of inositol polyphosphates, which may increase paracellular permeability.

Further studies will concentrate on the effect in our model of other proposed biochemical mechanisms of high-glucose-induced damage. These include increased activity of protein kinase C, increased production of nitric oxide, and damage from oxygen-free radicals, of which the last two may be synergistic.

Key Words
aldose reductase, blood–retinal barrier, diabetic retinopathy, retinal cell culture, tight junction

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