Cultured Retinal Pigment Epithelium Cells From Donors With Type I Diabetes Show an Altered Insulin Response

Michael V. Miceli*† and David A. Newsome‡

Retinal pigment epithelium (RPE) isolated from 13 of 49 eyes of donors with insulin-dependent diabetes produced viable, proliferating cultures. No difference was found in baseline glucose uptake and lactate production, as measured by $^{13}$C and $^1$H nuclear magnetic resonance, between the RPE cells from diabetic donors and those cultured from normal donors. Insulin-mediated stimulation of glucose uptake and lactate production was decreased significantly in the RPE cells from diabetic donors compared with those from normal controls. Oxygen consumption and the percentage of endogenous oxygen consumption from fatty-acid oxidation, determined by using a specific inhibitor, were similar in both groups. Cellular proliferation and endocytosis, measured by liposome uptake, also were stimulated by insulin similarly in both types of cells. These results show that at least one or more mechanisms of insulin action in the RPE, thought to be a noninsulin-dependent tissue, may be altered permanently by chronic insulin-dependent diabetes. This finding may have implications for the pathogenesis of disease in noninsulin-dependent tissues even in patients with tight glycemic control. Invest Ophthalmol Vis Sci 32:2847-2853, 1991

Complications resulting from diabetes mellitus vary and can affect all major organ systems. Retinal complications include increased permeability of the retinal microvessels,† neovascularization,‡ alterations in the interphotoreceptor matrix and basement membranes,§ and morphologic and biochemical changes of the retinal pigment epithelium (RPE).¶ The causes of the widespread effects of diabetes on organs and tissues are controversial. Proposed mechanisms include sorbitol-induced osmotic stress,∥ the inositol phosphate–protein kinase C hypothesis,** and the effects of protein glycosylation end products on enzyme systems.*** It also was suggested that growth factors, including insulin itself, may contribute to vessel wall thickening and end-stage proliferative disease.**** No proposed single theory explains all the observed effects. The mechanism of action of insulin is also controversial; it probably works through more than one mechanism.*****

We were interested in the effect of disease states on the metabolism of the RPE. The RPE plays a central role in the vision process, and conditions impairing normal RPE function can impair vision. If hyperglycemia or conditions associated with diabetes, such as hyperinsulinemia, result in altered phenotypic expression or other permanent changes in the RPE, such changes might be detected in the cultured cells. We cultured RPE cell populations from donor eyes and studied their growth characteristics and metabolism using nuclear magnetic spectroscopic and biochemical methods. These results showed that the acute effects of insulin on the stimulation of energy metabolism is altered selectively in RPE cells isolated from donors with type I diabetes compared with those isolated from normal human donors.

Materials and Methods

Donor Eyes

The RPE cells were isolated from whole globes or posterior poles less than 36 hr postmortem.**** Most of
the diabetic tissue was obtained from the National Disease Research Interchange (Philadelphia, PA). Based on the ocular and medical histories supplied, we classified eyes as from donors with insulin-dependent diabetes (IDDM) or IDDM with diabetic retinopathy (DR).

Donors classified as IDDM were affected for a minimum of 5 yr, and those classified as DR had visually detectable signs of retinopathy seen through a dissecting stereo microscope. In cases where the duration of IDDM was not known from donor records, evidence of DR had to be visible during the dissection for the data to be used.

Cell Culture

After the vitreous and the sensory retina were removed, the RPE cells were harvested by incubating the whole eyecup with 2.4% Dispase (Boehringer Mannheim, Indianapolis, IN) in calcium- and magnesium-free Hanks balanced salt solution (HBSS), containing 100 mM sorbitol. Freshly isolated cells were plated in Coon's modified Ham's F12, containing fetal bovine serum (FBS, 20%; Hyclone, Logan, UT) supplemented with penicillin (100 units/ml; Gibco, Grand Island, NY), streptomycin (100 Mg/ml; Gibco), glutamine (2.0 mM; Gibco), and ascorbic acid (0.25 mM; Gibco). After the cultures were established, the cells were expanded in this same medium with 5% FBS instead of 20% FBS. The cells released by exposure to 0.25% trypsin were pasaged in a standard fashion, using a 1:3 split. The fourth and fifth passage cells were grown in roller bottles. All cells used for metabolism experiments were fifth passage cells. All cell cultures used had an epithelioid morphology as judged by phase-contrast microscopy. Cell morphology was documented using a Nikon Diaphot inverted phase-contrast microscope equipped with a Nikon F-10 (Tokyo, Japan) camera. The cells were free from Mycoplasma contamination as determined by periodic assay (Mycoplasma Test Kit; Gen-Probe, San Diego, CA). Unless otherwise specified, all chemicals and reagents were from Sigma (St. Louis, MO). The data were analyzed statistically with Student's t-test done on a Compaq 386S (Houston, TX) computer using a statistical package (PC SAS; SAS Institute, Inc., Cary, NC).

Growth Curves

Fifth-passage cells were plated into 24-well plates in 1.0-ml growth media at a density of 5000–10,000 cells/well. Triplicate wells were counted every day for 7 days with media changes on days 3 and 6. Growth curves were sigmoidal, and doubling times were calculated from the log phase of growth.

Cell Perfusion

Glycolytic flux was measured on perfused cells trapped in agarose threads using a previously reported method. Briefly, the cells from six confluent roller bottles, day 1 postfeeding, were trypsinized, collected by centrifugation, counted, and mixed at 37°C with one-third volume HBSS containing 25 mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid) and 1.8% low gelling-temperature agarose. The cells were cast in 0.5-mm gel threads by extruding the liquid cell-gel mixture through 0.5-mm Teflon tubing (Pierce Chemical Co., Rockford, IL) which passed through an ice bath. The cell threads were collected directly into a 20-mm tube containing growth medium. The tube was fitted with an insert, and growth medium was perfused past the loosely packed threads at 1.5 ml/min. The perfusions were done under sterile conditions at 37°C.

After the cells were cast, they were perfused with growth medium at 37°C for 1 hr and with a modified Krebs-Ringer's bicarbonate buffer containing 135 mM NaCl, 5.0 mM NaHCO3, 1.2 mM MgCl2, 1.0 mM CaCl2, 4.5 mM KCl, 1.0 mM KH2PO4, 25 mM HEPES, pH 7.4, and 5 mM l-13C-glucose (Cambridge Isotope, Woburn, MA) in a total volume of 25 ml. The osmolarity of this solution was 305 mOsm, as measured by freezing-point depression. The length of the perfusion depended on the number of cells in the experiment and typically was 2 hr. After perfusion with labeled glucose, the perfusate was collected for quantitation of glucose and lactate and proton spectroscopy. In experiments with insulin, the same cells were perfused again with Krebs buffer containing 5 mM labeled glucose with 0.4 μg/ml bovine insulin.

Glycolytic Flux Measurements

The perfusates were lyophilized and 13C nuclear magnetic resonance spectra were obtained to quantify 13C metabolites. Spectra were obtained on an IBM NR200AF spectrometer (IBM Instruments, Inc., Rockford, IL) using a 10-mm sample tube. Spectrometer conditions were selected to give quantitative spectra. This included a 60° pulse, a 10-sec recycle delay, and inverse-gated decoupling for suppression of nuclear Overhauser enhancement. The 13C-glucose remaining in the perfusate was measured directly using a toluidine assay (Diagnostic Kit #635; Sigma). The 13C-lactate concentration then was determined by a ratio of areas to the glucose peak. The total lactate produced and an estimation of the hexose monophosphate shunt activity were determined by the method of Willis et al.

Proton spectra were obtained using a 5-mm magnetic resonance spectroscopy tube, a 45° pulse, and a
2-sec recycle time. The terminal methyl protons of the lactate were centered at 1.34 ppm with the accompanying 13C satellites located at approximately 1.66 and 1.02 ppm. The first-approximation hexose monophosphate shunt (HMPS) flux was calculated from the formula:

\[ A = \frac{(3 - 3r)}{(3 + 2r)} \]

where \( A \) is the percentage of glucose entering the HMPS and \( r \) is the ratio of 13C- to 12C-lactate measured from the proton spectrum.

At the end of the perfusions, the cell threads were washed with Krebs buffer and the cells collected for determination of total Lowry protein with bovine serum albumin as a standard. The color contributed to the protein assay by the HEPES was subtracted before the total proteins were calculated.

### Oxygen Consumption Measurements

Oxygen consumptions were measured at 37°C using a YSI oxygen monitor (Yellow Springs Instruments, Yellow Springs, OH) and a Clark-type polarographic electrode. Cells from one roller bottle (of identical passage and feeding history as the cells used in each perfusion experiment) were harvested on the same day as the perfusion experiment, counted, and washed twice with Krebs buffer containing no glucose. Endogenous oxygen uptake was measured in 3.0 ml of Krebs buffer without added substrate. After an initial rate was determined, 5 µl of a 25 mM solution of the inhibitor 2-tetradecyloxiranecarboxylic acid (McN3802; RW Johnson, Spring House, PA) in 100% ethanol was added. The McN3802 is an active site-directed irreversible inhibitor of carnitine palmitoyl transferase 1 that specifically inhibits long-chain fatty-acid oxidation. KCN (1 mM) inhibited O2 consumption by greater than 95%, indicating that the major end product (as expected) was lactate both mitochondrial and cytoplasmic. The cells were collected after the experiment for Lowry protein determinations and typically contained 2-3 mg of protein. The oxygen content of the Krebs solution was taken as 406 nmoles O2/ml.

### Liposome Uptake

Endocytosis was determined by measuring the increase of cell-associated radioactivity using liposomes made from dipalmitoyl phosphatidyl choline–dipalmitoyl phosphatidyl serine (Avanti Polar Lipids, Pelham, TN) and containing 1H-cholesteryhexadecyl ether (New England Nuclear Dupont, Wilmington, DE) as a nondegradable, nontransportable radioactive lipid marker. This system was well characterized and measures internalization of the radioactive liposome. The liposomes were made by the reverse-phase evaporation method and filtered through a 0.22-µm filter. The cells were grown in 12-well plates until just confluent. Uptake was measured in triplicate wells at 37°C in Krebs buffer containing 10 mM glucose with or without 0.4 µg/ml added insulin. The cells were incubated for 4 or 8 hr. After incubation, the cells were washed five times with ice-cold phosphate-buffered saline to remove bound but not internalized liposomes. The cells were dissolved in 500 µl of 0.5 N NaOH, and aliquots were removed to measure radioactivity and protein.

### Results

Cells were isolated from 49 diabetic globes (19 IDDM and 30 DR) and useful cultures obtained from 13 (three IDDM and ten DR). Useful cultured cells appeared 100% epithelioid in the first passage and maintained more than 80% epithelioid morphology through five passages. The 27% success rate was lower than usual in our laboratory for nondiabetic tissue (typically 40%). Because of the scarcity of the diabetic tissue, we used starting material up to 36 hr postmortem, a possible factor in the low success rate. However, a more likely cause was the average age of the diabetic donors, 63.7 ± 14.3 years (standard deviation). Successful cultures were produced from donors with an average age of 52.5 years; donors producing unsuccessful cultures had an average age of 66.9 years. The average age of the control donors used for comparison was 58.7 years. Average doubling times for the diabetic RPE populations was 2.2 ± 0.6 days for the diabetic cells and 2.7 ± 0.5 days for the normal cells (P > 0.05). Eight of the eyes had received laser treatment. Two produced successful cultures, and six did not, indicating that laser treatment did not seem to affect the rate of success. For our study, all diabetic cell populations were grouped together whether the donor eyes had retinopathy or not.

When we used 1-13C-glucose as the sole substrate, the major end product (as expected) was lactate both for normal and diabetic RPE cells (Fig. 1). The average glucose uptake–lactate production from these cells was 10.8 ± 1.1 and 19.3 ± 3.2 nmol min/mg protein (Table 1), almost identical to that of normal cells reported previously. Oxygen consumption in the diabetic RPE cells was also the same as in the normal RPE cells (Table 2). The HPMS activity was not significantly different between normal and diabetic RPE cells and averaged 13.4 ± 1.1%. As a result of the high endogenous oxygen consumption and the observation that most of the glucose used was not oxidized, the contribution of fatty-acid oxidation to the energy needs of the cells was estimated. To accomplish this, we used the com-
Insulin stimulated glucose uptake and lactate production in both normal and diabetic cells (Fig. 1, Table 1). In diabetic cells, insulin increased average glucose uptake just 5% and average lactate production, 12%. By contrast, in normal cells, insulin stimulated glucose uptake by 18% and lactate by 22%. These differences were significant at the \( P < 0.05 \) level. Dose-response experiments indicated that the maximum stimulation of glucose uptake occurred between 1.0 and 10 ng/ml of insulin.

Because the effects of insulin were assumed to be related to insulin's action as a growth factor, we reasoned that the physiologic response of insulin on other growth parameters also would be altered in diabetic cells. Insulin stimulated cell proliferation by a modest amount in all actively growing cultures. Surprisingly, this stimulation was not different for cells isolated from diabetic or normal donors (Fig. 3). Insulin also increased uptake of radioactive liposomes in 13 of 16 cultures tested, with an average increase of 16% (significant at the \( P < 0.02 \) level). Again, when comparing diabetic and normal cells, no significant difference was seen.

**Discussion**

The RPE seems to be affected in several ways by the hyperglycemic state, and morphologic changes in the RPE may be one of the first manifestations of disease in the eye. We report the first direct evidence that some metabolic effects mediated by IDDM in insulin-independent tissues are manifest long after removal of the cells from their hyperglycemic environment. The fact that some diabetic patients with tightly controlled blood glucose concentrations still exhibit progressive

<table>
<thead>
<tr>
<th>Glucose uptake (nmoles/min/mg)*</th>
<th>Lactate production (nmoles/min/mg)*</th>
<th>Glucose uptake plus insulin (nmoles/min/mg)*</th>
<th>Lactate production plus insulin (nmoles/min/mg)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diabetic (n = 12)</td>
<td>10.8 ± 1.1</td>
<td>19.3 ± 3.2</td>
<td>11.4 ± 1.0†</td>
</tr>
<tr>
<td>Normal (n = 9)</td>
<td>12.1 ± 2.4</td>
<td>20.6 ± 3.7</td>
<td>14.2 ± 2.1†</td>
</tr>
</tbody>
</table>

\( N \) values represent single experiments with separate populations of cells. * Values are averages ± SEM. † \( P < 0.05 \).
Table 2. Oxygen consumption in cultured normal human and diabetic RPE cells and the effect of inhibition of fatty acid oxidation

<table>
<thead>
<tr>
<th></th>
<th>O₂ consumption &lt;br&gt;endogenous (nmoles O/min/mg)*</th>
<th>O₂ consumption &lt;br&gt;plus McN3802 (nmoles O/min/mg)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diabetic</td>
<td>18.58 ± 0.95†</td>
<td>12.47 ± 0.83†</td>
</tr>
<tr>
<td></td>
<td>(n = 24)†</td>
<td>(n = 19)‡</td>
</tr>
<tr>
<td>Normal</td>
<td>19.41 ± 1.45†</td>
<td>13.27 ± 1.52†</td>
</tr>
<tr>
<td></td>
<td>(n = 9)</td>
<td>(n = 9)</td>
</tr>
</tbody>
</table>

* Values are averages ± SEM.
† Diabetic vs. normal: not statistically significant.
‡ N values represent 12 cell populations with duplicate determinations.
§ N values represent 12 cell populations with duplicate determinations in some cases.

Baseline measurements of glucose uptake on normal and diabetic cells were not distinguishable from those previously reported by us for normal RPE cells. The RPE cells in culture have high rates of glucose uptake and oxygen consumption with most of the glucose metabolized to lactate rather than oxidized to carbon dioxide. The use of a specific inhibitor of long-chain fatty-acid oxidation confirmed that a significant percentage of the endogenous oxygen consumption in RPE is from fatty-acid oxidation in both normal and diabetic cells. This percentage was not changed in the presence of 10 mM glucose, indicating that fatty acids are an important fuel source, even in the presence of glucose. This is not surprising because the RPE must metabolize the substantial amount of lipid ingested in spent rod and cone outer segments.

The results showing the effects of insulin are interesting and somewhat surprising. In cultured cells considered to be insulin dependent, insulin can stimulate glucose uptake by a factor of two. The stimulation of endocytosis by insulin is consistent with the observations of others that short-term decreases in cyclic adenosine monophosphate (cAMP) are associated with increases in phagocytosis in cultured rat RPE. One known effect of insulin is to decrease intracellular cAMP, and this should stimulate phagocytosis. No difference was found in phagocytic function between diabetic and normal cells; however, the effects of hyperglycemia on this function should be investigated. Our growth curves showed a smaller stimulation of proliferation by insulin than previously reported for RPE cells in culture. This may be a result of insulin already being present in the growth medium from the 5% FBS (average, 0.01 ng/ml).

One factor that may have affected the results of the glucose uptake was that these measurements were done on trypsinized cells. Trypsinization can increase or decrease the rate of glucose uptake in insulin-dependent tissues depending on the degree of enzyme treatment. It is possible that insulin stimulation may be different in cells that have not been trypsinized. Two findings that argue against this are that the stimulation of liposome uptake and cell proliferation done in nontrypsinized cells are similar in magnitude to the stimulation of glucose uptake. Furthermore, because both normal and diabetic cells were treated the same, the depressed stimulation of diabetic cells was not an artifact of harvesting. If the rate at which the insulin and argue that the effects of insulin are mediated through insulin receptors and not by insulin-like growth factor receptors as has been shown in other cell types. However, specific insulin receptors have not been found on RPE cells as of this writing.

The stimulation of endocytosis by insulin is consistent with the observations of others that short-term decreases in cyclic adenosine monophosphate (cAMP) are associated with increases in phagocytosis in cultured rat RPE. One known effect of insulin is to decrease intracellular cAMP, and this should stimulate phagocytosis. No difference was found in phagocytic function between diabetic and normal cells; however, the effects of hyperglycemia on this function should be investigated. Our growth curves showed a smaller stimulation of proliferation by insulin than previously reported for RPE cells in culture. This may be a result of insulin already being present in the growth medium from the 5% FBS (average, 0.01 ng/ml).

One factor that may have affected the results of the glucose uptake was that these measurements were done on trypsinized cells. Trypsinization can increase or decrease the rate of glucose uptake in insulin-dependent tissues depending on the degree of enzyme treatment. It is possible that insulin stimulation may be different in cells that have not been trypsinized. Two findings that argue against this are that the stimulation of liposome uptake and cell proliferation done in nontrypsinized cells are similar in magnitude to the stimulation of glucose uptake. Furthermore, because both normal and diabetic cells were treated the same, the depressed stimulation of diabetic cells was not an artifact of harvesting. If the rate at which the insulin

---

No. 11

RPE CELLS IN DIABETES / Miceli and Newsome

2851
Fig. 3. Stimulation of cell-associated radioactive liposomes and cellular proliferation by insulin. Liposomes were prepared as described in Materials and Methods. Insulin caused a 16% increase in uptake (significant at the \( P < 0.02 \) level). There was no significant difference between cells from normal and diabetic donors in liposome uptake. Cell proliferation was stimulated by 16%. There was no significant difference between normal and diabetic cells. N values represent separate populations of cells.

receptors are recycled to the surface is different between normal and diabetic cells, this dissimilarity may explain the differences seen in insulin stimulation of glucose uptake but not seen in phagocytosis or growth-curve experiments. Differences in internal trafficking between normal and downregulated insulin receptors were proposed to explain some of the effects of hyperinsulinemia in type II diabetes.\(^3\)\(^6\) Altered internal trafficking of receptors also may be important in long-term type I disease.

The finding of an altered insulin response in a non-insulin-dependent tissue also has implications for the general use of insulin therapy to treat diabetes. The goal of insulin therapy is to decrease hyperglycemia, and the effects of increasing serum insulin as insulin-dependent tissues become insulin resistant have unknown effects on tissues such as the RPE. This may be especially important in end-stage disease in the retina where hyperinsulinemia is implicated in the progression of vessel angiopathy and proliferative disease. Understanding all the mechanisms by which insulin acts on the cell is necessary to plan better treatment protocols and to determine the progression of diabetic disease. The effects of the hyperglycemic environment itself on RPE function and metabolism also warrant further investigation, especially those related to phagocytosis and lipid metabolism.

Key words: retinal pigment epithelium, diabetes, insulin, energy metabolism, endocytosis

Acknowledgments

The authors thank Dr. Michael R. Niesman for making the liposomes and his assistance with the uptake studies, the National Disease Research Interchange and the Southern Eye Bank for their help in obtaining the eye tissue, the nuclear magnetic resonance facility of the Department of Chemistry of Tulane University for providing equipment and assistance in the experiments, and Dr. G. Tutweiler and The R.W. Johnson Pharmaceutical Research Institute for the gift of the inhibitor McN3802.

References


