Energy Substrate Requirements of Rat Retinal Pigmented Epithelial Cells in Culture: Relative Importance of Glucose, Amino Acids, and Monocarboxylates

John P. M. Wood,1 Gly Chidlow,1 Mark Graham,1,2 and Neville N. Osborne1

PURPOSE. To determine the metabolic conditions that provide maintenance of viability for cultured rat RPE cells and to determine whether monocarboxylates such as lactate or pyruvate, which are known to exist at high concentrations in the subretinal space, can provide an alternative energy source to maintain cells when other nutritive supplies are limited.

METHODS. Cultured rat RPE cells (passage 2–4), in the absence of serum, were subjected to different metabolic challenges relating to glucose, amino acid, or oxygen deprivation. Lactate or pyruvate was added to some cells in each instance to determine whether cells could be maintained by using these substances as fuel sources for metabolic reactions. Cell viability was assessed after treatments, and in some cases proliferation rates and appearance of apoptosis-like DNA cleavage were also investigated by terminal deoxynucleotidyl-transferase-UTP-linked nick-end labeling (TUNEL). Western blot analysis was used to determine the expression of transporters for glucose and monocarboxylates in these cells.

RESULTS. RPE cell viability was partially reduced in the absence of glucose or with glycolytic inhibition. Lactate or pyruvate did not prevent these reductions. Inhibition of transaminase reactions with aminoxyacetic acid (AOAA) in the absence of glucose caused a complete loss of viability that was reversed by pyruvate or lactate. MCT inhibition was detrimental to RPE cell viability only at high concentrations (500 μM) in the presence of glucose but blocked the protective effect of pyruvate-lactate in the presence of AOAA at 1 μM.

CONCLUSIONS. Rat RPE cells require glucose as their primary metabolic substrate in culture, but can metabolize glutamine in its absence. When glucose and glutamine are limiting, RPE cells can metabolize monocarboxylates such as lactate or pyruvate. These data provide evidence that such cells are able to withstand various types of insult brought about by nutrient deprivation, by altering their pathways of energy production. (Invest Ophthalmol Vis Sci. 2004;45:1272–1280) DOI:10.1167/iovs.03-0693

The retinal pigmented epithelium (RPE) forms a monolayer of tightly linked cuboidal cells that juxtapose the posterior face of the retina.1–3 The apical surface of the RPE is separated from the neural retina by the subretinal space, and it is across this space that these cells mediate the transport of metabolic intermediates, waste products, ions, and fluid components between the chorio-capillaries and the retina and vice versa. For this purpose, the RPE expresses a variety of different transporter proteins on both its basal side, adjacent to Bruch’s membrane and the porous chorio-capillaries, and on its apical surface, in close opposition to the retinal photoreceptors.5 The combined movement of ions and fluid components between the retina and the RPE aids retinal attachment.2 Because the RPE forms the outer blood–retinal barrier and is essential for removal of waste products and entry of nutrients into the retina, any disturbance in normal physiological functioning of these cells necessarily has detrimental consequences for the retina. It is well documented that defects in RPE functioning may underlie various sight-threatening conditions, such as diabetic retinopathy, age-related macular degeneration, and proliferative vitreoretinopathy.7–5

The retina is known to produce high quantities of the glycolytic end product, lactate.5,7 Indeed, the concentration of lactate in the subretinal space is approximately 7 to 13 mM.8 Studies performed with retinal preparations in vitro have shown that Müller cells convert a large fraction of the glucose they take up from the blood supply to lactate, rather than oxidizing it through the Krebs’ cycle.7,9,10 Much evidence also exists to demonstrate that on release from the Müller cells, lactate is taken up by photoreceptors for use as a fuel in cellular energy production.9,10 This knowledge is bolstered by the fact that neurotransmission in the inner retina is predominantly fueled by glycolysis, whereas oxidative metabolism provides energy for outer retinal processes such as phototransduction.11 Indeed, lactate is the preferred Krebs’ cycle substrate for photoreceptors.12 This is also the case for other neurons during the neonatal period or when glucose availability is low—for example, during episodes of hypoglycemia, ischemia, or starvation or in ketosis.13 The phenomenon of glia-released lactate’s acting as a fuel for neuronal metabolism in the brain has been postulated and is termed the astrocyte-neuron lactate shuttle hypothesis (ANLSH).14,15 Measurements of lactate efflux into the vortex veins have indicated that most of this metabolite leaving the retina comes out through the RPE.16 This process has been shown to be activity dependent and to increase during dark adaptation.16

The facilitated transport of lactate and other monocarboxylates, including pyruvate, β-hydroxybutyrate, and acetoacetate, across the plasma membrane is mediated by a family of transporter proteins named the monocarboxylate transporters (MCTs).15 These transporters are saturable and proton coupled, to date, 14 distinct family members have been described.15,17 Movement of monocarboxylates by MCTs is stimulated solely by concentration gradients of both these compounds and by protons. No additional energy source appears to be required. Recent immunohistochemical studies have shown that MCTs are expressed by both retinal and RPE cells in vivo.18–21 MCT1, 2, and 4 are present in the retina,20,21 and MCT1 and -3 are expressed in RPE cells in vivo.18,19 The

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expression pattern of MCTs in the RPE is striking, with MCT3 confined to the basolateral membrane and MCT1 expressed on the apical surface. The presence of MCT4 has been demonstrated in very low amounts in the neonatal rat RPE by immunoblotting experiments, but the subcellular localization of this isoform remains unclear. It has been hypothesized that MCT1 is responsible for removal of lactate from the subretinal space and that MCT3 subsequently shuttles this monocarboxylate into the choroidal blood supply. Indeed, RPE preparations have been shown to transport lactate and protons. Suggs-tions that lactate movement across the RPE is accompanied by water flux supports the notion that MCTs are important in helping to maintain retinal attachment.

The exposure of the apical face of the RPE to high concentrations of lactate and the presence of MCTs on both surfaces of these cells indicate that this metabolite necessarily enters these cells. The question arises, therefore, as to whether lactate, under different circumstances, can be used as a substrate for energy production by the RPE. This question is due to the knowledge that lactate can be used by many cell types as a source of energy, particularly when glucose availability is low. The present series of investigations was performed therefore to determine what metabolic substrates are used by cultured rat RPE cells to maintain viability and also to determine specifically whether cultured RPE cells can preserve viability by metabolizing monocarboxylates such as lactate when added to the bathing medium, either under control conditions or when deprived of essential nutrients. Data obtained from these investigations should provide an insight into the susceptibility of RPE cells to nutrient deprivation and will provide information that may enable these cells and hence the neuroretina, to be protected from such insults, in vivo.

Materials and Methods

Culture of Rat Retinal Pigmented Epithelial Cells

Fetal bovine serum (FBS; European Community [EC] approved), Ham’s F-10 medium, glucose-pyruvate-lactate–free Dulbecco’s modified Eagle’s medium (DMEM), amphotericin B, sodium pyruvate, and trypsin (0.25% wt/vol) were from Invitrogen (Paisley, UK), and tissue culture flasks and 24-well plates were from Fahrenheit Laboratory Supplies (Northampton, UK). Polyornithine and fibronectin were from Collaborative Research (Bedford, MA). Poly-L-lysine (10 mg/mL), trypsin, and collagenase (pH 8.0), and the eyes were incubated in a shaking water bath at 37°C for 45 minutes. After incubation, BSS was equalized before the end of the appropriate treatment regimen, [3H]-thymidine was added (1 μCi/mL). At the end of the incubation, cells were washed twice with phosphate-buffered saline (PBS; 137 mM NaCl, 5.4 mM KCl, 1.28 mM NaH2PO4, 7 mM Na2HPO4, pH 7.4) and lysed with ice-cold 0.1 M NaOH. The amount of incorporated [3H]-thymidine was determined with liquid scintillation spectroscopy. Samples were taken from each well before counting radioactivity for protein determination, to equalize the radiolabel in each case.

Energy Substrates of Cultured Rat RPE Cells

The medium used for investigating the metabolic substrates required for maintenance of rat RPE cells in culture was glucose- and lactate-pyruvate–free DMEM. In all experiments, comparisons between FBS-containing and -free media were initially undertaken to determine the involvement of this nutrient additive in any system. Generally, though, FBS was left out of the medium in experimental situations because of its unknown constitution (e.g., unknown glucose and monocarboxylate concentrations). For glucose-dependence experiments, some cultures were incubated in glucose-free culture medium, some were treated with the glucose transport (GLUT1) inhibitor cytochalasin B (0.5 μM), and some were treated with the glycolytic inhibitor, iodoacetic acid (0.5 mM). To determine the importance of monocarboxylates, pyruvate or lactate was added or omitted in some cases and an inhibitor of monocarboxylate transport, α-cyano-4-hydroxycinnamate (4-CIN), was also used. Aminoxyacetic acid (AOAA) was used to inhibit transaminase reactions. Concentrations used for all compounds were as previously reported for the respective inhibitory activities. In some instances cultures were subjected to various periods of anoxia to inhibit aerobic metabolism, by placing them in a humid chamber at 37°C perfused with 95% N2-5% CO2 for 4 hours before sealing them for the appropriate amount of time, as reported previously.

Proliferation of RPE Cells

Cells used for proliferation studies were assessed to be approximately 70% confluent before treatment began, as described earlier. Six hours before the end of the appropriate treatment regimen, [3H]-thymidine was added (1 μCi/mL). At the end of the incubation, cells were washed twice with phosphate-buffered saline (PBS; 137 mM NaCl, 5.4 mM KCl, 1.28 mM NaH2PO4, 7 mM Na2HPO4, pH 7.4) and lysed with ice-cold 0.1 M NaOH. The amount of incorporated [3H]-thymidine was determined with liquid scintillation spectroscopy. Samples were taken from each well before counting radioactivity for protein determination, to equalize the radiolabel in each case.

Figure 1. Rat RPE cells in culture. (A) Cells at passage 3, as commonly used for the experiments outlined. The micrograph shows that cells lost their pigmentation, but retained a homogeneous appearance. (B) Cells labeled with anti-cytokeratin Ks 8.13 antibody, which is specific for RPE cells.
Cell Viability Studies

The assay used to assess cell viability as a measure of the potential for metabolic substrates to maintain RPE cells in culture was the 3(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay, modified from the method of Mosmann. This assay has been widely used in recent years to provide a quantitative assessment of cellular viability. It is generally accepted that MTT is reduced to form an insoluble, blue formazan product by accepting electrons from cellular reducing equivalents such as nicotinamide adenine dinucleotide phosphate (NADH), reduced nicotinamide adenine dinucleotide phosphate (NADPH), or succinate. It thus acts as a measure for both cytoplasmic and mitochondrial metabolic activity and hence is a useful means to determine whether metabolic substrates are used to provide respiratory energy. Studies in which MTT reduction in cells was assessed by microscope have determined that a percentage decrease in MTT reduction for a given group of cells tends to reflect a decrease in the ability of all cells present to reduce the dye (Wood P.M., unpublished observation, 2001). It does not reflect the complete loss of MTT-reducing capability in some cells, whereas others remain at control levels.

Briefly, cells were subjected to the appropriate treatments for the required times and then MTT was added to wells at a final concentration of 0.5 mg/mL for 1 hour further at 37°C. After this time, medium was removed from the cultures and reduced MTT (blue formazan product) was solubilized by adding 100 μL of dimethyl sulfoxide (DMSO) to each well. After plates were agitated for 15 minutes, the optical density of the solubilized formazan product in each well was measured using an automatic microplate reader (TiterTrek Plus M2S12, ICN Flow, Thame, UK) with a 570-nm test wavelength and a 690-nm reference wavelength.

Assessment of DNA Breakdown with the TUNEL Procedure

For the terminal deoxynucleotidyltransferase UTP-linked nick-end labeling (TUNEL) procedure, treated cells on coverslips were fixed in 4% paraformaldehyde for 20 minutes and then immersed in PBS containing 0.1% Triton X-100 (PBS-T). The labeling procedure was performed exactly as described previously. The labeled RPE cells on coverslips were washed in PBS, mounted on glass slides, and visualized with Nomarski optics. Some cells were treated after fixation but before TUNEL staining with DNase I (0.1 mg/mL) for 15 minutes at 37°C as a positive control.

Assessment of Transporter Expression in Cultured Rat RPE Cells by Immunoblot Analysis

Rat RPE cells (passage 3) were grown to confluence (approximately 2 × 10^6 cells), washed and harvested in PBS (35.5°C), and collected by centrifugation (80g for 8 minutes at 4°C). Cells were sonicated in buffer (20 mM Tris-HCl [pH 7.4], containing 2 mM EDTA, 0.5 mM EGTA, 1 mM dithiothreitol, 50 μg/mL leupeptin, 50 μg/mL aprotinin, 50 μg/mL pepstatin A, 50 μg/mL apronin and 0.1 mM phenylmethylsulfonyl fluoride). An equal volume of sample buffer (62.5 mM Tris-HCl [pH 7.4], containing 4% SDS, 10% glycerol, 10% β-mercaptoethanol, and 0.002% bromophenol blue) was added, and samples were incubated on ice for 30 minutes followed by 30 minutes of vigorous shaking. Electrophoresis of samples was performed as reported previously, using 10% polyacrylamide gels containing 0.1% SDS. Proteins were transferred to nitrocellulose and blots were stained as previously described for the presence of MCT1 and GLUT1, to demonstrate that the cells were able to take up monocarboxylates and glucose, respectively. The presence of actin was assessed in cell extracts as a positive control.

Immunocytochemistry

RPE cells grown to confluence on borosilicate glass coverslips were fixed in 4% paraformaldehyde for 10 minutes and then immersed in PBS-T. Labeling for the presence of MCT1 was achieved by incubating fixed cells overnight at 4°C with a chicken anti-rat MCT1 antibody (diluted 1:500 in PBS-T). After incubation with the primary antiserum, coverslips were washed in PBS-T and then incubated in PBS-T containing donkey anti-chicken conjugated to biotin (Vector Laboratories, Peterborough, UK: 1:100) and horse serum (1:100) for 30 minutes. Subsequently, to a further wash with PBS-T, immunoreactivity was visualized with a standard avidin-peroxidase detection kit (Vector Laboratories) with 0.1% (vol/vol) H2O2 and 0.1% (wt/vol) 3,3′-diaminobenzidine in PBS-T. After labeling, coverslips were mounted in PBS containing 1% glycerol and visualized by light microscopy.

Statistics

All experiments were performed with control samples in the same plates. Data were thus analyzed for significance using the Student’s paired t-test. In the main, data are expressed as the mean percentage of control value ± SEM.

Results

Rat RPE cells in culture formed a homogeneous population and were clearly labeled for RPE-specific cytokeratins at passage 3 (Fig. 1).

Glucose Deprivation Studies

Cultured rat RPE cells that were subjected to glucose deprivation exhibited a significantly reduced viability (68.9% ± 6.8% of the untreated control value) after 24 hours (Fig. 2A). This loss of MTT-reducing potential was not exacerbated up to 120 hours after glucose removal. There was no significant difference in the viability of cells incubated in medium containing 5 or 25 mM glucose up to 120 hours. The presence of 5 mM of either pyruvate or lactate in the medium did not prevent the reduction in viability of cells caused by glucose withdrawal (Fig. 2B).

The critical nature of altering glucose reserves in the medium is illustrated in Figure 3. After glucose deprivation for up to 24 hours, there was a small decrease in the viability of cells (approximately 30%; Figs. 2, 3). Reintroduction of 25 mM glucose to the medium after 24 hours of its deprivation initiated a process leading to death of the RPE cells (Fig. 3). By 72 hours after reintroduction of glucose to cells that had been deprived of the sugar for 24 hours, only 10.3% ± 2.3% of the control level of MTT-reducing potential could be detected in cultures (Fig. 3A). This process was characteristic of apoptosis, as defined by positive nuclear labeling by the TUNEL reaction (Fig. 3C).

Transport Inhibition

The glycolytic inhibitor IOA caused a dose-dependent reduction in viability of rat RPE cells over 24 hours, which was at its maximum at 6 μM (Fig. 4). The addition of monocarboxylates did not prevent the decreased viability. The glucose transport inhibitor 2-CrI, in the presence of glucose, also caused a dose-dependent decrease in cell viability after 24 hours or more (Table 1). The MCT inhibitor 4-CN had no effect on RPE cell viability at low concentrations but became slightly and significantly toxic when increased to 500 μM for 72 hours (Table 1).

Contribution of Hexose Monophosphate Shunt

Figure 5 illustrates the role of hexose monophosphate shunt reactions in maintaining RPE cell viability over 24 hours. The reduction in viability caused by blocking glycolysis in the presence of glucose with 10 μM IOA was not significantly enhanced by anoxia, which itself had no significant influence on viability after 24 hours. There was, however, an additive detrimental effect when combining anoxia for 24 hours with a...
medium containing no glucose. In such cultures, cell viability was almost completely lost. These data suggest that glucose removal has a greater detrimental effect than glycolytic blockade, which may indicate that this sugar is involved in additional cellular reactions.

**Transaminase Inhibition**

The pantransaminase inhibitor AOAA had no effect in the presence of glucose, but in the absence of the sugar, it dose dependently reduced cell viability to approximately 70% of the untreated value when glucose was absent. In comparison, there was no significant difference between cells incubated in either 5 or 25 mM glucose. The reduction of viability after 24 hours’ incubation in medium without glucose was not counteracted by 5 mM pyruvate or 5 mM lactate. *P < 0.05* compared with appropriate control cells untreated for the same period (i.e., medium not changed for the experiment although still containing 25 mM glucose by paired Student’s *t*-test analysis; *n = 12*).

**Proliferation Studies**

Incubation of nonconfluent cells in medium without serum, glucose, and monocarboxylates for 24 hours led to an incorporation of 0.52 picomoles [3H]thymidine per well. When glucose was present in the medium, it stimulated proliferation of cells, as measured by [3H]thymidine uptake, by 197.4% ± 11.6% (5 mM) and 205.9% ± 16.6% (25 mM) of the level of cells treated with glucose-free medium. Lactate (5 mM: 120.3% ± 16.3%) or pyruvate (96.9% ± 5.5%) had no significant effect on cell proliferation (Table 4).

**Transporter Expression in Cultured Rat RPE Cells**

Analyses of two distinct rat RPE cell lines by electrophoresis and Western blot analysis showed that MCT1 and GLUT1 were expressed, indicating that the cells had the potential for taking up monocarboxylates and glucose from the medium, respectively. Immunoblots probed for the presence of actin revealed that protein levels were similar in each sample (Fig. 7). Immunocytochemical studies revealed that cells were labeled in a homogeneous manner for MCT1 immunoreactivity (Fig. 7B). A micrograph shown is representative of cells from four different cultures labeled for MCT1.
Inhibitors), by paired Student’s t-test analysis (n = 9).

**DISCUSSION**

In this study, rat RPE cells in culture (passages 2–4) clearly used glucose as the primary energy source. However, metabolism of amino acids such as glutamine by these cells provided an alternative means of retaining viability. Furthermore, the monocarboxylates, pyruvate, or lactate were used as substrates, but only when glucose availability was low, and amino acid metabolism was inhibited. RPE cells, in vivo, are thought to use both oxidative and nonoxidative pathways of glucose metabolism,

3,32 although bovine RPE cells have been demonstrated to have a level of aerobic respiration that is only approximately half that of the retina.25 These data provide evidence that rat RPE cells can overcome deprivation of preferred energy substrates by switching modes of metabolic energy production, which suggests that such cells may be able to resist insults in vivo, such as hypoxia or hypoglycemia, by implementing alternative pathways of metabolism.

It is well known that some cell types reduce their rate of oxidative metabolism and convert to high rates of glycolysis in response to inhibition of mitochondrial respiratory processes by the Pasteur effect. Furthermore, inhibition of glycolysis implemented in response to an inhibition of mitochondrial respiratory activity. Although the effect of IOA was reduced slightly by concurrent anoxia, there was no detectable metabolic activity at all in cells incubated in a medium without glucose subjected to anoxia for 24 hours. *P < 0.05, when cells subjected to glucose and oxygen deprivation are compared with cells treated with IOA and anoxia, by paired Student’s t-test analysis (n = 8).

**TABLE 1. Effect of Transport Inhibitors on Viability of Cultured Rat RPE Cells**

<table>
<thead>
<tr>
<th>Treatment (µM)</th>
<th>Viability at 24 h</th>
<th>Viability at 72 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCB (50)</td>
<td>54.1 ± 6.7**</td>
<td>27.1 ± 5.3**</td>
</tr>
<tr>
<td>CCB (1)</td>
<td>93.4 ± 3.2</td>
<td>53.2 ± 6.5*</td>
</tr>
<tr>
<td>4-CIN (500)</td>
<td>97.3 ± 3.4</td>
<td>76.1 ± 7.6*</td>
</tr>
<tr>
<td>4-CIN (50)</td>
<td>101.3 ± 2.8</td>
<td>98.1 ± 3.2</td>
</tr>
<tr>
<td>4-CIN (1)</td>
<td>99.3 ± 4.6</td>
<td>99.2 ± 2.4</td>
</tr>
</tbody>
</table>

Data are expressed as percentage of control.

* P < 0.05, ** P < 0.01 compared with control cells treated for the same period of time with vehicle (1% v/v dimethyl sulfoxide for both inhibitors), by paired Student’s t-test analysis (n = 8–10).

**FIGURE 4.** Dose-dependent effect of the glycolytic inhibitor, iodoacetate (IOA), on reduction of rat RPE cell viability after 24 hours, in medium containing glucose. IOA had a similar detrimental effect in such medium whether 5 mM pyruvate, 5 mM lactate, or no monocarboxylates were present. The effect of IOA (5 µM) was slightly but significantly less destructive when pyruvate was present. *P < 0.05 compared with appropriate control cells in medium without IOA, by paired Student’s t-test analysis (n = 9).

**FIGURE 5.** Involvement of the hexose-monophosphate shunt for generation of a small proportion of cell energy in rat RPE cultures. Oxygen deprivation for 24 hours had no significant effect on cell viability, unlike IOA (10 µM) which reduced cells to approximately 25% of control metabolic activity. Although the effect of IOA was reduced slightly by concurrent anoxia, there was no detectable metabolic activity at all in cells incubated in a medium without glucose subjected to anoxia for 24 hours. *P < 0.05, when cells subjected to glucose and oxygen deprivation are compared with cells treated with IOA and anoxia, by paired Student’s t-test analysis (n = 8).

Because the amount of pyruvate passing into the Krebs’ cycle is low, the anaerobic lactate production constitutes a significant energy source. This is despite the fact that the energy yield from anaerobic lactate synthesis (2 moles adenosine triphosphate (ATP) per mole of glucose) is much less than that produced by oxidative metabolism (approximately 36 moles ATP per mole of glucose). The reasons that some cultured cells predominantly rely on nonoxidative pathways for glucose catabolism remains unclear. It has been suggested that prolonged culturing leads to an uncoupling of enzyme activities connecting glycolysis with the Krebs’ cycle, such as pyruvate dehydrogenase, phosphoenolpyruvate carboxykinase, or pyruvate carboxylase.35,36 It is also possible that oxygen availability is much reduced because diffusion to cells through the culture medium is uncontrolled and may therefore be extremely limited. In the case of the rat RPE cells examined in the present study, reliance on oxidative pathways of metabolism was essentially lost, as shown by the fact that an incubation for 24 hours in an anoxic environment (Fig. 6) had no significant effect on cell viability. Previous reports have shown that prolonged exposure to anoxia, particularly in the case of human RPE cells, leads to apoptotic cell death,26,37,38 but this effect was probably manifest through free radical intermediates, because it was blocked by flaviprine57 and melatonin,30 both of which possess free-radical-scavenging properties.

A phenomenon that has been widely described is the Pasteur effect. This is the compensatory increase in anaerobic glycolysis implemented in response to an inhibition of mitochondrial oxidative respiration. An elegant study by Winkler et al.39 has shown that both cultured rat retinal Müller cells (cell line RMC-1) and (human) RPE cells raise their rates of glycolysis in response to inhibition of mitochondrial respiratory processes by the Pasteur effect. Furthermore, inhibition of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) with 5 µM IOA in both cell types causes a decrease in the level of anaerobic but not aerobic ATP production with a resultant loss of cellular viability.39 These data show that, although such cells have the ability to withstand metabolic challenges resulting from a decrease in the oxygen supply (i.e., hypoxia, ischemia), they are ultimately dependent on an adequate supply of glucose for metabolic energy production. This is in agreement with the
present data that showed glucose to be the preferred substrate for RPE cell metabolism.

In the present study, the viability of rat RPE cells began to show a reduction after 6 hours of glucose deprivation, although this effect was only statistically significant at 24 hours. Cultured human Müller cells have been shown to maintain their aerobic ATP levels for 4 hours in the absence of glucose, but under anaerobic conditions or when challenged with IOA, ATP levels were diminished within this time. Moreover, these cells were completely resistant to experimental anoxia; and, furthermore, approximately 99% of the glucose used by these cells was metabolized to lactate under control conditions. These data imply that Müller cells in situ predominantly metabolize glucose, sparing oxygen for inner retinal neurons. This would probably be the case for the glycolytically active RPE cells, which could spare oxygen for photoreceptor metabolism. As Winkler stated, “Retinal Müller cells in culture are resistant to anoxia or absence of glucose, which provides a basis for understanding why Müller cells are less susceptible than neurons to ischemia or hypoglycemia.” The same reasoning holds for cultured RPE cells, which although susceptible to anoxia, maintain their viability for more than 24 hours in the absence of oxygen. Photoreceptors are also able to partially upregulate glycolysis in response to mitochondrial inhibition by the Pasteur effect, but, unlike RPE and Müller cells, these cells require glycolysis and oxidative respiration for energy production, because blockade of both processes together leads to a loss of receptor potential and a depletion of intracellular ATP. This would dictate that photoreceptors, although resistant to short episodes of mitochondrial inhibition, are also affected by a limited glucose supply. Moreover, reports have stated that photoreceptor metabolism can be maintained by utilization of lactate, previously released by Müller cells, as the end product of glycolysis. This has been questioned recently, however, by a study showing that retinal neurons and photoreceptors preferentially metabolize glucose, as long as the ambient supply of this sugar is adequate.

When rat RPE cells were deprived of glucose for 24 hours, cultures decreased to approximately 70% of their control viability levels. This effect was not further enhanced up to 120 hours of glucose deprivation, suggesting that alternative means of producing energy may have been implemented by glutaminolysis, in the absence of this sugar. Glucose was obviously the primary substrate for energy production in these cells, as complete viability was retained in the absence of glutamine, as long as glucose was present. Glucose is transported across cell membranes by specific, facilitated glucose transporters (GLUTs). The predominant transporter present in the RPE, on both the apical and the basolateral surface is GLUT1, as was shown to be expressed in these cells in the current study, although both GLUT1 and GLUT3 are expressed within the neuroretina. Comparison between two different glucose concentrations in the present study (5 and 25 mM) showed that both were equally effective in maintaining cells up to 120 hours of glucose deprivation, although this effect was only statistically significant at 24 hours. Cultured human Müller cells have been shown to maintain their aerobic ATP levels for 4 hours in the absence of glucose, but under anaerobic conditions or when challenged with IOA, ATP levels were diminished within this time. Moreover, these cells were completely resistant to experimental anoxia; and, furthermore, approximately 99% of the glucose used by these cells was metabolized to lactate under control conditions. These data imply that Müller cells in situ predominantly metabolize glucose, sparing oxygen for inner retinal neurons. This would probably be the case for the glycolytically active RPE cells, which could spare oxygen for photoreceptor metabolism. As Winkler stated, “Retinal Müller cells in culture are resistant to anoxia or absence of glucose, which provides a basis for understanding why Müller cells are less susceptible than neurons to ischemia or hypoglycemia.” The same reasoning holds for cultured RPE cells, which although susceptible to

TABLE 2. Influence of Transaminase Inhibitors on Viability of Cultured Rat RPE Cells in Glucose-Free Medium

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Viability at 24 h (%) of Control</th>
<th>Viability at 72 h (%) of Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>l-CS (1 mM)</td>
<td>74.6 ± 2.1</td>
<td>72.3 ± 6.3</td>
</tr>
<tr>
<td>AOAA (1 mM)</td>
<td>70.3 ± 4.6</td>
<td>27.4 ± 3.2‡</td>
</tr>
<tr>
<td>Glutamine-free medium (plus glucose)</td>
<td>98.3 ± 2.4</td>
<td>95.7 ± 4.9</td>
</tr>
<tr>
<td>Glutamine-free medium (minus glucose)</td>
<td>21.4 ± 6.7</td>
<td>16.9 ± 5.3</td>
</tr>
</tbody>
</table>

Data are expressed as percentage of control.

* P < 0.05 compared with control cells treated for the same period with vehicle (culture medium minus glucose), by paired Student’s t-test analysis (n = 8).

† P < 0.05 compared with cells treated with AOAA alone by paired Student’s t-test analysis (n = 8).

†* P < 0.05 compared with cells treated with AOAA plus pyruvate for the same period of time by paired Student’s t-test analysis (n = 8).

Anoxia-treated cells were placed in an anoxic gas chamber for the whole incubation period (72 hours).

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* P < 0.05 compared with control cells treated for the same period with vehicle (culture medium), by paired Student’s t-test analysis (n = 8).

† P < 0.05 compared with cells treated with AOAA alone by paired Student’s t-test analysis (n = 8).

†* P < 0.05 compared with cells treated with AOAA plus pyruvate for the same period of time by paired Student’s t-test analysis (n = 8).

** P < 0.01 compared with control cells treated for the same period with vehicle (culture medium minus glucose), by paired Student’s t-test analysis (n = 8).
In conclusion, the use of AOAA is a general inhibitor of transaminase reactions and had no effect. Furthermore, performing incubations in a medium without glutamine and glucose had an effect similar to that of AOAA, suggesting that glutamine was the essential component used by RPE cells. The data also indicate that the monocarboxylates pyruvate and lactate can be used by these cells, but only as an alternative to glutamine, as substrates for the Krebs cycle. In agreement with the present data, cultured Müller cells incubated with IOA could not maintain their ATP levels, even in the presence of lactate, pyruvate, glutamate or glucose.

The observation that the protective action of pyruvate or lactate in such situations was counteracted by concurrent deprivation anoxia, which would prevent oxidative pathways of metabolism. In agreement with the present data, cultured Müller cells incubated with IOA could not maintain their ATP levels, even in the presence of lactate, pyruvate, glutamate or glucose.

**Figure 7.** (A) Immunoblot analysis of transporter expression in cultured rat RPE cells (passage 3). Two distinct rat RPE cell lines were analyzed. The first lane represents one culture, and the second lane represents a different culture. The order of loading was the same for each protein analyzed. **(B)** Immunocytochemical localization of MCT1 immunoreactivity in cultured rat RPE cells (passage 3) showing the homogeneous distribution of label.

### Table 4. Proliferation of Rat RPE Cells in Serum-Free Culture

<table>
<thead>
<tr>
<th>Medium</th>
<th>Incorporation of [3H]thymidine (% of Control)</th>
</tr>
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<tbody>
<tr>
<td>Control</td>
<td>100 ± 12.9</td>
</tr>
<tr>
<td>+ 5 mM glucose</td>
<td>197.4 ± 11.6*</td>
</tr>
<tr>
<td>+ 25 mM glucose</td>
<td>205.9 ± 16.6*</td>
</tr>
<tr>
<td>+ 5 mM pyruvate</td>
<td>96.9 ± 5.8</td>
</tr>
<tr>
<td>+ 5 mM lactate</td>
<td>120.4 ± 16.3</td>
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</tbody>
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Control level of [3H]thymidine incorporation for cells incubated in serum-glucose- and monocarboxylate-free DMEM was 0.52 picomoles per well. 

P < 0.05 compared with control cells treated for the same period of time with medium lacking both glucose and monocarboxylates, by paired Student’s t-test analysis (n = 8).
Cells. It is well known that monocarboxylates pass through plasma membranes by specific transporters, the MCTs.\textsuperscript{15,17} RPE cells express a complement of MCTs (mainly MCT1 and -3, but can express trace amounts of MCT4), which are retained in culture.\textsuperscript{18–20} To demonstrate that rat RPE cells at passage 3 express transporters capable of taking up monocarboxylates, it was shown in the present study that MCT1 is expressed by these cells. Because it is thought that these cells organize themselves in culture with their apical surfaces pointing upward,\textsuperscript{55} it would seem reasonable to assume that the MCT isoform responsible for monocarboxylate entry in this case is MCT1, which is expressed on the apical face in vivo.\textsuperscript{19} The specific uptake of pyruvate or lactate by MCTs is further suggested by the blockade of the effects of these compounds by 4-CIN, a known MCT inhibitor.\textsuperscript{15,56} That lactate acts in a similar way to pyruvate indicates that cultured rat RPE cells also express one or more lactate dehydrogenase isoenzymes, as shown previously.\textsuperscript{52}

As previously discussed, cultured RPE cells may be expected to have an increased rate of glycolysis compared with cells in vivo. These cells therefore may be expected to express a distinct complement of transporters to clear the additional lactate. A recent report\textsuperscript{57} has demonstrated that cells (Xenopus laevis oocytes) that are highly glycolytic preferentially express the MCT4 isoform and, indeed, the transformed human RPE cell line ARPE-19 expresses MCT4 in large amounts,\textsuperscript{58} as distinct from RPE cells in vivo. Furthermore, in the present investigation, low concentrations of 4-CIN blocked lactate-pyruvate entry into cells, but were not toxic themselves. The higher concentration of 4-CIN (500 \mu M) was toxic to rat RPE cells after 72 hours. The results obtained with this MCT inhibitor may also be explained by differential expression of MCT isoforms with respect to cells in vivo. For example, MCT4, which is expressed at only trace levels by rat RPE cells, in situ, is blocked by a much higher concentration of 4-CIN than MCT1 (K_m \textsuperscript{s} is reported to be 991 ± 148 \mu M for MCT4 compared with 166 \mu M for MCT1 in transfected oocytes\textsuperscript{59}). This would be consistent with these cells expressing MCT4 to export lactate and blockade with the high concentration of 4-CIN leading to intracellular acidosis and gradual cell death. It is suggested, however, that rat RPE cells exposed to long-term culture may alter expression of their MCT complement to compensate for the altered metabolism shown in such cells. Determination of the expression of all MCTs in the RPE cells used in the present study may clarify the roles that these transporters play in monocarboxylate flux in such cells.

**Conclusions**

In summary, rat RPE cells in culture have been shown to rely primarily on glucose and secondarily on glutamine to provide metabolic substrates to maintain viability. In the absence of both glucose and glutamine, the cells can use the monocarboxylates, pyruvate, or lactate to this end. An important conclusion to be drawn from this study is that under physiological conditions in vivo, monocarboxylates are probably not used by RPE cells. The polarized localization of MCT1 and -3 on opposing faces of these cells is likely, therefore, to be solely for the purpose of shunting these compounds from the retina into the choroidal circulation. Bearing all these points in mind, however, these data indicate that RPE cells have the requisite machinery to metabolize monocarboxylates and are able to alter their substrates for energy production according to local substrate availability. This means that they have the capability to withstand both hypoxic and hypoglycemic insults. Furthermore, the data obtained in the present study lend support to the idea that, although useful, such culture preparations are not necessarily reflective of RPE cell function in vivo.

**References**

17. Halestrap AP, Meredith D. The SLC16 gene family: from monocarboxylate transporters (MCTs) to aromatic amino acid transporters and beyond. *Pflüger Arch.* In press.


