Phosphorus-31 NMR Spectroscopy of Cultured Human Retinal Pigmented Epithelial Cells

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Cultured human retinal pigmented epithelial cells were studied using phosphorus-31 nuclear magnetic resonance spectroscopy (P-31 NMR). Retinal pigmented epithelial cells from normal human donors were isolated and expanded using roller-bottle culture. The P-31 NMR spectrum of the intact living cells was obtained at 121 MHz by casting the cells in agarose threads and perfusing the threads with culture medium. The cells remained viable at 37°C in the NMR magnet for at least 24 hr, as determined by the stability of the phosphorus spectrum and by trypan blue dye exclusion at the end of the experiments. The intact cell spectrum showed resonances from phosphorylethanolamine, phosphorylcholine, inorganic phosphate, phosphodiesters, phosphocreatine, ATP, NAD+, and UDP-N-acetylglucosamine, with phosphorylethanolamine, phosphorylcholine, and ATP being the major metabolites present. The resonances were assigned by making a perchloric acid (PCA) extract of the intact cells and running under high-resolution conditions. The PCA extract spectrum also detected sugar phosphates, ADP and UTP, with the latter being approximately 20% of the nucleotide pool. These studies provide the basis for the study of normal and diseased human RPE cells by NMR spectroscopic methods. Invest Ophthalmol Vis Sci 28:70–75, 1987

NMR spectroscopy can be used to identify intracellular components noninvasively, and to follow the time course of changes in intracellular metabolites and intracellular pH in a physiologically intact system. 1–3 Phosphorus-31 NMR spectroscopy has been of particular utility because the P-31 nucleus is 100% naturally abundant and because the phosphorus metabolites detectable by NMR are directly involved in the energy reactions of the cell. Therefore, the P-31 profile can determine the steady-state concentrations of metabolites as an indicator of biosynthetic activity and open a window on the bioenergetics of the cell. Some cellular systems studied by phosphorus NMR include: Escherichia coli, 4 yeast, 5 whole human blood, 6 HeLa cell suspensions, 7 and Ehrlich ascites cells. 8 Phosphorus NMR studies of ocular structures include the lens, 9,10 cornea, 11 and the whole intact globe. 12 Part of the challenge in performing these studies has been in maintaining cells and tissues in a viable state during the course of the experiment, and not in an energy supply–demand imbalance. However, cells cast in agarose threads and perfused with culture medium can be maintained inside the NMR magnet for an indefinite period. 13,14

In this study, the authors report the first P-31 NMR spectra of cultured human retinal pigmented epithelium (RPE) cells. The authors are particularly interested in the retinal pigmented epithelium because of its importance in supporting the photoreceptor cells and the chorioretinal complex of the eye. Little is known about the biochemistry of human RPE and how its intermediary metabolism is altered in degenerative conditions of the retina such as retinitis pigmentosa. Here, the authors demonstrate the applicability of using cultured RPE and this system of casting cells in agarose threads for P-31 NMR analysis of human RPE metabolism.

Materials and Methods

Cell Culture

The culture of RPE is a well established procedure in our laboratory. 15–16 Cells were obtained from donor eyes less than 12 hr postmortem. Donors had no known history of ocular disease, and the eyes were free from retinal disease upon examination under the dissecting stereomicroscope. After removal of the anterior segment and the vitreous, the sensory retina was gently lifted out and detached from the optic nerve. A solution of 2.4% Dispase (Boehning-Mannheim; Indianapolis,

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IN) in Hank’s balanced salts, containing 100 mm sorbitol to maintain solution osmolarity, was added to the intact eye cup. The eye cup was incubated at 37°C for 30–40 min, with eyes from older donors requiring the longer incubation time. After incubation, cells were loosened by gentle pipetting. The cell suspension was placed into 60-mm polystyrene culture dishes, each containing 3 ml of Coon’s modified Ham’s F-12 nutrient medium (Gibco; Grand Island, NY) supplemented with 20% fetal bovine serum (FBS), 100 µg/ml penicillin, 50 µg/ml streptomycin, 2 mM glutamine, and 2 mM ascorbic acid. Cultures were maintained at 37°C in an atmosphere of 95% air, 5% CO₂ and 100% humidity. After 12–18 hr, depending upon the preparation, the cells which had not yet attached were removed and placed into a new dish. These “floater” cells were allowed to attach and were found to contain only one cell type as observed by phase contrast microscopy. The authors have found that the use of this differential attachment procedure effects a purification, because freshly harvested RPE cells tend to attach to culture dishes more slowly than any cells that may have inadvertently been harvested from the choroid. These starter cultures were then grown to confluence in Coon’s modified Ham’s F-12 containing 5% FBS and passed in a standard fashion using 0.25% trypsin. Cells were expanded using roller bottles and were typically 7–12th passage cells. All cell cultures used exhibited an epithelioid morphology upon examination by phase contrast microscopy. In some cases, the cells were returned to culture after cell perfusion studies and they exhibited identical morphologies after the cell perfusions. Cells were free from mycoplasma contamination, as determined by periodic assay (Mycoplasma Isolation Kit®, Flow Laboratories, Inc.; McLean, VA).

Cell Perfusion

Cell perfusions were performed by a modification of the method of Foxall et al.13 Approximately 2–3 × 10⁶ cells obtained from six roller bottles were used for each experiment. Cells were harvested using trypsin, washed by centrifugation in growth medium containing 20 mm HEPES and 10% FBS, and counted by hemocytometer to determine cell number. The cells were resuspended in 1.5 ml growth medium, to which was added 0.75 ml of liquid 1.8% low gelling-temperature agarose (Bio-Rad; Richmond, CA) in Hank’s balanced salts solution also containing 20 mm HEPES. The liquid cell–gel mixture was transferred to a 50-ml conical tube, fitted with a pressure cap and maintained at 37°C in a water bath. The tube was then subjected to a mild positive pressure from a variable-pressure pump and the mixture was extruded through 0.5-mm Teflon® tubing. The tubing passed through an ice bath, solidifying the gel. The cell-containing gel threads were cast directly into a sterile 20-mm NMR tube containing culture medium. After casting, the NMR tube was fitted with a Teflon® insert containing the perfusion lines (Fig. 1). Culture medium was perfused past the loosely packed threads at the rate of 1.5 ml/min using a peristaltic pump. The perfusate volume was 100 ml and was changed periodically depending upon the number of cells being perfused. All procedures were carried out under sterile conditions, and the cells could be returned to culture at the end of the experiment by gentle pipetting to disrupt the threads. At the end of the cell perfusions, cells were 80–90% viable as determined by trypan blue dye exclusion.

P-31 NMR

NMR spectra were obtained on a Brucker WM 300 spectrometer (Brucker Instruments; Manning Park, Billerica, MA) operating at 121.49 MHz for phosphorus. Spectra of intact cells in a 20-mm NMR tube were collected using 4K data points and a 5000 Hz spectral width, with proton broadband decoupling. Typical
pulse conditions were a 1-sec recycle time and a 45° tip angle (see figure legends for exact pulse conditions). The cells were maintained at 37°C while in the magnet using the variable-temperature unit in the Bruker probe. Perchloric acid (PCA) extracts were run using 16K data points and a 4-sec recycle time in a 15-mm NMR tube. These extracts were made at the end of the experiments for identification of phosphorus metabolites by disrupting the cell threads using a motor driven Teflon-glass homogenizer in 10 ml total volume and then adding 1/10 volume of 70% PCA to the ice-cold homogenate. The mixture was immediately centrifuged at 4°C for 5 min and then neutralized with 5M KOH to a pH of approximately 8. The resulting supernatant was lyophilized and redissolved in 5 ml of 20 mM EDTA and 20% D2O in water. Peak identification and concentration determinations were made by adding small volumes (typically 25 µl) of known concentrations of pure compound (Sigma; St. Louis, MO) to the extract and rerunning under the same conditions. Compounds were considered identified if shift positions matched exactly (±0.01 ppm) at pH 8.5, 8.0, 7.4, 7.0, and 6.0.

Results

A P-31 NMR spectrum of intact RPE cells in agarose threads is shown in Figure 2. The spectrometer conditions were chosen so that the major peaks are close to fully relaxed. Zero parts per million was assigned to the resonance from 85% orthophosphoric acid.

Results

A P-31 NMR spectrum of intact RPE cells in agarose threads is shown in Figure 2. The spectrometer conditions were chosen so that the major peaks are close to fully relaxed. The perfusate contained approximately 50 /µM inorganic phosphate (Pi); peak 4, glycerolphosphorylcholine (GPC); and peak 5, phosphocreatine (PCr). Peaks 6, 7, and 10 arise mainly from the gamma, alpha, and beta phosphates of ATP, respectively, although UTP and ADP are also present in significant amounts. Peak 8 is from NAD+, in addition to a diphosphodiester. The latter also gives rise to peak 9, which has been identified as UDP-N-acetylgalactosamine. Noteworthy in this spectrum is the low level of inorganic phosphate relative to the nucleotide triphosphates. Also, the inorganic phosphate which is both intracellular and extracellular phosphate is seen as one peak, indicating that the intracellular and extracellular pH was the same to within 0.2 pH units. Similar spectra have been run under a variety of extracellular pH values (6.5–8.0), and the intracellular pH calculated from the shift of the inorganic phosphate was always the same as the measured extracellular pH. Furthermore, there has never been more than one P; peak or any evidence of splitting, indicating that there was no large pH difference between intracellular and extracellular environments under our conditions. It is possible, however, that the intracellular phosphate is very low and therefore the observed phosphate peak is from the 50 /µM extracellular phosphate contributed by the 5% bovine serum.

The P-31 NMR spectrum of RPE cells perfused in agarose threads is stable over time (Fig. 3). This is important, in order to ensure that the cells are in a physiologically stable state.

Definitive peak identification was made by preparing PCA extracts of the RPE cells at the conclusion of the experiments. This was necessary due to the resolution limitations of the intact cell spectra. A representative PCA extract spectrum is shown in Figure 4. The peak linewidths were approximately 4 Hz in the extracts as compared with 40–80 Hz in the intact cells. The shift position of the observable phosphate metabolites at pH 8.05 and their relative concentrations in the extract are given in Table 1. These data are typical of PCA extract spectra reported for other cell types. However, to our knowledge, UTP has not been reported in NMR spectra of nonmalignant cells or tissues, and the identification of UDP-N-acetylgalactosamine in extract spectra has heretofore not been reported in the literature.

Discussion

These experiments combine the techniques of human cell culture and in vivo and in vitro P-31 NMR to study cellular metabolism. Our use of the agarose thread technique allows measurements to be made at 37°C on a cell population that maintains its viability and, to all indications, its initial physiologic state. Previous P-31 NMR experiments on cellular suspensions had to rely on measurements made at less than ambient
Fig. 3. Successive spectra of RPE cells at 37°C showing stability of phosphorus metabolites in our system. One-hour spectra were accumulated using a 45° pulse and a 1-sec recycle time. Every other spectrum is shown. Note that the area of the beta ATP peak is constant during the course of the experiment. This preparation contained approximately $200 \times 10^6$ cells.

Fig. 4. P-31 NMR of a PCA extract from approximately $250 \times 10^6$ cells. Spectrum was run at room temperature with spinning using a 4-sec recycle time and a 45° pulse angle. The solution pH was 8.05. This spectrum is the accumulation of 7200 pulses. The exact shift positions and concentrations are given in Table 1. $^a$ = not assigned; $^b$ = tentative.
of the phosphate peaks. The values obtained range from approximately 20% of the nucleotide pool. UTP has also been reported in the P-31 NMR spectrum of cultured HeLa cells7 and in experimental tumors.21,26 To our knowledge, this is the first report of UTP detected by P-31 NMR in nonmalignant cultured mammalian cells, and we believe that it reflects a specific differentiated function of these cells. Along this line, we also reproducibly observed a diphosphodiester giving rise to the two sets of doublets at -10.60, -10.80 and -12.33, -12.51 ppm. We have identified this peak as arising from UTP. In our cells, UTP represents approximately 20% of the nucleotide pool. UTP has also been reported in the P-31 NMR spectrum of cultured HeLa cells7 and in experimental tumors.21,26 To our knowledge, this is the first report of UTP detected by P-31 NMR in nonmalignant cultured mammalian cells, and we believe that it reflects a specific differentiated function of these cells. Along this line, we also reproducibly observed a diphosphodiester giving rise to the two sets of doublets at -10.60, -10.80 and -12.33, -12.51 ppm. After running a number of UDP-diphosphosugars involved in polysaccharide biosynthesis, the only compound that exhibited equivalent shift positions to the unknown compound was UDP-N-acetylglucosamine. To our knowledge, UDP-N-acetylglucosamine has not been previously reported in the literature in NMR spectra of cells or tissues. However, this compound may be the unidentified diphosphodiester reported by other investigators in other cell lines.7,18 The presence of a significant amount of UDP-N-acetylglucosamine and UTP probably reflects the significant biosynthetic activity of RPE in producing glycoproteins and glycosaminoglycans. These compounds are found in Bruch’s membrane and the interphotoreceptor matrix, two extracellular matrices to which the RPE contributes, and on the cell surface of RPE.27,28 It has been shown that glycosaminoglycans

### Table 1. Shift positions and metabolite concentrations in the PCA extract of Figure 4

<table>
<thead>
<tr>
<th>Compound</th>
<th>Chemical shift</th>
<th>Concentration (μmol)</th>
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<tbody>
<tr>
<td>NA</td>
<td>5.22</td>
<td>—</td>
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<tr>
<td>Glucose-6-PO₄</td>
<td>5.04</td>
<td>0.12</td>
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<tr>
<td>NA</td>
<td>4.69</td>
<td>—</td>
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<tr>
<td>F-1-6 DiPO₄</td>
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<tr>
<td>PE</td>
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<td>0.04</td>
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<tr>
<td>PC</td>
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<td>0.76</td>
</tr>
<tr>
<td>Pi</td>
<td>3.13</td>
<td>1.04</td>
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</tr>
<tr>
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<tr>
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<tr>
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<td>-5.04, -5.22</td>
<td>0.24</td>
</tr>
<tr>
<td>ADP</td>
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<td>0.38</td>
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<tr>
<td>ADP</td>
<td>-9.80, -9.98</td>
<td>0.38</td>
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<tr>
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<td>-10.21, -10.36</td>
<td>1.03</td>
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<tr>
<td>UDP-N-acetylglucosamine</td>
<td>-10.60, -10.81, -12.33, -12.51</td>
<td>0.24</td>
</tr>
<tr>
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</tr>
<tr>
<td>ATP</td>
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<td>0.79</td>
</tr>
<tr>
<td>UTP</td>
<td>-20.61, -20.77, -20.92</td>
<td>0.24</td>
</tr>
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</table>

*Tentative.  
NA = not assigned.

temperatures 7,8,17,18 Some employed intermittent bubbling with oxygen19 or the use of hydrogen peroxide to keep the organelles from becoming hypoxic.19 The procedure used in our study has the advantage of allowing for a more thorough NMR investigation (for example, for determinations of reliable first order relaxation times [T1] data at physiologic temperatures). We have obtained preliminary T1 data on RPE cells in order to determine the degree of relative saturation of the phosphate peaks. The values obtained range from 1.5 sec for the monoester peaks to 0.3 sec for the peaks of ATP. However, the values must be considered tentative. A more thorough investigation of the relaxation times of these metabolites is being done.

The spectra of intact cells allows one to determine the steady-state concentrations of the metabolites over time, although some of the peaks arise from more than one compound. In order to identify these metabolites, the low molecular weight–compounds observable by NMR were extracted with 7% PCA from the intact cells. This technique reveals several important metabolites. The major monoesters in these cells are phosphorylethanolamine and phosphorylcholine. Although it is widely thought that these metabolites are intermediates in lipid metabolism, there is little consensus as to whether these compounds accumulate as precursors, as breakdown products, or are metabolic controls in these processes.20,21 One or both of these metabolites has also been identified in high concentrations in cultured tumor cells,8,18,22 in solid tumors,21,23 in neonatal brain,24 and in developing dog brain.25 It is possible that these compounds arise from increased catabolism of lipids in necrotic tumors and from increased lipid synthesis in brain. However, neither of these explanations suggests why these compounds are found in cultured RPE. We have noted that the relative concentrations of these compounds vary from preparation to preparation. A similar phenomenon was noted with the concentration of phosphorylcholine and phosphorylethanolamine in a lymphoid cell line.18 We are currently investigating this observation in RPE.

The major peaks in the upfield region of the extract are from phosphocreatine and ATP which give rise to major peaks in the intact cells. The presence of phosphocreatine indicates that these cells contain creatine kinase, since the only known pathway for PCr synthesis is via this enzyme. Creatine kinase is found in high concentrations in other tissues, most notably heart, muscle, and brain, although it is by no means ubiquitous. Further studies are needed to determine the activity of this enzyme in RPE, and to say whether it is of the brain (BB) or muscle (MM) isozyme.

Also notable was the presence of another compound with a shift position similar to that of ATP. It can be seen in Figure 4 as an upfield shoulder on the two gamma peaks of ATP at -5.04 and -5.22 ppm, and is partially resolved from the beta ATP peaks at -20.61, -20.77, and -20.92 ppm. We have identified this peak as arising from UTP. In our cells, UTP represents approximately 20% of the nucleotide pool. UTP has also been reported in the P-31 NMR spectrum of cultured HeLa cells7 and in experimental tumors.21,26 To our knowledge, this is the first report of UTP detected by P-31 NMR in nonmalignant cultured mammalian cells, and we believe that it reflects a specific differentiated function of these cells. Along this line, we also reproducibly observed a diphosphodiester giving rise to the two sets of doublets at -10.60, -10.80 and -12.33, -12.51 ppm. After running a number of UDP-diphosphosugars involved in polysaccharide biosynthesis, the only compound that exhibited equivalent shift positions to the unknown compound was UDP-N-acetylglucosamine. To our knowledge, UDP-N-acetylglucosamine has not been previously reported in the literature in NMR spectra of cells or tissues. However, this compound may be the unidentified diphosphodiester reported by other investigators in other cell lines.7,18 The presence of a significant amount of UDP-N-acetylglucosamine and UTP probably reflects the significant biosynthetic activity of RPE in producing glycoproteins and glycosaminoglycans. These compounds are found in Bruch’s membrane and the interphotoreceptor matrix, two extracellular matrices to which the RPE contributes, and on the cell surface of RPE.27,28 It has been shown that glycosaminoglycans
are synthesized in significant amounts by RPE in organ culture\textsuperscript{29} and in primary cell culture.\textsuperscript{30} Cultured RPE has been shown to synthesize and secrete chondroitin sulfate, dermatan sulfate and hyaluronic acid.\textsuperscript{30} N-acetylgalactosamine is an important component of hyaluronic acid. UDP-N-acetylgalactosamine can also be epimerized to UDP-N-acetylgalactosamine, an important precursor of dermatan sulfate and chondroitin sulfate. The necessary enzymes for these reactions have not yet been identified in RPE.

In summary, we feel that the application of NMR to the study of cultured human RPE cells provides new information on the physiology and biochemistry of these cells. The use of a perfused whole-cell system allowed us to determine that the observable phosphates were not changing during the spectral accumulations, and demonstrated that these cells can be maintained viable in the NMR magnet for an extended period of time. This should set the stage for further NMR studies on RPE aimed at investigating their intermediary metabolism, an aspect of these important ocular cells now not well understood.

Key words: phosphorus-31 NMR, retinal pigmented epithelium, cultured human ocular cells, cell metabolism

References