Roles of Glucose in Photoreceptor Survival*

Andrei O. Chertov‡, Lars Holzhausen‡, Iok Teng Kuok§, Drew Couron§, Ed Parker§, Jonathan D. Linton‡, Martin Sadilek, Ian R. Sweet†, and James B. Hurley‡†

From the Departments of ‡Biochemistry, §Medicine, Diabetes, Obesity Center of Excellence, ¶Ophthalmology, ¶‡Chemistry, University of Washington, Seattle, Washington 98195

Received for publication, July 5, 2011, and in revised form, August 4, 2011 Published, JBC Papers in Press, August 12, 2011 DOI 10.1074/jbc.M111.279752

The aim of this study is to understand the energy requirements of photoreceptor neurons. Glucose withdrawal causes photoreceptor death. Mitochondrial fuels and autophagy can enhance survival. The energy requirements of photoreceptors will contribute to understanding the basis of retinal disease.

Vertebrate photoreceptor neurons have a high demand for metabolic energy, and their viability is very sensitive to genetic and environmental perturbations. We investigated the relationship between energy metabolism and cell death by evaluating the metabolic effects of glucose deprivation on mouse photoreceptors. Oxygen consumption, lactate production, ATP, NADH/NAD+, TCA cycle intermediates, morphological changes, autophagy, and viability were evaluated. We compared retinas incubated with glucose to retinas deprived of glucose or retinas treated with a mixture of mitochondrion-specific fuels. Rapid and slow phases of cell death were identified. The rapid phase is linked to reduced mitochondrial activity, and the slower phase reflects a need for substrates for cell maintenance and repair.

Degeneration of photoreceptor neurons (PR) causes blindness in a variety of types of human retinal diseases, including retinitis pigmentosa, Leber’s congenital amaurosis, and Oguchi disease (1–4). A challenge to understanding these diseases has been the diversity of factors involved in PR degeneration. These include physical stresses, such as too much light, or mutations in one of over 40 different genes. Inherited defects in the genes encoding phototransduction proteins like rhodopsin, phosphodiesterase, guanylyl cyclase, or its regulatory protein arrestin, rhodopsin kinase, and the cGMP-gated channel all can cause PR degeneration. Yet mutations in another protein essential for phototransduction, transducin, do not cause degeneration (5). The pathways by which PRs die also are diverse and not completely understood. Caspase-mediated apoptosis is a primary pathway for irreversible PR degeneration (6, 7), yet other modes of cell death also can occur (8–12).

Energy metabolism may be a critical juncture that links PR function and survival. Vision is lost after only 10 s when the supply of blood to the eye is blocked (13). A decrease in available glucose leads to PR death and subsequent loss of vision in an age-related manner (14). Also, mouse models of PR degeneration show evidence of cell death because of nutrient deprivation (15). This is likely due to the unique nature of PR metabolism. Energy metabolism of PRs resembles that of rapidly dividing cells, such as cancer cells (16). Glycolysis produces ~50% of the ATP in PRs (17, 18). Even when O2 is available, >80% of glucose taken up by a PR is used for anaerobic glycolysis, i.e. it is converted to lactate. Both rod-dominated (19–21) and cone-dominated (22) retinas use glucose in this manner. Glucose enters PRs through the Glut-1 transporters located in the inner segment of PRs (23). There it is phosphorylated by hexokinase 2 (24) and then diffuses to other parts of the cell.

The metabolism of glucose supports several key activities that are likely to be essential for PR survival. ATP production is an important outcome of glucose metabolism. An insufficient rate of ATP production appears to cause degeneration of some types of neurons (25). Glucose metabolism keeps mitochondria active, which may prevent them from initiating apoptosis (26, 27). Glucose also contributes to synthesis of GlcNAc, which is required for asparagine (N)-linked protein glycosylation and subsequent trafficking to the cell surface. Proper trafficking of transporters and growth factor receptors is required for glucose uptake (28) and cellular responses to pro-survival signals (29). Glycosylation of rhodopsin is a particularly important factor that influences PR viability (30). O-Linked protein glycosylation also is important as it plays a role in intracellular signaling (31, 32). Glucose also is needed for production of cytosolic NADPH, which can inhibit caspase-mediated apoptosis (33, 34), support anabolic activity (24, 35), and help to maintain appropriate levels of reactive oxygen species (36). Finally, glucose deprivation activates autophagy (37), which has been described as a pro-survival activity (15, 38, 39).

To understand the connection between PR metabolism and degeneration, we performed a series of experiments to identify the metabolic needs of PRs. We investigated how the different pathways of glucose metabolism contribute to PR viability. We confirmed that glucose deprivation causes mitochondrial de-
polarization, depletion of ATP, and cell death. Unexpectedly, we found that short term cell survival correlates with mitochondrial activity but not with ATP levels. A slower mode of cell death occurs in the absence of glucose even when mitochondrial activity is sustained by mitochondrion-specific fuels. We present evidence that glycosylation enhances PR viability and that autophagy plays a pro-survival role during prolonged glucose deprivation. This study identifies some of the essential metabolic requirements of PRs and helps to explain the relationship between metabolic dysfunction and PR degeneration.

**EXPERIMENTAL PROCEDURES**

**Animals—**C57BL mice (6–8 weeks old) were purchased from The Jackson Laboratory (Bar Harbor, ME). The mice were housed in a central animal facility at 21 °C (12:12 h light/dark cycle). Unless otherwise specified, all experiments were carried out under ambient light conditions.

**Reagents—**All chemicals and reagents were from Sigma unless otherwise specified.

**Retinal Dissections—**The mice were euthanized by cervical dislocation. The eyes were removed and hemisected with small scissors. Retinas were separated from retinal pigment epithelium in Krebs-Ringer/HEPES/bicarbonate (98.5 mM NaCl, 4.9 mM KCl, 2.6 mM CaCl₂, 1.2 mM MgSO₄·7H₂O, 1.2 mM KH₂PO₄, 26 mM NaHCO₃, 20 mM HEPES, 5 mM glucose). When a sodium salt was added to the buffer, less NaCl was added to keep a constant Na⁺ concentration. The mito-fuel mix contained 10 mM pyruvate, 1 mM glutamine, 5 mM malate, 1 mM leucine.

**Oxygen Consumption—**A flow culture system was used to measure oxygen consumption rate and cytochrome c reduction while collecting outflow fractions for subsequent measurement of lactate (40, 41). The Krebs-Ringer/HEPES/bicarbonate was supplemented with 0.1% fraction V bovine serum albumin and 1% penicillin/streptomycin/fungizone (Invitrogen). Isolated retinas were quartered with a scalpel, and tissue from four retinas was loaded into each chamber as described previously for pancreatic islets (42) except that no Cytodex beads were added. Oxygen tension was measured by lifetime phosphorescence detection (using a system custom made by Tau Theta, Inc.) of an oxygen-sensitive dye (platinum tetrapentafluorophenyl porphyrin) (Frontier Science, Logan, UT) that was painted on the inside of the perfusion chamber (43). Absorbance by cytochrome c was measured by light transmission at 550 nm through the layer of retinal tissues as described previously (42). Cytochrome c reduction experiments were only conducted in light. Lactate was measured using a glucose/glucose oxidase assay kit (Invitrogen) according to the manufacturer’s instructions except that lactate oxidase was substituted for glucose oxidase. OCR and lactate production rate were calculated as the flow rate (≈65 μl/min) times the difference between inflow and outflow levels of oxygen or lactate.

**ATP Measurements—**Isolated mouse retinas were incubated in Krebs-Ringer/HEPES with various metabolic fuels for 90 min. At the end of each experiment, the retina was homogenized in 300 μl of 100% DMSO. The retinal extracts were frozen on dry ice and lyophilized overnight. After resuspension in 200 μl of water, the retinal extracts were centrifuged at 16,000 × g, and the supernatants were preserved. ATP was measured with an ATP bioluminescence assay kit and Biotek plate reader.

**GC-MS Analysis of Metabolites—**Isolated mouse retinas were incubated as for ATP experiments. Following incubation, two retinas were homogenized in 100 μl of a 700:200:50 mixture of methanol/chloroform/water and placed on dry ice. Retinal extracts were centrifuged at 16,000 × g, and 80 μl of the supernatant was transferred into glass inserts. The extracts were dried under vacuum and derivatized with 70 μl of freshly prepared methylhydroxylamine HCl in pyridine (20 mg/ml) and incubated at 37 °C for 90 min. Retinal extracts were further derivatized with 70 μl of N-tert-butyldimethylsilyl-N-methyltrifluoroacetamide at 70 °C for 30 min. GC-MS was carried out using the Agilent 5973 MSD/6890 GC (Agilent Corp, Santa Clara, CA). An Rtx-5MS (30 m × 0.25 mm × 0.5 μm film, Restek, Bellefonte, PA) column was used for GC separation of metabolites. Ultra high purity helium was the gas carrier at a constant flow rate of 1 ml/min. Each sample had 1 μl injected in split-less mode by the Agilent 7683 autosampler. The temperature gradient started at 100 °C with a hold time of 4 min and then increased at 5 °C/min to 300 °C where it was held for 5 min. The temperatures were set as follows: inlet 250 °C, transfer line 280 °C, ion source 230 °C, and quadrupole 150 °C. Mass spectra were collected from m/z 50 to 600 at 1.4 spectra/s after a 6.5-min solvent delay. The peaks were analyzed using Agilent data analysis software. Metabolite peak identities were previously defined with standards (44) and verified by mass after each experiment. Data are expressed as ion signal strength for each metabolite normalized to glucose samples.

**NADH Measurements—**Isolated mouse retinas were incubated in Krebs-Ringer/HEPES/bicarbonate solution supplemented with 5 mM glucose, “mix,” or no nutrient for 90 min. Following incubation, both retinas from the same animal were homogenized in 300 μl of homogenization buffer (Abcam ab65349) and then frozen on dry ice. The homogenates were centrifuged at ~60,000 × g for 1 h. The supernatant was used to measure NAD⁺, NADH, using a thiazolyl blue-based cycling assay (45) modified to be used with a plate reader.

**Electron Microscopy—**Isolated mouse retinas were incubated in Krebs-Ringer/HEPES/bicarbonate solution supplemented with 5 mM glucose, mix, or no nutrient for 4 h. Then the retinas were fixed by immersion in 2% glutaraldehyde (Electron Microscopy Sciences) in 0.1 M sodium cacodylate buffer, pH 7.4, overnight at 4 °C. The tissue was then washed in buffer and further fixed in 1% osmium tetroxide in cacodylate buffer for an hour prior to en bloc staining with uranyl acetate (1%). Subsequently, the tissue was dehydrated in a graded ethanol series, embedded in Epon-araldite resin, sectioned, and stained with lead citrate.

**LC3 Western Blot—**Isolated mouse retinas were incubated in Krebs-Ringer/HEPES/bicarbonate solution supplemented with 5 mM glucose, mix, or no nutrient for 4 h. To prevent lysosomal degradation of activated LC3, retinas were treated with 30 μM chloroquine. Two mouse retinas were homogenized together in 100 μl of RIPA buffer (50 mM Tris–Cl, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS) with pro-
tease inhibitor mixture (Roche Applied Science). Retinal homogenates were then lysed with a syringe and centrifuged at 500 × g for 5 min. Supernatants were transferred to new tubes, and 2 μl were withdrawn for determination of protein concentration (Bio-Rad DC assay). Samples were then mixed with 5× loading dye (60 mM Tris-Cl, pH 6.8, 2% SDS, 10% glycerol, 5% β-mercaptoethanol, 0.01% bromophenol blue) and boiled 5 min. Proteins were separated by SDS-PAGE (Bio-Rad 15%). After transfer of proteins onto PVDF (Millipore), membranes were blocked for 1 h at room temperature in Odyssey blocking buffer (LI-COR Biosciences). Membranes were incubated overnight at 4 °C with monoclonal anti-LC3B antibody (Cell Signaling Technology 3868S) diluted 1:500 in blocking buffer. Primary antibody was detected with IRDye-800CW goat anti-rabbit secondary antibody (LI-COR Biosciences) diluted 1:500 in blocking buffer. Fluorescence was quantified using an Odyssey Imaging System.

Cell Death in Outer Retina—Cell death in the outer retina was assessed using propidium iodide (PI) (Molecular Probes), which enters dead cells but not viable cells. As indicated under “Results,” the incubation solutions were also supplemented with 5 mM glucose, mix (10 mM pyruvate, 5 mM malate, 1 mM L-leucine, and 1 mM L-glutamine), 15 mM GlcNAc, a combination of those, or no nutrient at all. For experiments evaluating the role of autophagy and protein glycosylation, 10 mM 3-methyladenine (3-MA) was added to inhibit autophagy, and 1 μg/ml tunicamycin was added to prevent protein glycosylation, respectively. Isolated retinas were incubated for 45 and 90 min and 4 or 8 h at room temperature at ambient light conditions and loaded with 100 μg/ml propidium iodide for the last 45 min of the incubation. After the incubation, retinas were washed three times for 3 min each wash in Krebs-Ringer/HEPES/bicarbonate with 5 mM glucose.

For imaging, retinas were flattened on nitrocellulose filter paper (inner retina facing the objective) and imaged in Krebs-Ringer/HEPES/bicarbonate 5 mM glucose with the Olympus FluoView-1000 confocal microscope using the Olympus XLUM Plan FL N 20x/NA1.00, water immersion objective. Image stacks of the retina (636 × 636 μm, 1-μm z-step size) were recorded. PI fluorescence was excited at 564 nm and imaged at 508–552 nm emission. TMRM fluorescence was excited at 559 nm and imaged at 570–620 nm. Signals were separated using an SDM560 emission dichroic mirror. Enhanced GFP specifically labels rod PRs and was used for orientation and to determine the image stack size. A first image stack was obtained in Krebs-Ringer/HEPES/bicarbonate solution supplemented with 5 mM glucose to verify mitochondrial location. Solutions were then exchanged to Krebs-Ringer/HEPES/bicarbonate solution supplemented with mix (10 mM pyruvate, 5 mM malate, 1 mM L-leucine, and 1 mM L-glutamine), 5 mM glucose, and 5 μM FCCP (Sigma) or no nutrient at all. Image stacks of the same spot were recorded at 0-, 11-, 26-, and 41-min time points. 100 nM TMRM was present throughout the experiment.

The analysis was performed with Image J (National Institutes of Health). The ellipsoid region, which is the region where the majority of mitochondria are located in PRs, was identified as the peak region of TMRM fluorescence. The TMRM fluorescence signal of single optical slices was averaged and summed over 13 optical slices in the peak region (encompassing 12 μm of the peak region). All time points were normalized to the 0 time point. To correct for bleaching artifacts, normalized TMRM values were divided by TMRM values obtained from glucose control retinas. Control retinas were loaded and treated the same way but were imaged in Krebs-Ringer/HEPES/bicarbonate solution that contained 5 mM glucose in the presence of 100 nM TMRM at each time point. To correct for background signal, data are shown as % loss of TMRM signal such that the 41-min FCCP data point is treated as complete loss of signal.

Isolated Mitochondria—Isolated mouse retinas were incubated in Krebs-Ringer/HEPES/bicarbonate with various metabolic substrates for 90 min and then homogenized in a glass-Teflon Dounce homogenizer in 1 ml of mitochondria isolation buffer. The buffer consisted of 225 mM mannitol, 75 mM sucrose, 1 mM EGTA, and 5 mM HEPES, pH 7.0. Six retinas were combined for each condition. Homogenized retinas were centrifuged at 300 × g for 4 min at 4 °C, and the supernatant was transferred to fresh tubes. The pellet was re-extracted in 1 ml of mitochondria isolation buffer and once again centrifuged at 300 × g for 4 min at 4 °C. The supernatants were then combined and centrifuged at 16,000 × g for 15 min at 4 °C. Following centrifugation, the pellet was retained and resuspended in 1 ml...
of mitochondria isolation buffer with 1 mg/ml fatty acid-free BSA. After a second 15-min spin at 16,000 × g, the pellet was resuspended in 200 μl of mitochondria isolation buffer with 1 mg/ml fatty acid-free BSA. Oxygen consumption was measured in an Oroboros Oxygraph-2K as the slope of the rate of O₂ decrease in the chamber. The respiration buffer consisted of 150 mM KCl, 10 mM KH₂PO₄, 1 mM MgCl₂, pH 7.4. To start the experiment, 50 μl of mitochondria were injected into each chamber. Respiration was initiated by serial addition of pyruvate/malate and ADP. The final concentrations of substrates were 1.5 mM pyruvate, 0.5 mM malate, and 0.25 mM ADP. After addition of each component, a base line was allowed to stabilize for 5 min, except for after addition of ADP when the data were recorded for 15 min. Data points were gathered every 30 ms. Data shown are an average of the stable base line. Mitochondria from each condition were tested in triplicate.

RESULTS

O₂ Consumption and Lactate Production Decline Rapidly upon Withdrawal of Glucose from Isolated Mouse Retinas—To understand why retinas require glucose for survival, we first determined how withdrawal of glucose from isolated mouse retinas affects O₂ consumption and secretion of lactate. Isolated mouse retinas were placed in a perifusion apparatus in a Krebs-Ringer/HEPES/bicarbonate solution with 5 mM glucose (43). The OCR and lactate levels were measured to evaluate respiration and glycolysis in the retina.

A transient increase of OCR occurred shortly after withdrawal of glucose (Fig. 1A). This likely reflects increased respiration driven by cytosolic ADP accumulation as glycolysis slows. Subsequently, OCR wanes. The rate of lactate production declines more rapidly (Fig. 1B) than OCR. When this experiment was performed at 25 °C, OCR and lactate production partially recovered upon re-introduction of glucose. When
Roles of Glucose in Photoreceptor Survival

**FIGURE 2.** Mito-fuel mix maintains OCR and cytochrome c reduction without glucose. A, OCR was measured in isolated mouse retinas in the presence of mito-fuel mix (mix: 10 mM pyruvate, 5 mM malate, 1 mM glutamine, and 1 mM leucine) during glucose withdrawal at 25 °C. In the presence of glucose (Glc), mito-fuel mix elevated OCR. Without glucose, mito-fuel mix increased OCR. OCR could be restored to pre-glucose withdrawal levels. B, addition of mito-fuel mix decreased lactate production. C, cytochrome c was kept reduced during glucose withdrawal by the mito-fuel mix. For each panel, data shown are averages of four experiments. Error bars represent S.E. For OCR and lactate measurements, the first point was treated as 100% and used to normalize subsequent data points.

The same experiment was carried out at 37 °C, glucose withdrawal resulted in a greater irreversible loss in OCR and lactate production (Fig. 1, C and D).

**Exogenous Mitochondrial Fuels Can Support OCR without Glucose**—We attempted to prevent OCR from decreasing upon glucose withdrawal by adding exogenous fuels that can be used by mitochondria but cannot enter glycolysis. To accomplish this, the perifusate was supplemented with a mix of 10 mM pyruvate, 5 mM malate, 1 mM glutamine, and 1 mM leucine. Addition of the “mito-fuel” mix in the presence of glucose increased the OCR by ~10% (Fig. 2A) and decreased lactate production by ~20% (Fig. 2B). When glucose was removed from the perifusate, respiration increased another ~15%. The mito-fuel mix also prevented irreversible loss of OCR when glucose was withdrawn. These results show that retinal mitochondria can use exogenously provided mitochondrial-specific fuels to remain functional. These data also show that mitochondria in the mouse retina do not function at their maximal capacity in the presence of glucose alone.

**Glucose Withdrawal Results in PR Death That Can Be Averted by the Mito-fuel Mix**—A portion of the loss of O2 consumption that occurs upon glucose withdrawal is irreversible. This suggests that glucose withdrawal causes cell death. We used the dye propidium iodide to quantify cell death. Live cells exclude PI (47), but it readily enters dead cells, binds to nucleic acids, and fluoresces. We incubated mouse retinas with PI to evaluate PR viability in the presence either of glucose, the mito-fuel mix alone, or no fuel. The retinas were imaged by confocal microscopy, and PI-positive nuclei specifically in the PR layer were quantified (Fig. 3A). Retinas incubated for 90 min at 20 °C without glucose had six times the number of PI-positive nuclei compared with retinas incubated with glucose (Fig. 3B). The mito-fuel mix prevented nearly all of the PI staining induced by incubating retinas without glucose.

**Depletion of ATP during 90 Min of Glucose Withdrawal Is Not the Cause of Cell Death**—As neurons age, their ATP-generating capacity decreases, and they become more susceptible to death (25). It has been suggested that the ability of a neuron to sustain a sufficient level of ATP determines its ability to survive. We tested the hypothesis that low ATP levels are the cause of cell death during glucose withdrawal. We compared the amount of ATP in isolated mouse retinas in the presence of glucose and in the absence of glucose with and without the mito-fuel mix. The removal of glucose resulted in an ~70% loss of ATP after 90 min of withdrawal. Remarkably, retinas incubated with the mito-fuel mixture alone were unable to sustain ATP any better than retinas incubated with no substrate at all (Fig. 3C). Because significant cell death does not occur under these conditions, cytosolic ATP may not be a dominant factor required for PR viability.

To confirm this, we investigated the role of ATP by depleting it even further using 2-deoxyglucose (2DG). Previous studies (48) showed 2DG is taken up into cells and phosphorylated by hexokinase and ATP. Because the product of this reaction cannot enter subsequent steps of glycolysis, 2DG severely depletes intracellular ATP. Consistent with those earlier studies, we find that 2DG lowers the ATP content in mouse retinas incubated with the mix to less than 5% of its normal level (Fig. 3C). Remarkably, PR survival under this condition is nearly as good as in the presence of glucose (Fig. 3, A and B). These results indicate that the mito-fuel mix is suppressing cell death by a mechanism that is independent of ATP.

To ensure that PI entry into cells accurately reports irreversible cell death, we allowed retinas to recover from glucose withdrawal by restoring 5 mM glucose to the culture medium after a 45-min period of glucose withdrawal (Fig. 3D). After 6 h of recovery, PI was added, and staining was measured. The results are very similar to PI staining without the recovery period. This confirmed that cell death occurring during the first 45 min is irreversible. It also shows that cells that were stressed, but did not die, can fully recover.

**Mito-fuel Mix Compensates for Loss of Tricarboxylic Acid Intermediates Caused by Glucose Withdrawal**—To evaluate the effects of glucose withdrawal on global cellular metabolism, we measured a variety of cytosolic and mitochondrial metabolites.
Mouse retinas were isolated and incubated with metabolic fuels for 90 min. We then extracted the retinas with a 70:20:5 mixture of methanol, chloroform, and water and used gas chromatography-mass spectrometry (GC-MS) to quantify TCA cycle intermediates, amino acids, and products of glycolysis. Fig. 4 shows that withdrawal of glucose alters the levels of several of the measured metabolites. Glutamine and lactate are the most severely depleted, whereas aspartate increases severalfold upon glucose withdrawal.

To verify that the exogenous mito-fuel mixture we added is taken up by retinal cells, we measured metabolite levels in retinas treated with the mito-fuel mix. Several TCA intermediates increased, including succinate, fumarate, and citrate that were not in the mix. Alanine, derived from pyruvate, and glutamate, derived from glutamine and \( \alpha \)-ketoglutarate, also increased. We confirmed that mito-fuel mix components do not feed into glycolysis because serine, which is synthesized from a glycolytic intermediate 3-phosphoglycerate, remains at low levels in the presence of the mix without glucose. Remarkably, pyruvate from the mix is not converted into lactate in the absence of glucose. This indicates that the cytosolic NADH/NAD\(^+\) ratio is too low under these conditions to sustain the conversion of pyruvate into lactate.
Roles of Glucose in Photoreceptor Survival

**Figure 4.** Mito-fuel mix can reverse depletion of TCA cycle intermediates caused by glucose withdrawal. Cellular metabolites were measured by GC-MS from mouse retinal homogenates (see "Experimental Procedures"). All amino acids, except aspartate, and TCA intermediates were decreased in retinas incubated without glucose (No Glc). TCA intermediates were elevated in retinas incubated without glucose but in the presence of mito-fuel mix (Mix) that contained pyruvate, malate, glutamine, and leucine. Metabolite levels are averages from four retinas exposed to glucose, no glucose, or mito-fuel mix for 90 min. The metabolite levels shown in the graph are normalized to levels in the glucose control retinas. Error bars represent S.E., and * indicates that a value is significantly different (p < 0.05) from the glucose control.

**Figure 5.** Mito-fuel mix increases NADH, mitochondrial polarization, and mitochondrial health in absence of glucose. A, NAD^+ -NADH were isolated from mouse retinas incubated with different metabolic conditions for 90 min. Glucose withdrawal (No Glc) led to a decrease in NADH. The mito-fuel mix (Mix) increased NADH beyond levels found with glucose. NADH reduction levels are an average of duplicate measurements from two separate retinas. Error bars represent the range, and * indicates that the value is significantly different (p < 0.02) from the glucose control. B, TMRM confocal microscopy was used to measure mitochondrial polarization in isolated mouse retinas. Removal of glucose caused a loss of TMRM signal that was almost as fast as treatment of retinas with the uncoupler FCCP (5 μM). The mix kept the TMRM signal at 50% of starting value. TMRM curves are an average of five separate experiments. Error bars represent S.E. Repeated measure ANOVA analysis showed the following: p < 0.02 for time having the same effect for all metabolites, that p < 0.08 for the effects of the metabolites being the same, and p < 0.0001 for time having no effect on the results. The value for the mix at 41 min is significantly different (p < 0.05) from the 41-min values for both no glucose and FCCP. C, mitochondria were isolated from mouse retinas after 90 min of incubation in different metabolic conditions. Removal of glucose led to a decrease in respiration ability of isolated mitochondria. The mix prevented some of the loss of mitochondrial respiration ability. O2 consumption measurements for each condition are an average of two (mix) to five (Glc) separate experiments. Error bars represent the range (for n = 2) or S.E. (for n > 2), and * indicates that a value is significantly different (p < 0.05) from the glucose control.

*Mito-fuel Mix Elevates NADH Levels, Increases Mitochondria Membrane Polarization, and Prevents Mitochondrial Damage—*How does the mito-fuel mix enhance PR viability? Mitochondria can initiate cell death (26), so we tested whether mitochondrial activity might be an important influence on PR survival in our experiments. We made three types of measurements to evaluate effects of glucose withdrawal on mitochondria. We measured the NADH/NAD^+ ratio, mitochondrial membrane potential, and respiratory activity of isolated mitochondria.

NAD^+ is reduced to NADH in the cytoplasm by glycolysis, and it is reduced in the mitochondrial matrix by the TCA cycle. We used an enzyme cycling assay to measure the percentage of total NAD in its reduced state in whole retina homogenates. The results in Fig. 5A indicate that glucose depletion may favor the oxidized state NAD^+. The mito-fuel mix substantially increases the reduction of NAD. Both GC-MS analysis (Fig. 4) and perfusion assay (Fig. 1) show that the amount of cytosolic NADH in the absence of glucose is too low to convert pyruvate into lactate or oxaloacetate into malate even when the mito-fuel mix is added. Therefore, all the NADH made when the mito-fuel mix was added in the absence of glucose must be in the mitochondrial matrix. This indicates that NADH was made by the TCA cycle faster than in glucose so that a higher steady state level of NADH/NAD^+ is established.

We also evaluated the effect of higher flux through the TCA cycle on mitochondrial membrane potential. We used confocal microscopy to monitor fluorescence from the membrane potential sensitive dye, tetramethylrhodamine methyl ester (TMRM). Retinas from Nrl-GFP mice were used in these experiments to identify the PR layer. Nrl-GFP transgenic mice express GFP only in rod photoreceptors (46). As expected, TMRM fluorescence from the cell bodies of PRs in mouse retinas decreases in response to the mitochondrial uncoupler FCCP, which validates TMRM as an approximate reporter of mitochondrial membrane potential (Fig. 4B). TMRM fluorescence also decreases when glucose is withdrawn. The presence of the mito-fuel can partially prevent the decrease in TMRM fluorescence. This is a qualitative analysis because the loading of TMRM depends on potentials across both the mitochondrial...
inner membrane and the cellular plasma membrane (49). Nevertheless, the results show that the mito-fuel alone can sustain the mitochondrial membrane potential. The residual decrease in TMRM accumulation under these conditions likely reflects loss of plasma membrane potential caused by depletion of ATP (Fig. 3C).

We also evaluated the effect of glucose withdrawal on the stability of mitochondria. Mitochondria were isolated from mouse retinas that had been incubated for 90 min with various metabolic fuels. The ability of the isolated mitochondria to consume O$_2$ was measured in an Oroboros Oxygraph. Mitochondria isolated from retinas that had been deprived of glucose during the 90 min of incubation had a respiration rate 40% lower than mitochondria from retinas incubated with glucose (Fig. 5C). Mitochondria isolated from retinas incubated without glucose but with the addition of the mito-fuel mixture had only a 20% decrease in respiration rate.

**Mito-fuel Mix Alone Cannot Sustain Photoreceptors during Long Periods of Glucose Withdrawal**—The experiments described so far show that the mito-fuel mix promotes PR survival during glucose withdrawal for 90 min. This enhanced survival correlates with increased membrane potential but not with sustained ATP levels. To determine whether the mix can sustain PRs for longer times, we used PI to monitor cell death after 4 and 8 hr of glucose deprivation (Fig. 6A). After these incubation times, the mito-fuel mix was substantially less effective at reducing cell death than it was after only 90 min. Although sustained mitochondrial activity enhances short term survival, an additional type of activity provided by glucose metabolism is required for longer term survival.

**N-Acetylglucosamine Enhances PR Survival**—One possible mechanism by which glucose specifically enhances survival is through protein glycosylation. GlcNAc, a substrate for protein N- and O-linked glycosylation, is required for proper function and trafficking of membrane proteins and for cellular signaling. Synthesis of GlcNAc requires glucose, so we evaluated the effect of supplying GlcNAc to glucose-deprived retinas (Fig. 6B). The addition of 15 mM GlcNAc completely suppressed cell death after 90 min of glucose deprivation. As with the mix, the effect was most pronounced only at 90 min. It was much less effective after 4 and 8 hr of glucose withdrawal. The combination of GlcNAc and the mito-fuel mix did not further decrease cell death either alone or in combination with the mito-fuel mix.

We made several types of measurements to determine how GlcNAc prevents PR death. It does not contribute to oxygen consumption (Fig. 7A), lactate production (Fig. 7B), or cytochrome c reduction (Fig. 7C). It also does not restore ATP in retinas incubated without glucose (Fig. 7D). These findings show that the glucose portion of GlcNAC cannot be used for glycolysis. To test whether GlcNAc promotes survival by supporting N-linked protein glycosylation, we measured PI-positive nuclei in retinas incubated with GlcNAc and tunicamycin, an inhibitor of N-linked glycosylation. Tunicamycin blocks only ~10% of the short term (90 min) protective effect GlcNAc. However, after 4 hr of glucose withdrawal, it blocks all of the protection by GlcNAc (Fig. 7E).

![Figure 6. Mito-fuel mix not sufficient to prevent cell death without glucose over longer starvation periods.](image-url)

**Autophagy Prolongs PR Survival during Glucose Deprivation**—Macro-autophagy can delay neurodegeneration (50). Mouse PRs have components of the biochemical apparatus needed for macro-autophagy (51), and chaperone-mediated autophagy occurs during retinal degeneration (15). Therefore, we evaluated autophagy in mouse retinas deprived of glucose.

Using transmission electron microscopy (Fig. 8, A–C), we found that the PR cytoplasm is more disorganized and has less overall density when retinas are deprived of glucose. Mitochondria appear shriveled in glucose-deprived retinas and swollen in retinas treated with the mito-fuel mix. However, we observed double-walled vesicles, characteristic of ongoing autophagy, even in the presence of glucose as if macroautophagy occurs constitutively under our retina culture conditions (data not shown).
To confirm this, we evaluated autophagy more quantitatively by immunoblot analysis of the ubiquitously expressed microtubule-associated protein light chain 3 (LC3) (Fig. 8D). Mouse retina homogenates were probed with an anti-LC3 antibody, and the ratios of activated to nonactivated LC3 were compared. To slow autophagosome degradation, retinas were treated with the lysosome inhibitor chloroquine. Consistent with the EM analysis, we found evidence for active autophagy even in the presence of glucose or the mito-fuel mix. Glucose deprivation increased the LC3-II/LC3-I ratio by \( \frac{30}{100} \) (Fig. 8E).

Autophagy may enhance PR survival by breaking down non-essential components of the cell to convert them to substrates for energy production and anabolic activity. To test this, we used 10 mM 3-methyladenine (3MA) to block autophagy (52) during glucose deprivation and quantified cell death by PI-positive nuclei (Fig. 8F). 3MA did not alter cell viability in glucose-deprived retinas incubated with the mito-fuel mix at 90 min. However, at 4 h, retinas incubated with the mito-fuel mix and 3MA were indistinguishable from those incubated without any substrates. This shows that macroautophagy can provide essential material to PR cells to delay cell death during starvation.

**DISCUSSION**

In this study we sought to identify the most basic metabolic requirements of PRs in the retina and to understand why PRs die when these requirements are not met. Some of the methods we used allowed us to evaluate the specific effects of nutrients or nutrient deprivation on photoreceptors. Propidium iodide uptake was measured by microscopy, so we could use it as a specific indicator of photoreceptor cell death. We also specifically examined photoreceptors when we measured their morphology by electron microscopy. Other types of measurements evaluated effects of nutrients on all of the cells in the retina, including neurons and glia. Those types of measurements...
included O₂ consumption, lactate production, NADH, ATP, TCA intermediates, amino acids, and autophagy. Nevertheless, some of the findings from whole retina, such as the absence of lactate production during glucose deprivation and the extraordinarily low levels of ATP in the presence of 2-DG reflect unambiguously the metabolic activity of photoreceptors.

**FIGURE 8.** Glucose withdrawal depletes organelles from the cytosol and increases the incidence of organelles within vesicular structures. EM images of photoreceptor inner segments were taken after 4 h of incubation with glucose (Glc), no glucose (No Glc), or mito-fuel mix (Mix). 

A, glucose-treated retinas contained large mitochondria and strong electron density in the cytosol. B, glucose-deprived retinas contained many small and fractured mitochondria. Some mitochondria were also found within vesicular structures. Cells from these retinas showed a loss of electron density in the cytosol. 

C, mito-fuel mix-treated retinas contained large mitochondria but did appear to have lost some electron density in the cytosol. D, autophagy is active in all retinas but increases in response to glucose withdrawal. Mouse retinas were isolated and then incubated with glucose (G), no glucose (NG), or mito-fuel mix (M) for 4 h prior to homogenization in RIPA buffer. All retinas were treated with chloroquine (30 μM) to prevent autophagosome degradation. Retinal extracts were then separated on a 15% polyacrylamide gel. After transferring proteins onto PVDF, the membrane was probed with anti-LC3 II antibody. Activated LC3 was found in all three conditions. 

E, amount of LC3 I and LC3 II were quantified from the Western blots and are expressed as a ratio of LC3 II to LC3 I for each condition. Retinas deprived of all metabolites had the highest ratio. 

Bar graphs are ratio averages from three separate experiments. Error bars represent S.E., and none of the differences are statistically significant (p > 0.05). Inhibition of autophagy accelerates cell death during glucose deprivation. F, PI-positive nuclei in mouse retinal outer nuclear layer were measured using confocal microscopy. PI (100 μg/ml) was added to incubation buffer in the last 45 min of the incubation. For each condition, the images were averaged into z-stack projections. The autophagy inhibitor 3MA did not increase PI-positive nuclei in retinas incubated with glucose. 3MA also did not have an effect on retinas incubated with the mito-fuel mix for 90 min. 3MA treatment in retinas incubated with the mito-fuel mix resulted in a phenotype that was indistinguishable from retinas incubated with no metabolic substrates (No Glc) at 4 and 8 h. Each data point is an average of at least eight (636 × 636 μm) spots from two separate retinas. Error bars represent S.E. Repeated measure ANOVA analysis showed that p < 0.0001 for a time had the same effect for all metabolites, that p < 0.0001 for the effects of the metabolites was the same, and p < 0.0001 for a time had no effect on the results.
Roles of Glucose in Photoreceptor Survival

Two Phases of Cell Death

We identified two modes of cell death caused by glucose deprivation as follows.

Rapid Cell Death Caused by Mitochondrial Failure—Removal of glucose from the retina in the absence of any other nutrient causes mitochondrial depolarization and irreversible loss of OCR. This phase of cell death can be prevented by supplementing retinas with fuels for oxidative phosphorylation. Surprisingly, this beneficial effect was not due to elevation of cellular ATP. Mitochondrial polarization and activity are more important than ATP for preservation of cell viability during 90 min of glucose deprivation.

Slower Form of Cell Death That May Reflect Loss of Anabolic Building Blocks for Cellular Maintenance—Mitochondrial fuels cannot prevent this process. The onset of this form of cell death can be delayed by autophagy, which redistributes nonessential cytoplasmic material into material essential for maintenance of cellular integrity.

Short Term Survival

Previous studies showed that retinas require a constant supply of glucose because they do not have large energy reserves (53, 54). Consistent with this, we found temperature-dependent, irreversible loss of O₂ consumption within minutes of removing glucose from the perfusion medium (Fig. 1). This suggests that cells could be damaged by depletion of cellular metabolites. We asked whether the damage could be prevented by mitochondrion-specific fuels. We found that a mix of pyruvate, malate, leucine, and glutamine can prevent loss of O₂ consumption during 90 min of glucose withdrawal (Fig. 2).

We measured cell death in the PR layer of the retina by quantifying fluorescent nuclei of cells that cannot exclude PI. Removal of glucose for 90 min caused an ~6-fold increase in PI-positive nuclei (Fig. 3). The mito-fuel mix suppresses this cell death but does not restore ATP to its glucose level. This confirms the important contribution of glycolysis to ATP production in the retina (17, 18). This series of experiments also demonstrates that high ATP concentrations are not always required for photoreceptor survival. Remarkably, the mito-fuel mix prevents cell death even when 2DG is used to deplete ATP below 5% of its normal level.

The rapid phase of PR cell death in response to glucose deprivation occurs only when mitochondria are inactive. Glucose withdrawal depletes TCA cycle intermediates, lowers the NADH/NAD⁺ ratio, and depolarizes mitochondria, whereas the mito-fuel mix elevates TCA intermediates, increases NADH/NAD⁺, and sustains the mitochondrial membrane potential.

We also found that short term survival of PRs can be enhanced by GlcNAc even when mitochondria are inactive. We showed by several criteria that exogenous GlcNAc does not contribute to glycolysis or respiration, so GlcNAc must be enhancing PR survival by supporting either N- or O-linked glycosylation of proteins. The importance of N-linked glycosylation of rhodopsin for PR function and survival is well established (55, 56). N-Linked glycosylation also is needed for cell surface expression of proteins needed for metabolism and signaling (28, 29, 57). We found that GlcNAc can support short term survival in the absence of glucose or the mito-fuel mix, and this effect is insensitive to tunicamycin. GlcNAc also enhances long term PR survival. In contrast to the short term effect, the long term activity of GlcNAc appears to require N-linked glycosylation because it is blocked by tunicamycin. The differences in tunicamycin sensitivity of short and long term survival indicate that a different type of activity of GlcNAc, such as O-linked glycosylation, is responsible for its ability to enhance short term survival (58).

Long Term Survival

Active mitochondria alone are not sufficient for long term survival of PR cells. The mito-fuel mix is less effective at blocking cell death beyond 90 min, suggesting that other aspects of glucose metabolism are required for long term survival.

A previous study showed that autophagy can be activated in PRs in response to stress (39). Our EM analysis of photoreceptors from retinas incubated 4 h with glucose, no fuel, or mito-fuel mix revealed that glucose withdrawal causes changes in mitochondrial morphology and loss of electron density in the cytosol. However, we detected what appear to be autophagic vesicles under all conditions. Immunoblot analysis of retinal extracts confirmed that the activated form of LC3 is present after 4 h of incubation, regardless of the type of fuel provided to the retina. Autophagy enhances long term survival of PRs, as the autophagy inhibitor, 3MA, increased the number of PI-positive PRs in retinas provided with the mito-fuel mix. This finding confirms that glucose is needed to provide more for the retina than fuel for mitochondrial activity and ATP production. The requirement for autophagy in the absence of glucose indicates that under normal conditions glucose also provides substrate for synthesis of building blocks required for cell maintenance and repair.

These studies show that mitochondrial activity, protein glycosylation, and anabolic building blocks are fundamental needs of PR cells. Genetic or environmental stresses that make it impossible for these needs to be met will cause PRs to die.

Acknowledgments—We thank Barry Winkler, Ken Lindsay, and Jack Saari for insightful discussions and Alok Tewari for advice on statistical analyses.

REFERENCES

1. Farber, D. B., and Lolley, R. N. (1974) Science 186, 449–451
2. Fuchs, S., Nakazawa, M., Maw, M., Tamai, M., Oguchi, Y., and Gal, A. (1995) Nat. Genet. 10, 360–362
3. Ramamurthy, V., Roberts, M., van den Akker, F., Niemi, G., Reh, T. A., and Hurley, J. B. (2003) Proc. Natl. Acad. Sci. U.S.A. 100, 12630–12635
4. Yamamoto, S., Sippel, K. C., Berson, E. L., and Dryja, T. P. (1997) Nat. Genet. 15, 175–178
5. Mlyngaragam, G. H., McGee, T. L., Berson, E. L., and Dryja, T. P. (2006) Mol. Vis. 12, 1496–1498
6. Travis, G. H. (1998) Am. J. Hum. Genet. 62, 503–508
7. Wenzel, A., Grimm, C., Samardzija, M., and Remé, C. E. (2005) Progr Retin. Eye Res. 24, 275–306
8. Doonan, F., Donovan, M., and Cotter, T. G. (2005) Invest. Ophthalmol. Vis. Sci. 46, 3530–3538
9. Goebel, D. J., and Winkler, B. S. (2006) J. Neurochem. 98, 1732–1745
