Phosphatase control of 4E-BP1 phosphorylation state is central for glycolytic regulation of retinal protein synthesis

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Gardner TW, Abcouwer SF, Losiewicz MK, Fort PE. Phosphatase control of 4E-BP1 phosphorylation state is central for glycolytic regulation of retinal protein synthesis. Am J Physiol Endocrinol Metab 309: E546–E556, 2015. First published July 21, 2015; doi:10.1152/ajpendo.00180.2015.—Control of protein synthesis in insulin-responsive tissues has been well characterized, but relatively little is known about how this process is regulated in nervous tissues. The retina exhibits a relatively high protein synthesis rate, coinciding with high basal Akt and metabolic activities, with the majority of retinal ATP being derived from aerobic glycolysis. We examined the dependency of retinal protein synthesis on the Akt-mTOR signaling and glycolysis using ex vivo rat retinas. Akt inhibitors significantly reduced retinal protein synthesis but did not affect glycolytic lactate production. Surprisingly, the glycolytic inhibitor 2-deoxyglucose (2-DG) markedly inhibited Akt1 and Akt3 activities, as well as protein synthesis. The effects of 2-DG, and 2-fluorodeoxyglucose (2-FDG) on retinal protein synthesis correlated with inhibition of lactate production and diminished ATP content, with all these effects reversed by provision of α-mannose. 2-DG treatment was not associated with increased AMPK, eEF2, or eIF2α phosphorylation; instead, it caused rapid dephosphorylation of 4E-BP1. 2-DG reduced total mTOR activity by 25%, but surprisingly, it did not reduce mTORC1 activity, as indicated by unaltered raptor-associated mTOR autophosphorylation and ribosomal protein S6 phosphorylation. Dephosphorylation of 4E-BP1 was largely prevented by inhibition of PP1/PP2A phosphatases with okadaic acid and calyculin A, and inhibition of PPM1 phosphatases with cadmium. Thus, inhibition of retinal glycolysis diminished Akt and protein synthesis coinciding with accelerated dephosphorylation of 4E-BP1 independently of mTORC1. These results demonstrate a novel mechanism regulating protein synthesis in the retina involving an mTORC1-independent and phosphatase-dependent regulation of 4E-BP1.

retinal protein synthesis; glycolysis; Akt/mTOR pathway; protein phosphatases

NORMAL RETINAL FUNCTION requires high cellular metabolic activity (3, 47) to perform phototransduction, regenerate photoreceptor outer segments, maintain electrical gradients across membranes of unmyelinated neuronal axons, and conduct bidirectional macromolecular transport through ganglion cell axons to the lateral geniculate nucleus of the thalamus. Moreover, unlike skeletal muscle, liver, or adipose tissue, the retina has little capacity to locally store protein, glycogen, or lipids to respond to fluctuations of metabolic demands, which makes it highly dependent on glucose availability. We (5) previously suggested that the high metabolic activity required to maintain visual function coupled with a low intrinsic storage capacity may compromise the retina’s adaptability to metabolic stress.

The metabolic features of the retina include an exceptionally high dependence on glycolysis, with 90% of retinal ATP derived from glycolysis vs. 50% in brain (49, 50). This feature parallels the high basal insulin receptor and Akt kinase activities in the retina, which are approximately twice that of insulin-sensitive tissues such as liver and skeletal muscle (38). Akt is a central regulator of the Warburg effect observed in tumors (40), which also exhibit a high rate of aerobic glycolysis to generate macromolecules required for cellular proliferation. These observations suggest that postmitotic cells of the retina operate under metabolic conditions resembling the Warburg effect that typically characterizes proliferating embryonic (2) or malignant cells (46). Warburg’s group first observed this similarity and disregarded retinal aerobic glycolysis as an experimental artifact (22). However, subsequent studies confirmed that retinal ATP levels, ion transport, and continued electrical activity are highly dependent on aerobic glycolysis (4, 49–51). Cusson et al. (7) recently hypothesized that the retinal Warburg-like effect could be attributed to the generation of photoreceptor outer segments, a process resembling the G1 growth phase in proliferating cells. Also consistent with that hypothesis, the profile of oxygen partial pressure across the retina shows high levels near the choroid with a dramatic decline across the photoreceptor inner segments and very low levels across the inner retina (8). These data suggest that the photoreceptor inner segments, with their relatively high rate of oxidative phosphorylation, consume most of the oxygen diffusing from the choroidal circulation, thus requiring cells in the inner retinal layers to exist in a relatively hypoxic environment that would favor reliability on glycolysis. These similarities between neoplastic and retinal cells, recently summarized by Ng et al. (35), led us to hypothesize that the dependence on glycolysis and relatively high basal Akt activity in the normal adult mammalian retina are related, such that Akt maintains a high basal rate of glycolysis to sustain retinal biosynthetic requirements and/or ATP production. As a consequence, diabetes-induced reduction of retinal insulin receptor and Akt activity (37, 39) may lead to reduced biosynthetic capacity essential for retinal physiology and thus compromise cell viability. In addition, altered metabolite profiles in the retina during diabetes may further disturb essential anabolic pathways and limit adaptive mechanisms (1).

We (13, 14) showed recently that both retinal insulin receptor signaling and excess glucose concentrations regulate retinal cell death and protein synthesis in diabetic rats and mice, thus revealing a clear interplay between growth factor and nutrient regulation of cell viability. In the present study, we undertook a specific assessment of the regulatory function of the Akt/mTOR pathway...
and glycolysis on retinal protein synthesis. Retinal protein synthesis was quantified using an ex vivo approach previously shown to yield equivalent results to in vivo analysis but allowing the controlled use of pharmacological agents. Thus, we were able to measure retinal protein synthesis in response to chemical inhibitors of Akt activity and glycolysis. Collectively, the data indicate that retinal protein synthesis is highly dependent on mTOR complex 2 (mTORC2), Akt1/3, and glycolysis. Inhibition of glycolysis using glucose analogs blocked retinal protein synthesis while causing a rapid dephosphorylation of eukaryotic initiation factor 4E binding protein-1 (4E-BP1) that, surprisingly, did not coincide with diminished mTORC1 activity but was largely prevented by inhibitors of protein phosphatases PP1, PP2A, and PPM1. This is one of the first studies to investigate the relationship of glycolysis to anabolic function in postmitotic neural tissue and thus provides new insights at the intersection of neurobiology and metabolism to understand the vulnerability of the retina to metabolic disturbances.

MATERIALS AND METHODS

Animals. All experiments were conducted in accordance with the Association for Research in Vision and Ophthalmology Resolution on the Care and Use of Laboratory Animals, and with approval of the University of Michigan Animal Care and Use Committee. Adult male Sprague-Dawley rats (Charles River, MA) were used in all experiments. Rats were housed under a 12:12-h light-dark cycle with free access to standard rat chow and water. At the time of isolation of the retinas, the rats were anesthetized with injection of 100 mg/kg pentobarbital sodium ip and euthanized by decapitation following motor reflex loss for rapid dissection of retinas. Retinas were either immediately placed in the appropriate culture medium or frozen in liquid nitrogen and stored at −80°C until analysis.

Protein synthesis analysis. Retinas were incubated in l-methionine-free Dulbecco’s modified Eagle’s medium (DMEM) containing 25 mM glucose for 30 min at 37°C in a 95% O2-5% CO2 atmosphere-free Dulbecco’s modified Eagle’s medium (DMEM) containing 1% FBS incubated. After methionine depletion, retinas were transferred into l-methionine-free DMEM containing 1 μCi/mmol l-[35S]methionine. Following incubation in the specific appropriate conditions described for each experiment, isolated rat retinas were snap-frozen in liquid nitrogen. Radiolabeled methionine incorporated into trichloroacetic acid-precipitable protein was immediately analyzed using dabsylation of the amino acid and radioactivity measurement. Protein determinations were made using the biuret method. Rates of protein synthesis were calculated using the method of Garlick et al. (15).

Protein activity measurements. For mTOR activity analysis, retinas were incubated for 2.5 h with inhibitors in DMEM containing 1% FBS at 37°C in 95% O2-5% CO2. Retinas were rinsed with cold Tris-buffered saline and flash-frozen. mTOR activity was then analyzed using the KLISA mTOR Activity Assay (EMD Millipore) according to the manufacturer’s instructions. Akt1 and Akt3 activity analyses were performed as previously described (42) on ex vivo retinas subjected to inhibitors for 30 min in DMEM containing 1% FBS at 37°C in 95% O2-5% CO2. Retinas were rinsed with 1× PBS and flash-frozen.

Metabolite measurements. Rate of glycolysis was estimated by measuring lactate accumulation in media of ex vivo retinas after incubation in conditions equivalent to those described above for protein synthesis experiments, with the exception of including unlabeled methionine throughout the experiment. Lactate concentrations were measured in 1:8 diluted media using the l-Lactate assay kit from BSRC (Biomedical Research Service Center, University at Buffalo) per their instructions.

Ex vivo retinas incubated as described above were otherwise processed to analyze ATP content. Following incubation, retinas were quickly rinsed with cold phosphate-buffered saline (PBS) and flash-frozen. Frozen retinas were homogenized in ice-cold PBS, lysates were diluted 1:5, and ATP concentrations were assayed according to the manufacturer’s microplate protocol (ATP Bioluminescence Assay Kit HS II, Roche).

Dephosphorylation analysis. For the in vitro phosphatase activity analysis, ex vivo retinas were incubated for 15 min with or without 25 mM 2-deoxy-d-glucose (2-DG) in DMEM with 1% FBS at 37°C in 95% O2-5% CO2. Retinas were rinsed with cold PBS and flash-frozen. Retinas were homogenized by sonication in phosphate lysis buffer [50 mM Tris, pH 7.0, 100 mM NaCl, 1 mM DTT, 1% Triton X-100, 0.1% deoxycholate, 0.5 mM EGTA, 0.1 mM EDTA, with protease inhibitor cocktail (Roche)] and then diluted 1:2 with either PP2A phosphatase buffer (50 mM Tris, pH 7.0, 100 mM NaCl) or total phosphatase buffer (50 mM Tris, pH 7.0, 100 mM NaCl, 1.5 mM CaCl2, 1.5 mM MgCl2, 0.3 mM MnCl2, 1.5 mM NiCl2, and 1.5 μM calmodulin). Aliquots of lysates were incubated at 37°C for 0, 7.5, 15, 30, 60, and 120 min. Lysates were then flash-frozen and analyzed by Western blot as described below.

For analysis of the effect of phosphatase inhibitors on ex vivo dephosphorylation, retinas were preincubated with 1.5 μM calcium or 1 μM okadaic acid in DMEM with 1% FBS for 30 min at 37°C in 95% O2-5% CO2 followed by addition of 25 mM 2-DG for an additional 30 min. Retinas were rinsed with cold PBS and flash-frozen. Lysates were then analyzed by Western blot.

Western blot analysis. Retinas were homogenized by sonication in mTOR lysis buffer (from K-LISA mTOR activity Assay, EMD Millipore). Protein concentrations were measured with the BCA (bicinchoninic acid) protein assay (Bio-Rad, Hercules, CA), and all samples were adjusted for equal protein concentration. Retinal lysates were used for immunoblot analysis as previously described (30), using the following antibodies (Table 1): phospho-4E-BP1 from Millipore (Billerica, MA), PKKc, raptor, rictor, mTOR, phospho-mTOR (Ser2448), 4E-BP1, phospho-4E-BP1 (Thr37/46), S6 ribosomal protein, phospho-S6 (Ser235/236), and phospho-S6 (Ser240/244) from Cell Signaling (Boston, MA). Results were normalized by reprobing the same membrane with an antibody against β-actin (Millipore). Quantification was performed by measuring fluorescence of Cy3- and Cy5-conjugated secondary antibodies using a Typhoon FLA9000 Imager (GE Healthcare Lifesciences) or luminescence reaction (ECL Prime, GE Healthcare Lifesciences) of HRP-conjugated antibodies, using a FluorChem M Imager (ProteinSimple). Digital images were quantified using ImageQuant TL (GE Healthcare Lifesciences).

Table 1. Information on the antibodies used in this study

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Coimmunoprecipitation. For immunoprecipitation of mTORC complexes, 500 µg of total retinal lysates (prepared as for immunoblotting) were incubated 2 h at 4°C with a selected mouse monoclonal antibody targeting rictor or raptor (Santa Cruz Biotechnology, Santa Cruz, CA). Protein G-Sepharose beads (GE Health Care, Piscataway, NJ) were then added and incubated for 1 h at 4°C. The immune complex was then collected by centrifugation at 1,500 g, washed three times with ice-cold mTOR buffer, and eluted from beads by addition of electrophoresis sample buffer and heating at 70°C for 10 min. Immunoblotting was then performed with antibodies to p-Ser2481-mTOR, total mTOR, raptor, and/or rictor.

Reagents. Glycolytic (2-DG, 2-FDG), oxidative phosphorylation [antimycin A (AA)], and phosphatase inhibitors (calyculin A, okadaic acid, and cadmium), l-fructose, l-mannose, and d-mannose were from Sigma. Akt inhibitors (triciribine, Akt inhibitor VIII, and Akt inhibitor XII) and protein biosynthesis inhibitor (cyclohexamide) were purchased from Calbiochem.

Statistical analysis. ANOVA models with heterogeneous variances, adjusted for the replication of the experiment, were fitted to the data to assess differences between the experimental groups (in most cases: basal vs. 2-DG or 2-FDG and other treatments). Means ± SE and statistically significant differences are reported. Analyses were performed using non-repeated-measures ANOVA followed by the SNK test for multiple comparisons or t-test for a single comparison. Statistical correlations were computed by Pearson correlation calculations with a two-tailed P value (GraphPad Prism 6).

RESULTS

Retinal Akt activity is a key regulator of retinal protein synthesis. To test the hypothesis that high Akt activity is essential to maintain retinal protein synthesis, we examined the effect of Akt inhibitors on metabolic labeling of newly synthesized protein in ex vivo retinal cultures and compared it to cyclohexamide, a well-known inhibitor of protein biosynthesis. All three isoforms of Akt are expressed in the retina, although we have previously shown that unlike other insulin-sensitive tissues Akt1 and Akt3 are the most prominent (38). Since no isoform-specific inhibitor is available, we used multiple inhibitors with differing IC₅₀ values for the three isoforms to test their respective effects on retinal protein synthesis. Triciribine was used as a pan-specific inhibitor of all Akt isoforms, whereas Akt inhibitor XII and Akt inhibitor VIII (data not shown; EMD-millipore) were used as more isoform-selective Akt inhibitors (55). Doses effectively inhibiting Akt2 alone (1 µM Akt inhibitor XII) did not impair protein synthesis in ex vivo retinas. In contrast, doses affecting all three isoforms (30 µM Akt inhibitor XII and 2 µM triciribine) decreased retinal protein synthesis by up to 50% (Fig. 1A). This response coincided with a greater than 60% reduction in Akt3 activity. Interestingly, the effect of Akt inhibition on protein synthesis...
did not coincide with a reduction in lactate production (Fig. 1B) or retinal ATP content (data not shown), suggesting that Akt3 inhibition did not reduce retinal protein synthesis due to a loss of energy production. To test the relative independence of Akt and energy production, we tested the impact of blocking glycolysis and oxidative phosphorylation on retinal Akt activity. Inhibition of glycolysis with 2-DG or, to a lesser extent, inhibition of oxidative phosphorylation with AA, reduced Akt1 and Akt3 kinase activities (Fig. 1, C and D). These findings suggest that Akt3, and possibly Akt1, plays a central role in controlling retinal protein synthesis and that reduction of its activity is sufficient to reduce protein synthesis without extensive energy depletion.

**Glycolysis inhibition reduces retinal protein synthesis.** Inhibition of glycolysis and oxidative phosphorylation each diminished Akt3 activity, and direct inhibition of Akt3 reduced protein synthesis in dose-dependent fashions (Fig. 2A). 2-DG and the related glycolytic inhibitor 2-FDG are competitive inhibitors of glucose transporters and hexokinase, while the resulting products of hexokinase, 2-DG-6-phosphate and 2-FDG-6-phosphate, act as noncompetitive inhibitors of hexokinase (binding at the allosteric site) and as potent competitive inhibitors of phosphoglucone isomerase (20, 25, 52). Metabolism of D-mannose depends on hexokinase but bypasses phosphoglucone isomerase, which is inhibited by 2-DG-6-phosphate and 2-FDG-6-phosphate. Addition of D-mannose at 25 mM effectively reversed inhibition of protein synthesis by both 2-DG at 12.5 mM and 2-FDG at 6.25 mM (Fig. 2B). In contrast, addition of nonmetabolizable 25 mM L-mannose had no effect, whereas addition of fructose had a small but significant positive effect on protein synthesis in the presence of 2-DG (data not shown). As expected, both intracellular ATP (Fig. 2C) and the accumulation of extracellular lactate (Fig. 2D) were greatly reduced by both glycolytic inhibitors, and D-mannose prevented these reductions in manners that correlated directly with its effect on retinal protein synthesis. These results suggest that the effects of 2-DG on retinal protein synthesis are indeed due to its ability to prevent glycolytic flux, primarily through inhibition of phosphoglucone isomerase. Importantly, AA, which inhibits oxidative phosphorylation, had no impact by itself on either ATP content (Fig. 2C) or lactate production (Fig. 2D), sug-

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**Figure 2. Glycolysis and oxidative phosphorylation are critical for retinal protein synthesis.** A: protein synthesis was assessed in ex vivo retinas subjected to specific glycolytic [2-deoxyglucose (2-DG)] or oxidative phosphorylation inhibitors [antimycin A (AA)], demonstrating that glycolysis and oxidative phosphorylation are necessary for retinal protein synthesis in an additive manner (n ≥ 4/group; *significantly different from basal level: ***P < 0.001; **significantly different from 12.5 mM 2-DG or 2.5 μM AA alone: ###P < 0.001). B: reduction of protein synthesis by 2 glycolytic inhibitors [2-DG and 2-fluoro-2-deoxy-d-glucose (2-FDG)] was largely prevented by addition of D-mannos, but not L-mannose, demonstrating that this effect is due specifically to phosphoglucone isomerase inhibition (n ≥ 4/group; *significantly different from basal level: ****P < 0.001; *significantly different from 12.5 mM 2-DG or 2.5 μM AA alone: ###P < 0.001).
suggesting that glycolysis can sustain retinal protein synthetic activity, at least temporarily, under conditions of impaired oxidative phosphorylation. Together, these results clearly demonstrate the critical role of glycolysis in retinal anabolic activity (Fig. 2).

2-DG does not affect AMPK or eIF2α phosphorylation but causes dephosphorylation of 4E-BP1. We next determined whether the effects of glycolysis inhibition on protein synthesis was specific or part of broader stress responses. Depletion of ATP and accumulation of ADP and AMP cause activation of AMP-activated protein kinase (AMPK), which can subsequently inhibit protein synthesis by repressing mTORC1 phosphorylation of eIF4E binding protein-1 (4E-BP1) (6) and by directly phosphorylating the elongation factor eEF2 (19). In addition, under aerobic conditions, 2-DG can cause tumor cell toxicity through inhibition of N-glycosylation, causing endoplasmic reticulum (ER) stress, which is reversed by provision of d-mannose (24). Protein synthesis would be expected to be inhibited in response to ER stress by PERK-mediated phosphorylation of eIF2α, as part of the unfolded protein response (UPR) (11). We therefore examined the impact of treatment with 25 mM and 100 mM 2-DG on the phosphorylation of AMPK-Thr172 (indicative of its activation), eEF2-Thr56, eIF2α-Ser51, and 4E-BP1-Thr37/46 (Fig. 3). Because these effects are highly dynamic, they were examined in a time-course study. Activating AMPK phosphorylation was only slightly and transiently increased in ex vivo retinas, both with and without 2-DG treatment, with no apparent difference between control and 2-DG-treated retinas (Fig. 3, A and B). eIF2α phosphorylation increased slightly in control retinas within the first hour, whereas it was diminished in 2-DG-treated retinas. Similarly, phosphorylation of eEF2 was unaffected throughout the duration of the experiment in control retinas, whereas it started decreasing in response to 2-DG at the 2-h mark (Fig. 3, C and D).

In contrast, studies revealed a surprising and dramatic reduction of 4E-BP1 phosphorylation during the first hour of incubation under all treatment conditions, with the decrease being greatly accelerated by 2-DG in a dose-dependent fashion (Fig. 3A). After 1 h, a marked difference in 4E-BP1-Thr37/46 phosphorylation was observed between control and 2-DG-treated retinas, with it being nearly undetectable in the 2-DG-

Fig. 3. Glycolysis inhibition of retinal protein synthesis is not due to stress-induced initiation or elongation arrest. Time courses of AMPK, initiation factor eIF2α (A), binding protein 4E-BP1 (B), and elongation factor eEF2 (C) phosphorylation were assessed in lysates from ex vivo retinas subjected or not to glycolytic inhibitors (2-DG). The pattern of phosphorylation of AMPK does not show any increase in the 2-DG-treated retina. Glycolytic inhibition did not lead to a dramatic increase of phosphorylation of eIF2α compared with the untreated retinas (A), whereas 4E-BP1 phosphorylation was dramatically reduced within the 1st hour following glycolysis inhibition (B; *phospho-bands quantified; *co-running with total). C: consistent with the absence of induction of AMPK, glycolytic inhibitors did not induce any rapid increase in phosphorylation of eEF2 but rather a decrease at the latest time analyzed (n = 3/group).
treated retinas. These data suggest that inhibition of protein synthesis by 2-DG coincided with 4E-BP1 dephosphorylation rather than UPR activation and that 2-DG treatment caused dephosphorylation of 4E-BP1 in an AMPK-independent manner.

Glycolysis inhibition modestly alters mTOR signaling in the retina. In response to anabolic stimuli, protein synthesis is increased by Akt-mediated mTORC1 activation that in turn phosphorylates 4E-BP1 and alleviates its suppressive effect on eIF4E (26). Because 2-DG diminished both Akt activity and 4E-BP1 phosphorylation, we examined its effects on the mTOR pathway. We compared the effects of 2-DG, 2-FDG, and Akt inhibitors on phosphorylation of 4E-BP1 at Thr37/46 and S6 ribosomal protein (the other best-characterized substrate of mTORC1) at Ser240/244 and Ser235/236 compound sites. Both 2-DG and 2-FDG caused dramatic (>90%) loss of 4E-BP1 phosphorylation, whereas Akt inhibitors had no effect (Fig. 4A), even though the Akt inhibitors were present at concentrations that significantly inhibited protein synthesis (Fig. 1). Surprisingly, 2-DG, 2-FDG, and Akt inhibitors all had no specific effect on S6 phosphorylation (Fig. 4B). Although the amount of phosphorylated S6 was diminished in retinas treated with the glycolysis inhibitors, it reflected an equivalent reduction in the total amount of S6 protein. Further analysis of the time-dependent dephosphorylation of 4E-BP1 (Fig. 4C) and its correlation with retinal ATP levels (Fig. 4D) demonstrated that retinal ATP reduction followed a smooth decay that commenced within 5 min in both 2-DG-treated and untreated retinas and continued for 150 min. The decay was significantly accelerated in the presence of 2-DG. 4E-BP1 dephosphorylation exhibited a 5-min lag and then decayed more rapidly than ATP, with the majority of loss occurring over the next 25 min. Control retinas exhibited depletion of ATP and loss of 4E-BP1 phosphorylation, and both were greatly accelerated by 2-DG treatment. However, although 4E-BP1 dephosphorylation starts later than ATP depletion, a direct correlation between ATP loss and 4E-BP1 dephosphorylation was not observed (control: $r^2 = 0.7$, 2-DG: $r^2 = 0.75$; both nonsignificant).

Both 4E-BP1 and S6 showed different susceptibilities to glycolysis and Akt inhibition, so we directly analyzed total (mTORC1 and mTORC2) mTOR kinase activity per the method used in our recently published study (13). Total mTOR activity was significantly reduced by ~25% by 2-DG treatment (Fig. 5). In contrast, treatment of retinas with Akt inhibitors and AA at doses that significantly inhibit protein synthesis (Fig. 1) had no significant effects on total mTOR activity. The effects of 2-FDG on active mTOR in mTORC1 and mTORC2 complexes were further examined by assaying phosphorylation of the mTOR autoactivation site Ser2481 (13) following raptor and rictor protein coimmunoprecipitation, respectively (Fig. 6A). Similar to the effects of 2-DG on total mTOR activity, 2-FDG lowered raptor- and rictor-associated mTOR phosphorylation by 9% ($P < 0.047, n = 7$) and 33% ($P = 0.047, n = 7$), respectively. Rapamycin predictably affected only raptor-associated mTOR. Akt inhibitors seem to selectively reduce raptor-bound mTOR phosphorylation, but only

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**Fig. 4.** Glycolysis inhibition specifically decreased 4E-BP1 phosphorylation, whereas Akt inhibition did not. Analysis of phosphorylation of known regulators of protein synthesis and downstream targets of mTORC1, 4E-BP1 (A; *phospho-bands quantified; #co-running with total) and S6 ribosomal protein (B) show that glycolysis had a much more dramatic effect on 4E-BP1 phosphorylation than Akt inhibition. Interestingly, both glycolysis and Akt inhibition reduced the content of S6 ribosomal protein without reducing its relative phosphorylation ($n \geq 3$ group). Time course of 4E-BP1 phosphorylation (C) as well as correlated ATP levels (D) were assessed in lysates from ex vivo retinas subjected or not to glycolytic inhibitors [2 μM triciribine (Tri), 25 mM 2-DG, 6.25 mM 2-FDG; $n \geq 2$ group; statistically significant correlation, ***$P < 0.001$].
when all three isoforms were inhibited (Fig. 6A). We then tested the effects of 2-DG and 2-FDG on phosphorylation of the mTORC2 substrate PKCα on Ser657. The inhibitors had a slight effect on PKCα phosphorylation, whereas in the same retinas they completely abolished phosphorylation of Akt at Ser473 (also an mTORC2 substrate) and Thr308 (a PDK-1 substrate) (Fig. 6B). A high dose (10 μM) of Torin-1, expected to completely inhibit mTORC2 (16), had similar effects on PKCα and Akt phosphorylation, whereas rapamycin (an inhibitor of mTORC1) had no effects. Akt inhibitors also diminished Akt phosphorylation in the order: Akt2 < Akt1/2 < Akt1/2/3 but did not affect PKCα phosphorylation.

This signaling pathway is further demonstrated by the relationship between 4E-BP1 phosphorylation and mannose supplementation. α-Mannose greatly prevented the reduction in ATP and lactate production by retinas but only partially prevented the glycolytic inhibitor-induced reduction in retinal protein synthesis (Fig. 2). Interestingly, mannose also partially restored 4E-BP1 phosphorylation, further demonstrating that glycolytic inhibition of retinal protein synthesis may be, at least partially, energy independent (Fig. 7A).

**Glycolysis regulates 4E-BP1 through phosphatase activity.** 4E-BP1 phosphorylation progressively decreases in untreated ex vivo retinas, and glycolysis inhibition accelerates the process, leading to almost complete dephosphorylation by 1 h (Fig. 3). In addition, glycolysis inhibitors 2-DG and 2-FDG exhibited dramatic effects on protein synthesis and 4E-BP1 phosphorylation while exhibiting very limited effects on other measures of mTORC1 activity. We therefore examined the possibility that loss of 4E-BP1 phosphorylation was the result of accelerated dephosphorylation rather than decreased mTORC1 kinase activity. To test this hypothesis, we performed coinubation with inhibitors of either the PPM1 family (cadmium) or the PP1/PP2 family (okadaic acid) of phosphatases, both of which have been suggested to regulate the phosphorylation of 4E-BP1 in other conditions (29, 33). Addition of either phosphatase inhibitor, cadmium or okadaic acid, normalized 4E-BP1 phosphorylation under normal and glycolysis-inhibited conditions (Fig. 7B), confirming that both

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**Fig. 5.** Inhibition of glycolysis but not Akt or oxidative phosphorylation reduces retinal mTOR signaling. Total mTOR activity was assessed in ex vivo retinas subjected to specific glycolytic (2-DG), oxidative phosphorylation (AA), or Akt inhibitors. Retinal mTOR activity measurement demonstrated that mTOR activity was significantly reduced in the retina only in response to glycolytic inhibition (n = 4/group; *significantly different from basal level; **P < 0.01).

**Fig. 6.** Glycolysis inhibition dramatically alters Akt signaling while only slightly affecting mTORC2 in the retina. A: specific activity of mTORC1 and mTORC2 complexes was assessed by immunoprecipitation of rictor and raptor followed by assessment of bound mTOR activity using an antibody against p-Ser2481. Glycolytic inhibitors moderately reduced both raptor-associated mTOR phosphorylation (L, left) and rictor-associated mTOR phosphorylation (A, right), consistent with the partial reduction in total retinal TOR activity. B: analysis of PKCα phosphorylation, a specific target of mTORC2, confirmed the impact of glycolysis inhibition, but not Akt inhibition, on this pathway. Additionally, analysis of Akt activation using the consensus sites Thr308 and Ser373 showed that both were dramatically affected by glycolysis and specific Akt inhibition (L, original lysate; No ab, no antibody control; IP: C, control condition; Rap, 100 nM rapamycin; 2-DG, 25 mM; 2-FDG, 6.25 mM; n = 7/group).
families of phosphatases are key regulators of 4E-BP1 phosphorylation in the retina. Interestingly, the pattern of phosphorylation suggests that okadaic acid, and thus PP1/PP2 phosphatases, primarily and more effectively affect the slower running phospho-isoform of 4E-BP1, whereas cadmium, and thus PPM1 phosphatases, seem to affect the faster migrating phospho-isoform. These results suggest perturbation of sequential 4E-BP1 dephosphorylations by different classes of phosphatases, with a slightly greater role of the PP1/PP2 family.

Overall, these data suggest that inhibition of glycolysis affects retinal protein synthesis through Akt and 4E-BP1, but by using alternative mechanisms of regulation, especially involving accelerated dephosphorylation of 4E-BP1, rather than deactivation of mTORC1.

**DISCUSSION**

The mechanisms that regulate protein synthesis in the normal mammalian retina have received little attention. This study tested the hypothesis that high basal protein synthetic rate observed in normal retinas is linked to proportionately high Akt activity and glycolytic rate. The results reveal the following. 1) Inhibition of Akt activity (particularly Akt1 and Akt3) significantly reduced protein synthesis without affecting glycolytic lactate production; 2) glycolysis inhibition with glucose analogs reduced retinal protein synthesis with concurrent reductions in lactate production and ATP content, effects that were reversed by provision of D-mannose; 3) glycolysis inhibition reduced Akt and total mTOR activities; 4) glycolysis inhibition caused rapid dephosphorylation of the translation initiation factor 4E-BP1 but had no effect on other measures of mTORC1 activity; and 5) loss of 4E-BP1 phosphorylation was largely prevented by PP1/PP2 or PPM1 inhibition. Together, these data reveal a previously unrecognized interplay between the Akt/mTOR pathway and glycolysis to control retinal anabolic activity and its regulation, presumably through 4E-BP1 dephosphorylation.

Akt plays a central role in control of anabolism in insulin-sensitive tissues such as liver and skeletal muscle (43, 53), but much less is known about its role in the retina. The retina is unique in that it must balance the requirement for acute vision against structural features that require a dual metabolic environment. The ganglion cells and inner plexiform layer reside in a relatively hypoxic environment with a Po2 ≈ 25 mmHg, whereas the photoreceptors are in a relatively oxygen-rich environment with a Po2 ≈ 80 mmHg (47). The retina has a high rate of glucose uptake, and 90% of glucose is metabolized to lactate by glycolysis (49, 50), providing the majority of retinal ATP. This high rate of glucose uptake is in line with a high anabolic activity, as reflected by the rate of protein synthesis, which is approximately twice that of skeletal muscle (13), and a high basal rate of Akt activity compared with muscle and liver (38). The present results suggest that Akt (principally Akt1 and Akt3) regulates retinal protein synthesis and that inhibition of glycolysis influences protein synthesis through a mechanism involving Akt but independent of mTORC1. Of note, these results in normal ex vivo retinas compare closely with the reduction of Akt and mTOR activities we observed in retinas from diabetic rats and mice (13, 39) and the lack of effect of diabetes on retinal mTORC1 activity. These results also strongly show the relevance of the ex vivo retina method for the investigation of in vivo pathophysiologival mechanisms of disease.

The sensitivity of retinal protein synthesis to inhibition of Akt was not due to energy depletion, because lactate production and ATP levels were unaffected by Akt inhibitors. More likely, the high level of Akt activity makes the retina highly dependent on glycolysis (48). Evidence for this concept has been shown in hematopoietic cells transfected with Akt that become dependent on glycolysis and are thusquisitively sensitive to glycolytic inhibition with 2-DG and undergo apoptosis (12). Thus, the retina exhibits many features of the Warburg effect, including high Akt activity and dependence on glycolysis for anabolic activities and ATP generation, as summarized recently by Ng et al. (35). The major distinction between embryonic or malignant cells and the retina is that neurons and glial cells are postmitotic. A link between glycolysis and protein synthesis in the central nervous system, specifically the brain, was first established by Lipton et al. (28), who showed...
that glycolysis was necessary for increased hippocampal neuron protein synthesis in response to neuronal activity stimulated by increased extracellular potassium.

In ex vivo retinal cultures, diminished protein synthesis caused by glucose analogs was closely correlated with inhibition of lactate production and lowering of ATP levels in the tissue (Fig. 2). Provision of α-mannose in the presence of 2-DG supported glycolysis and ATP levels as well as protein synthesis. It seems likely that retinal tissue is therefore shutting down protein synthesis in response to ATP depletion. Such a response may be an effort to conserve ATP and avoid an energy crisis. Inhibition of oxidative phosphorylation with AA also effectively blocked protein synthesis without affecting glycolysis. The additive effect of 2-DG and AA on protein synthesis and lactate production supported this supposition (Fig. 2A). However, the time course analysis of ATP and 4E-BP1 phosphorylation (Fig. 4) suggests that while ATP depletion begins 5 min prior to the decrease in phosphorylation of 4E-BP1, the decrease in 4E-BP1 phosphorylation is then much more rapid than ATP depletion. It cannot be excluded that rapid 4E-BP1 dephosphorylation is triggered once ATP falls below a certain level and then follows with an independent kinetics. Indeed, a direct test of the role of ATP depletion by its prevention is impractical in the ex vivo retinas, as exogenous additions of ATP or pyruvate do not rescue intracellular ATP levels. Alternatively, the absence of correlation between ATP depletion and 4E-BP1 dephosphorylation in the early phase of the time course suggests that 4E-BP1 dephosphorylation is largely ATP independent early on. Supporting the involvement of specific alternative mechanisms, established mechanisms of dampening protein synthesis in response to ATP depletion were not in evidence in the retinal tissue. For example, Maus et al. (32) found that inhibited protein synthesis in neuronal cultures treated with 2-DG or inhibitors of mitochondrial respiration coincided with ATP depletion, diminished mTOR activity, AMPK activation, and eEF2 phosphorylation. We found no evidence of increased AMPK activation or eEF2 phosphorylation in 2-DG-treated retinas (Fig. 3).

We also found that 2-FDG caused the same level of protein synthesis inhibition as 2-DG did at a twofold higher concentration (Fig. 2C). In addition, we did not find any evidence of eIF2α phosphorylation (Fig. 3), which would halt global protein translation in response to ER stress (11). These results are consistent with a mechanism of inhibition of protein synthesis by glucose analogs that involves inhibition on glycolysis rather than protein glycosylation. 2-DG and 2-FDG act as competitive inhibitors of glucose transporters and hexokinase, whereas the resulting products of hexokinase, 2-DG-6-phosphate and 2-FDG-6-phosphate, act as noncompetitive inhibitors of hexokinase (binding at the allosteric site) and as potent competitive inhibitors of phosphoglucose isomerase (20, 25, 52). 2-FDG is a more potent inhibitor of glycolysis than 2-DG because fluorine mimics hydrogen at the 2′ carbon so that the 2-FDG analog more closely resembles D-glucose (52). In turn, 2-DG is the better inhibitor of protein glycosylation because it is a better analog of α-mannose than 2-FDG and therefore forms 2-DG-GDP and 2-DG-dolichol, which competitively inhibit the transfer of mannose to N-acetyl-glucosamine (52). Under aerobic conditions 2-DG, but not 2-FDG, can cause tumor cell toxicity through inhibition of N-glycosylation, causing ER stress, which is reversed by provision of α-mannose (24). However, high levels of 2-FDG can inhibit subsequent protein glycosylation steps by competing with glucose (52). In this context, the absence of ER stress in response to both glucose analogs is consistent with the absence of impact on retinal cell survival (data not shown). The fact that α-mannose was able to effectively reverse the effects of 2-DG in the absence of ER stress further suggests that the glucose analogs are acting primarily by inhibition of phosphoglucose isomerase and not through inhibition of glucose transport or hexokinase, as glycolysis of α-mannose also depends on these latter activities. α-Mannose, enters cells via glucose transporters (18, 41) and is phosphorylated by hexokinase, but it enters into glycolysis via a separate feeder pathway, converging at fructose 6-phosphate via the isomerization of mannose 6-phosphate by phosphomannose isomerase (34).

To our knowledge, this is the first report of mTORC1-independent and AMPK-independent regulation of protein synthesis in response to inhibition of glycolysis. Zheng et al. (56) showed that 2-DG treatment of mouse embryo fibroblasts caused progressive loss of 4E-BP1 and p70 S6 kinase (S6K1) phosphorylation. However, these authors concluded that dephosphorylation of the two mTORC1 substrates was due to mTORC1 deactivation in response to energy depletion-induced AMPK activation, because AMPK negatively regulates mTORC1 by phosphorylating both TSC2 and raptor (44). This mechanism serves to halt cell cycle progression of mitotic cells in response to low energy (17). mTORC1 and AMPK may also counterregulate protein synthesis in postmitotic cells, as activation of AMPK blocks the Akt/mTORC1-dependent stimulatory effect of brain-derived neurotrophic factor (BDNF) on neuronal protein synthesis (21). Counterregulation of protein synthesis by mTORC1 and AMPK may also occur through the negative regulation of eEF2 by eEF2 kinase (eEF2K). eEF2K is inhibited by S6K1 downstream of mTORC1, while eEF2K is directly activated by AMPK (31, 45). This seems to be a protective stress response mechanism, since tumor cells lacking eEF2K are sensitive to nutrient deprivation and chemotherapeutic agents that target cellular energy metabolism (27, 44). The present results suggest that these responses are deficient in the retina, and therefore dephosphorylation of 4E-BP1 by phosphatase action may serve as an additional protection to prevent an energy crisis in retinal cells by shutting down 5′ cap-dependent translation, an effect that could be associated with previously reported changes in O-GlcNAcylation of 4E-BP1 in response to glucose levels (10). Such a response to an energy crisis could occur in the ischemic retina, for example during central retinal vein occlusion, central retinal artery occlusion, and retinal detachment.

Diverse phosphatases have been shown to be involved in the regulation of the Akt/mTOR signaling cascade in various cell types and tissues (9). For example, Akt-Thr408 is a substrate of PP2A (23, 36), and serine/threonine protein phosphatase PP2A plays a critical role in regulating mTOR activity in PC-3 prostate cancer cells (54). These data are consistent with our results and a potential central role of phosphatase proteins PP1/PP2 or PPM1 in the regulation of retinal metabolism via control of Akt and 4EBP1 phosphorylation.

In summary, this study demonstrates the importance of Akt and 4E-BP1 in the regulation of retinal metabolism and suggests a central role for protein phosphatases in the regulation of 4E-BP1 in response to glycolytic inhibitors. While further
studies are needed to determine the precise cell type, mediators, and mechanism involved in this response, the present work provides experimental support for the caution raised by Ng et al. (35) that glycolytic inhibitors for cancer chemotherapy could compromise retinal function.

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DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

REFERENCES


