Reductive carboxylation is a major metabolic pathway in the retinal pigment epithelium

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Edited by Martin Friedlander, The Scripps Research Institute, La Jolla, CA, and accepted by Editorial Board Member Jeremy Nathans October 24, 2016 (received for review March 20, 2016)

The retinal pigment epithelium (RPE) is a monolayer of pigmented cells that requires an active metabolism to maintain outer retinal homeostasis and compensate for oxidative stress. Using \textsuperscript{13}C metabolic flux analysis in human RPE cells, we found that RPE has an exceptionally high capacity for reductive carboxylation, a metabolic pathway that has recently garnered significant interest because of its role in cancer cell survival. The capacity for reductive carboxylation in RPE exceeds that of all other cells tested, including retina, neural tissue, glial cells, and a cancer cell line. Loss of reductive carboxylation disrupts redox balance and increases RPE sensitivity to oxidative damage, suggesting that deficiencies of reductive carboxylation may contribute to RPE cell death. Supporting reductive carboxylation by supplementation with an NAD\textsuperscript{+} precursor or its substrate \(\alpha\)-ketoglutarate or treatment with a poly(ADP ribose) polymerase inhibitor protects reductive carboxylation and RPE viability from excessive oxidative stress. The ability of these treatments to rescue RPE could be the basis for an effective strategy to treat blinding diseases caused by RPE dysfunction.

Reductive carboxylation | RPE | metabolism | age-related macular degeneration | oxidative stress

The retinal pigment epithelium (RPE) is a monolayer of postmitotic cells situated between the photoreceptors of the retina and the choroidal blood supply. The interaction of the RPE and photoreceptors is critical to maintaining vision. Functions of the RPE include phagocytosis of shed photoreceptor outer segments, recycling of retinoids, production and secretion of cytokines and chemokines, and mediating the exchange of nutrients and metabolites between the choroid and photoreceptors (1, 2). RPE cells provide crucial metabolic support for the retina.

RPE dysfunction can lead to photoreceptor death and retinal degenerative disease, such as age-related macular degeneration (AMD), which is the leading cause of irreversible vision loss in the elderly human population (3–5). RPE cells are exposed to ongoing oxidative stress from the combined effects of light, choroidal \(O_2\), polyunsaturated fatty acids, and retinoids (6). The resulting impairment in RPE energy metabolism and function by oxidative stress is one likely mechanism for the pathogenesis of AMD (3, 7–11).

Mitochondria support the active energy metabolism of the RPE (10–12). A recent report showed that RPE is less stable and less able to support the retina when it is forced to rely on glycolytic rather than mitochondrial metabolism (13). Another recent report supports the importance of mitochondria in RPE by showing that bolstering mitochondrial activity makes these cells more resilient to oxidative damage (14).

In mitochondria, citrate can be generated from acetyl CoA and oxaloacetate as part of the TCA cycle. However, under hypoxic conditions, some cells also produce citrate via reductive carboxylation of \(\alpha\)-ketoglutarate (\(\alpha\KG\)) through the action of NADPH-dependent isocitrate dehydrogenases (IDH) (15–17). Reductive carboxylation occurs in a small cohort of cells from liver, heart, brown adipocytes, and quiescent fibroblasts (18–20), where it supports redox homeostasis and synthesis of lipids, nucleotides, and urea (16, 18).

Reductive carboxylation of \(\alpha\KG\) to isocitrate by NADPH and CO\(_2\) can be catalyzed by IDH1 and IDH2, two of three isoforms of IDH. Overexpression of IDH1 or IDH2 can enhance protection from oxidative damage, whereas interference with their activities causes oxidation of glutathione and accumulation of reactive oxygen species (21–24). Loss of IDH1 and IDH2 activity also slows lipid synthesis and cell growth (25). IDH2 KO mice have dysfunctional mitochondria, poor redox homeostasis, and accelerated heart failure (26). Other studies have highlighted the importance of pyridine nucleotides, NAD\textsuperscript{+}/NADPH, and NADP\textsuperscript{+}/NADP\textsuperscript{H} in reductive carboxylation (15, 16). Pyridine nucleotides have diverse metabolic roles in differentiation, survival, and protection from oxidative stress (27).

We report here that RPE cells have an exceptionally high capacity for reductive carboxylation. They acquire it with maturation and use it for viability, redox balance, mitochondrial function, and response to oxidative stress. Reductive carboxylation in RPE is compromised when oxidative stress becomes excessive. Remarkably, supplementing with either an NAD\textsuperscript{+} precursor or an inhibitor of poly(ADP ribose) polymerase (PARP) protects reductive carboxylation and RPE cell viability from the effects of oxidative stress.

Results

There is an Unusually High Metabolic Flux Through Reductive Carboxylation in RPE. We quantified \textsuperscript{13}C-labeled metabolites from RPE cells that were fed \textsuperscript{13}C-glutamine for 1 h. The

Significance

In the vertebrate eye, a monolayer of cells, called the retinal pigment epithelium (RPE), is between the choroidal blood supply and the retina. The RPE provides metabolic support for the retina, including delivery of glucose and other nutrients. Here, we show that reductive carboxylation of \(\alpha\)-ketoglutarate, a type of metabolism that supports growth and survival of cancer cells, is a prominent feature of RPE cells. We show that extreme oxidative stress can overwhelm the reductive carboxylation pathway. However, we also found that the RPE can be protected from extreme oxidative stress by supplementation with an NAD\textsuperscript{+} precursor or \(\alpha\KG\).


The authors declare no conflict of interest.

This article is a PNAS Direct Submission. M.F. is a Guest Editor invited by the Editorial Board.

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1604572113/-/DCSupplemental.
Reduced carboxylation can be catalyzed by the NADP- and C(29) (Fig. S4 and Fig. S1) and NADPH within the cell. M4 and M5 are the two most abundant species of labeled citrate produced in these experiments (Fig. S2A). We performed this analysis with human fetal retinal pigment epithelium (hRPE) cells, human induced pluripotent stem cell (iPSC)-derived RPE cells, ARPE-19 cells (a human RPE cell line), cultured murine Müller glia, human cardiac endothelial cells (hCardiac ECs), HeLa cells (a cancer cell line), isolated mouse retina, and slices of murine neuronal tissues (cerebellum, hippocampus, and olfactory bulb). M4 is the major form of citrate made in the retina, other neuronal tissues, hCardiac ECs, and cancer cells (HeLa). In striking contrast, M5 is the major form in RPE (Fig. 1B).

The rate at which labeled citrate accumulates (Fig. S2B) shows that reductive carboxylation is more active than oxidation in hRPE, whereas oxidation is more active than reductive carboxylation in mouse retina. We also evaluated two other pathways that can generate cytosolic NADPH. Both malic enzyme activity (formation of M3 pyruvate from U-13C-glutamine) (Fig. S2B) and pentose phosphate pathway (PPP) activity [formation of M1 pyruvate from 1,2-13C-glucose (26)] (Fig. S2C) are minor compared with reductive carboxylation in hRPE cells.

Comparison of M5 citrate and M5 αKG in cells and tissues incubated with 13C-glutamine shows that reductive carboxylation is more active in RPE than in other cells and tissues (Fig. S2D). M3 malate, fumarate, and aspartate are made from M5 citrate produced by reductive carboxylation in the matrix and exported to the cytosol (Fig. S1). These indicators of reductive carboxylation are sixfold more abundant in RPE than in retina (Fig. S2F).

NADP-Dependent IDH2 Catalyzes Reductive Carboxylation in RPE Cells. Reductive carboxylation can be catalyzed by the NADP-dependent enzymes IDH1 and IDH2, which are distributed in the cytosol and mitochondria, respectively (Fig. S3A). IDH1 and IDH2 are approximately twofold more abundant in hRPE than in human fetal retina (Fig. S3B and C). In adult human tissue, IDH1 and IDH2 are abundant in RPE but nearly undetectable in the choroid (Fig. 1C). Oxalosomal OA, a competitive inhibitor of NADP-dependent IDH (20), inhibits reductive carboxylation of αKG to citrate in hRPE (Fig. 1D). M3 malate, M3 fumarate, and M3 aspartate are approximately threefold less abundant when OMA is present, and metabolites from the oxidative pathway increase slightly (Fig. S3 D and E). Inhibition of IDH activity by OMA increases the number of cells positive for MitoSOX red staining (Fig. S3F). However, OMA treatment has only minor effects on the steady-state levels of NADPH, NADPH, oxidized glutathione (GSSG), and glutathione (GSH) (Fig. S3 G and H). Interpretation of these findings is complicated by the absence of information about flux through the NADPH redox cycle and the distributions of pools of NADP+ and NADPH within the cell. Additional studies will be required to determine whether inhibition of IDH1 and IDH2 influences redox flux through NADPH, whether it causes compensatory changes from other NADPH pathways, and whether it affects cytosolic and mitochondrial distributions of NADPH and reduced glutathione.

We used siRNAs to diminish expression of IDH1 or IDH2 in hRPE cells (Fig. S4 A–C). Knocking down IDH2 restricts reductive flux to citrate (Fig. 1E), and it decreases formation of M3 malate, M3 fumarate, and M3 aspartate (Fig. 1F). There is no effect on the M2 isotopologues, indicating that IDH knockdown does not affect oxidative flux in the mitochondria (Fig. 1G). There is no effect of knocking down IDH1 on reductive flux from αKG to citrate (Fig. 1E). We suggest that this is because IDH1 does not catalyze the reductive reaction in the scheme in Fig. 2A and Fig. S1. Knocking down IDH1 makes more cytosolic M5 citrate available to form M3 malate and M3 aspartate (Fig. 1F and upper right of Fig. S1).

Reductive Carboxylation Contributes to Redox Homeostasis in RPE. Loss of reductase homeostasis can perturb mitochondrial bioenergetics and glucose metabolism. Together with nicotinamide nucleotide transhydrogenase (NNT), IDH2 helps convert mitochondrial NADH into NAD+ (29) (Fig. 2A). Knocking down IDH2 increases [NADH] and decreases [NAD+], whereas knocking down IDH1 only increases [NADH] (Fig. 2 B and C and Fig. S4D). Inhibiting both IDH1 and IDH2 by OMA substantially lowers the NAD+/NADH ratio (Fig. S4E). Consistent with the lowering of NAD+/NADH, we found that inhibiting IDH increases AMP/ATP and ADP/ATP (Fig. 2D and Fig. S4F). To examine whether loss of IDH activity influences glycolysis, we
NADP-dependent IDH activity influences NAD/NADH, mitochondrial bioenergetics, glycolysis, and lipid synthesis in hRPE. (A) Schematic illustrating NAD metabolism by NADP-dependent IDH. Blue arrows in mitochondria. Production of NAD+ (red) comes from reductive carboxylation in mitochondria and separately in the cytosol. (B and C) The effect of IDH1 or IDH2 knockdown on NAD+ and NADH/NAD levels relative to control (n = 3). (D) Knockdown of both IDH1 and IDH2 increases the AMP/ATP ratio. (E) Schematic illustration of U-13C glucose metabolism. Black circles represent the labeled carbons. (F and G) Knockdown of IDH2 inhibits glucose metabolism in hRPE. Decreased levels of M3 pyruvate and M2 citrate were determined by isotope analysis after incubating hRPE with 5 mM U-13C glucose for 15 min. (H) Schematic illustrating the role of IDH in palmitate synthesis from U-13C glucose. Black circles represent the labeled carbons. ACL, ATP citrate lyase. (I) The effect of IDH1 or IDH2 knockdown on the relative abundance of palmitate isotope distributions in hRPE cells (arbitrary units) derived from U-13C glucose incubated with hRPE for 48 h. (J and K) Knockdown of IDH1 increases labeled malate and aspartate from U-13C glucose, whereas IDH2 knockdown decreases labeled malate and aspartate. M3 and M1 isotope distributions include carbons that complete more than one TCA cycle. Mean ± SD (n = 3). *P < 0.05 vs. SiCon (nontarget siRNA control).

Reductive Carboxylation Contributes to Fatty Acid Synthesis in RPE. Fatty acids are made from acetyl-CoA produced by ATP citrate lyase (Fig. 2H). We incubated RPE cells with 2 mM U-13C glucose in DMEM with 1% FBS for 48 h and then, quantified 13C in palmitate. The RPE culture medium was replaced with DMEM, because standard RPE culture medium contains unlabeled glutamate and aspartate. Cells carbonylate acetyl-CoA into malonyl-CoA, which then contributes two carbons to each elongation cycle of fatty acid synthesis. Knockdown of IDH2 inhibits M2, M4, and M6 palmitate formation, whereas knockdown of IDH1 increases M2 palmitate (Fig. 2I and Fig. S4G). Inhibition of IDH1 causes accumulation of cytosolic citrate, which is broken down by citrate lyase into acetyl-CoA and oxaloacetate. Knockdown of IDH1 also causes malate and aspartate to accumulate (Fig. 2J and K). These findings support the model shown in Fig. 2H.

Reactive Carboxylation Increases as RPE Cells Mature. When RPE cells mature and differentiate, they shift to mitochondrial oxidative metabolism to support their specialized functions (30). We analyzed reductive carboxylation in hRPE cells at different stages of maturity in culture. RPE cells were either plated at varying densities and cultured for 1 wk or plated at a fixed density and cultured for up to 8 wk. Mature RPE cells have hexagonal morphology, tight junctions, and melanin pigment granules. These characteristics start to appear in hRPE seeded at 169,000 in a 35-mm dish after 1 wk in culture, although they are most pronounced when the cells are seeded at a density of 327,000 (Fig. S5 A−F). Similar hexagonal morphology and pigmentation appear in hRPE seeded at 100,000 and cultured for 4 wk and become more pronounced after 8 wk in culture (Fig. S5 G−L). Along with increasing cellular maturity, reductive flux from αKg to citrate increased by approximately twofold (Fig. S5 K and L) as did the M3 intermediates from the reductive pathway (Fig. S4M). M2 metabolites from the oxidative pathway did not increase, except at the very highest cell density (Fig. S4N).
phosphate (DHAP), and it increases levels of aspartate, α-hydroxybutyrate (3HBA), and GSSG. Remarkably, PJ34 completely blocks these effects (Fig. 3K).

GAP and DHAP accumulate, because GAPDH requires NAD⁺. Depletion of NAD⁺ by PARP also might affect other NAD⁺-dependent dehydrogenases (Fig. S6B). PJ34 counteracts the effect of H₂O₂ on lactate/pyruvate and 3HBA/acetoacetate ratios (Fig. S6 C and D). It also counteracts the effect of H₂O₂ on αKG/citrate (Fig. S6E), which influences reductive flux in cancer cells (35).

We treated hfRPE with 1 mM H₂O₂ in the absence or presence of PJ34 and monitored cell death by lactate dehydrogenase (LDH) in the culture medium (Fig. S7A) and by cell density (Fig. S7B–E). Cells exposed to H₂O₂ died within 48 h, but concurrent treatment with 100 nM PJ34 completely blocked H₂O₂-induced cell death. This finding indicates that RPE cell death from H₂O₂ is caused by depletion of NAD⁺ by PARP.

Oxidative Damage in RPE Is Enhanced When Reductive Carboxylation Is Inhibited. Because reductive carboxylation can produce NADPH needed to reduce glutathione, we asked whether impairment of the reductive pathway makes RPE cells more vulnerable to oxidative stress. We cultured hfRPE cells for 10 d with or without knockdown of IDH1 and IDH2. We then treated them with 1 mM H₂O₂ for 24 h and stained them with ethidium homodimer to quantify cell death (Fig. S8 A–F). IDH2 siRNA alone induces cell death threefold more than control siRNA. H₂O₂ alone increases cell death 65-fold (Fig. S8G). H₂O₂ with IDH1 knockdown did not cause additional death, but together with IDH2 knockdown, it increased death 95-fold. The findings suggest that IDH2 can help to protect RPE from oxidative stress.

αKG Protects RPE from Oxidative Stress. We reasoned that enhancing reductive carboxylation by providing excess αKG as a substrate for IDH2 could enhance resistance of RPE cells to H₂O₂. Fig. S8 H and I shows that 1 mM dimethyl α-ketoglutarate (dmαKG) protects hfRPE cells completely from the damaging effects of 1 mM H₂O₂.

An NAD⁺ Precursor Protects RPE from Oxidative Stress. Blocking PARP activity restores NAD⁺ and it overrides the effect of H₂O₂ on reductive carboxylation (Fig. 3). Another way to restore NAD⁺ is to supplement the culture medium with nicotinamide mononucleotide (NMN), an NAD⁺ precursor. Remarkably, supplementation with NMN completely prevents RPE cell death induced by H₂O₂ (Fig. 4 A–E), and it doubles the ratio of NAD⁺/NADH (Fig. 4F). NMN also counteracts the effect of H₂O₂ on glycolysis (Fig. S6D). However, it does not counteract the stimulatory effect of H₂O₂ on the low level of PPP activity present in RPE (Fig. S6E). Taken altogether, our findings indicate that IDH2-mediated reductive carboxylation is a pathway enriched in RPE cells that might enhance their resistance to oxidative stress (Fig. 4G).

Discussion

We report here that reductive carboxylation plays a major role in the energy metabolism of RPE cells. Reducing power stored in NADH in the mitochondrial matrix can be transferred to citrate by NNT and IDH2 (29). Citrate produced in the matrix can be transported to the cytoplasm, where IDH1 can use it to reduce cytosolic NADPH to NADP⁺ and enhance protection from oxidative stress (13, 14). We favor the idea of the IDH2-mediated pathway shown in Fig. 4G, because knockdown of IDH2 inhibits incorporation of 13C from glutamine into fatty acids. However, additional studies will be required to evaluate the contribution from an IDH1-mediated pathway (16) that can generate mitochondrial NADPH, such as occurs in tumor cell spheroids (17) and pyruvate dehydrogenase-deficient cells (36).

The high level of activity of reductive carboxylation in RPE may help to explain the importance of mitochondria in supporting RPE function and enhancing protection from oxidative stress (13, 14). The RPE provides glucose to the retina by transporting it from the choroid, and therefore, this process would be most efficient if the RPE consumes as little glucose for itself as possible. We suggest that reductive carboxylation is a way for RPE cells to generate NADPH without consuming glucose (Fig. 4G). Consistent with this suggestion, other pathways that produce cytosolic NADPH, the PPP, and malic enzyme, appear to be less active than reductive carboxylation in RPE cells.

RPE cannot survive long-term deficiency of NAD⁺ (37, 38), and a mutation in nicotinamide nucleotide adenyltransferase 1 (NMNAT1), the enzyme that converts NMN into NAD⁺, causes severe retinal degeneration in humans (39–41). We have shown that, when oxidative stress is excessive, PARP depletes NAD⁺.
This depletion blocks glycolysis and reductive carboxylation, because they require pyridine nucleotides.

An important implication of our findings is that failure of reductive carboxylation in the RPE could be a key factor in the pathogenesis of RPE-related diseases, such as AMD. Dysfunction of RPE mitochondria and increasing susceptibility to oxidative stress with age are linked to AMD (8, 11). Oral antioxidant supplements have been shown to have only modest effects on AMD disease progressions (42). In our studies, NMN, PJ34, and dmsKG restore reductive carboxylation and protect RPE cells from oxidative damage very effectively, and therefore, they may represent promising therapeutic targets for AMD.

Our findings about RPE metabolism suggest a hypothesis to explain why RPE dysfunction in diseases, like AMD (8), DNA damage in RPE mitochondria accumulates with age (8), and mismatches between respiratory complex subunits encoded by the mitochondrial and nuclear genomes can increase oxidative stress (43). Single-strand breaks in DNA can stimulate PARP to make PAR (44), and PARP activity is required for DNA repair in RPE cells (32). The negatively charged PAR facilitates DNA repair by loosening the structure of chromatin (45). NAD⁺ is consumed by PARP, but NAD⁺ also is a required substrate for Sirt1, a nuclear enzyme that deacetylates histones (46). Sirt1 activity slows when NAD⁺ is depleted by PARP (47), leaving histones more acetylated, which loosens chromatin structure even further.

Loosening of chromatin by accumulation of PAR and acetylated histones is important for DNA repair, but it also can allow genes that normally are inaccessible within heterochromatin to become more generally accessible to polymerases and transcription factors (48). We suggest that photoreceptors, specialized for aerobic glycolysis (49, 50), become less glycolytic, whereas RPE cells that rely on mitochondria for reductive carboxylation develop a more conventional metabolism. Loss of the unique and complementary metabolic features of these tissues may compromise the essential symbiotic relationship between the RPE and the retina. An attractive feature of this hypothesis is that each of its predictions will be testable with currently available technology.

Inhibiting PARP rescues RPE from oxidative stress. Inhibiting PARP also blocks DNA repair (32), and therefore, it is unlikely that PARP inhibitors could be an effective long-term therapy. However, we found that supplementation with an NAD⁺ precursor also is a very effective way to protect RPE cells from oxidative stress. This approach to therapy is likely to be more successful than PARP inhibition, because it can restore glycolysis, reductive carboxylation, and sirtuin activity without compromising DNA repair.

NAD⁺ declines with age (47, 51), and supplementation with NAD⁺ precursors can delay aging, liver disease, and vascular dysfunction (52–54). NAD⁺ precursors, such as nicotinamide riboside, can be used safely in humans (55, 56). In the absence of animal models for AMD, our findings highlight the need for clinical trials that evaluate NAD⁺ precursors for their ability to prevent and treat AMD.

Our study focused on identification and quantification of reductive carboxylation in RPE. Follow-up studies on RPE metabolism should investigate relative contributions of reductive carboxylation, malic enzyme activity, and the PPP to NADPH production. It also is important to determine how these pathways are regulated and how they are influenced by stress, aging, and disease states.

In summary, we found that reductive carboxylation is a prominent feature of healthy RPE cells. This metabolic pathway enhances the ability of RPE to maintain its cellular redox potential, and it contributes to lipid synthesis and possibly, resistance to oxidative stress. We found that excess oxidative stress depletes NAD⁺ in RPE cells, and supplementation with NAD⁺ precursors restores pyridine nucleotides and rescues RPE from cell death.

**Materials and Methods**

**Cell Culture.** Dissections of fetal tissue (16–18 wk gestation) to isolate RPE were performed following a previously published protocol (57). Use of fetal RPE cells as described did not qualify as human subjects research, as determined by the University of Washington Institutional Review Board. Isolated fetal RPE formed a confluent, pigmented monolayer of hexagonal cells after ~4 wk in culture. ARPE-19 cells were cultured in the RPE medium.

Cardiac ECs were obtained from Lonza and cultured according to the manufacturer’s protocol. Müller glial cells were isolated from mice as reported (58) and cultured in Neurobasal medium with 10% (vol/vol) FBS.

**Stable Isotope-Labeled Metabolite Analysis.** RPE cells and all other cells or tissues (Fig. 1B) were changed into Krebs-Ringer bicarbonate medium (59) with 5 mM glucose and 2 mM U-¹³C glucose for 1 h or 5 mM U-¹³C glucose for 15 min. The decrease in glucose concentration in the media during the course of those experiments was negligible. For fatty acid analysis, RPE cells were labeled with 2 mM U-¹³C glucose in DMEM with 1% FBS for 48 h. RPE cells were homogenized, and the metabolites were extracted, dried, derived with N-tert-butyldimethylsilyl-N-methyltrifluoroacetamide (TBDMDS), and analyzed by GC-MS (Agilent 7890/5975C) as described in detail (60–62).

Additional details and other materials and methods are in SI Materials and Methods. Research involving human subjects described in SI Materials and Methods was approved by the University of Washington Institutional Review Board, and informed consent was obtained.
We thank the University of Washington Vision Research Center (to S.W.), the National Institutes of Health (to A.R.), EY00641 (to J.B.H.), EY17883 (to J.B.H.), EY19174 (to J.R.C.), and EY001730 (to National Eye Institute Vision Research Core); the Bill & Melinda Gates Foundation (J.R.C.); and an unrestricted grant from Research to Prevent Blindness (to J.D. and J.R.C.).

ACKNOWLEDGMENTS. We thank the University of Washington Vision Research Center (to S.W.), the National Institutes of Health (to A.R.), EY00641 (to J.B.H.), EY17883 (to J.B.H.), EY19174 (to J.R.C.), and EY001730 (to National Eye Institute Vision Research Core); the Bill & Melinda Gates Foundation (J.R.C.); and an unrestricted grant from Research to Prevent Blindness (to J.D. and J.R.C.).