Stimulation of AMPK prevents degeneration of photoreceptors and the retinal pigment epithelium

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Retinal degenerative diseases are generally characterized by a permanent loss of light-sensitive retinal neurons known as photoreceptors, or their support cells, the retinal pigmented epithelium (RPE). Metabolic dysfunction has been implicated as a common mechanism of degeneration. In this study, we used the drug metformin in a gain-of-function approach to activate adenosine monophosphate-activated protein kinase (AMPK). We found that treatment protected photoreceptors and the RPE from acute injury and delayed inherited retinal degeneration. Protection was associated with decreased oxidative stress, decreased DNA damage, and increased mitochondrial energy production. To determine whether protection was a local or a systemic effect of metformin, we used AMPK retinal knockout mice and found that local expression of AMPK catalytic subunit α2 was required for metformin-induced protection. Our data demonstrate that increasing the activity of AMPK in retinal neurons or glia can delay or prevent degeneration of photoreceptors and the RPE from multiple types of cell-death triggers.

neuroprotection | metabolism reprogramming | mitochondria | oxidative stress | photoreceptor degeneration

Retinal degenerations are characterized by a progressive loss of photoreceptors or their support cells, the retinal pigmented epithelium (RPE). Retinitis pigmentosa (RP) is a group of inherited retinal diseases that have been linked to mutations in over 84 unique genes or genetic loci (1). Studies have shown that oxidative stress and metabolic dysregulation are involved in rod and cone photoreceptor death in RP (2). Several studies have suggested that developing therapies designed to increase glycolysis and increase glucose availability to photoreceptors may improve photoreceptor survival and function to halt disease progression (3–5). Age-related macular degeneration (AMD) is the most common form of retinal degeneration, and is a multifactorial disease that affects the macular region of the retina. More than 30 million individuals worldwide are affected by vision loss from AMD (6). Mutations in more than 20 genes and loci have been associated with increased risk of AMD (7, 8). However, age and smoking remain the two highest risk factors, which suggests that oxidative stress and reactive oxygen species production play a pivotal role in early disease progression (8). Mechanisms of AMD that have been studied extensively include elevated oxidative stress, altered mitochondrial bioenergetics, dysregulated RPE metabolic metabolism, and increased inflammation (9–11). These results suggest that regulating metabolic activity could improve survival and function of rods, cones, and the RPE in patients with retinal degeneration.

Adenosine monophosphate-activated protein kinase (AMPK) is an evolutionarily conserved serine/threonine kinase that functions as a heterotrimeric protein composed of a single catalytic α-subunit, regulatory β-subunit, and AMP-binding γ-subunit. Because there are two α-, two β-, and three γ-isoforms in mammals, there are 12 possible combinations and therefore 12 unique AMPK complexes (12). However, isotypes have different tissue distributions, suggesting that not all AMPK complexes exist in any one cell type. AMPK is a cellular energy sensor that regulates metabolic homeostasis and is activated by nutrient deprivation, low energy states, fasting, or hypoxia (13, 14). Once activated, AMPK is able to restore energy balance by inhibiting protein translation and fatty acid synthesis and is also able to switch on energy production by increasing glycolysis and promoting mitochondrial biogenesis (15, 16). The combined reduction in energy expenditure and increased energy production represents a significant metabolic reprogramming within cells. Metformin (1,1-dimethylbiguanide hydrochloride) is a Food and Drug Administration-approved drug and is widely prescribed for type 2 diabetes to control serum glucose levels. Metformin is thought to work through activation of AMPK signaling (17). Because of its ability to regulate metabolism, metformin is actively studied for protective therapies in aging, neurodegenerative disease, and cancer. Studies have shown that metformin protects cells in vivo from brain ischemia/reperfusion injury, cardiovascular disease, and statin-associated muscle symptoms (18–20).

In this study, we used metformin to determine whether stimulation of the AMPK pathway protects photoreceptors and the RPE from retinal degeneration. We tested metformin in three different mouse models of degeneration: a light-induced degenerative model, the *Pde6b*^{rd10} inherited retinal degeneration model, and a model of sodium iodate-induced RPE and retinal injury. We found that metformin was able to protect photoreceptors from light damage, delay rod and cone degeneration in the Rd10 model, and increase the resistance of the RPE to the injury. We also show that metformin's mechanism of protection was associated with increased mitochondrial biogenesis and reduced oxidative stress. Finally, using AMPKα conditional knockout mice, we observed that metformin-mediated protection required the expression of AMPKα2, but not AMPKα1, in the neural retina,

Significance

In this paper, we show that stimulation of adenosine monophosphate-activated protein kinase (AMPK) by metformin is able to protect photoreceptors and the retinal pigmented epithelium (RPE) in three different mouse models of retinal degeneration, including acute bright light damage, *Pde6b*^{rd10} inherited retinitis pigmentosa, and sodium iodate-induced RPE injury. The study suggests that activators of AMPK can have broad-spectrum protection in the retina to prevent loss of vision from acute injury, inherited retinal degeneration, and age-related macular degeneration.

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suggesting localized protection to the retinal cells. Our results suggest that targeting metabolic regulation by metformin has therapeutic potential to preserve vision, independent of the cause of disease.

Results

Metformin Protects Photoreceptors from Light-Induced Retinal Degeneration. In humans, the maximum daily metformin dose is 2,550 mg. Using a well-established conversion of human to mouse dose (*SI Appendix, Methods and Materials*), the maximum mouse dose was determined to be 524 mg/kg (21, 22). The human recommended maintenance dose (2,000 mg/d) was equivalent to 411 mg/ kg in mice. Based on these conversions, we dosed mice with a range of metformin from 300 to 500 mg/kg by s.c. injections. Metformin treatment did not alter retinal structure or function (Fig. 1 and *SI Appendix*, Fig. S1), demonstrating that the drug was not harmful to the retina. Since metformin can cross the blood–brain barrier (23), we chose a once-daily s.c. injection for delivery. To determine whether metformin could protect photoreceptors from an acute injury, we used a well-established light damage (LD) model to induce the death of rod photoreceptors (24). Albino mice exposed to 4,000 lx of white fluorescent light for 4 h had a loss of 50 to 75% of retinal photoreceptors in untreated mice and mice treated with vehicle (Fig. 1 and *SI Appendix*, Fig. S1). Mice given the highest dose of metformin (500 mg/kg) once per day for 7 d before light damage were almost fully protected from light damage, with significant preservation of both retinal structure and photoreceptor function (Fig. 1 and *SI Appendix*, Fig. S1). Metformin-induced protection was dose-dependent (Fig. 1). To determine whether metformin-induced protection required multiple days of treatment, mice were given single, daily metformin doses for 1, 2, 4, or 7 d before light exposure. We found that full protection from LD required at least 4 d of metformin injections (*SI Appendix*, Fig. S2).

Metformin-Induced Protection Is Mediated by Local Signaling and Acts Through the AMPK α2-Subunit. To determine whether protection was a function of local activity within the retina or systemic metabolic changes, we used intravitreal injection to deliver metformin directly to the eye 2 d before light damage. Intravitreal delivery prevented photoreceptor death and preserved photoreceptor function in light-damaged mice, suggesting that protection was dependent on local activity in the retina (*SI Appendix*, Fig. S3). To determine whether metformin crosses the blood–retina barrier, retinas were collected 2 h after s.c. metformin injections (300 mg/kg) and the concentration of metformin in the retina was measured by liquid chromatography-mass spectrometry (LC-MS) (25). Daily metformin injections yielded the same retinal levels of metformin 2 h post injection (Fig. 2*A*). Following a single injection, metformin levels in the retina remained constant for up to 8 h, and then declined rapidly (Fig. 2*B*). In addition, the single daily systemic injection of metformin resulted in rapid and sustained phosphorylation of AMPK in the retina (Fig. 2*C*). These findings confirmed that metformin can cross the blood–retina barrier to activate AMPK in retinal cells and is cleared from the retina over time. These results are consistent with metformin pharmacology in humans, where metformin's half-life in serum is approximately 5 h and it is excreted in urine (26).

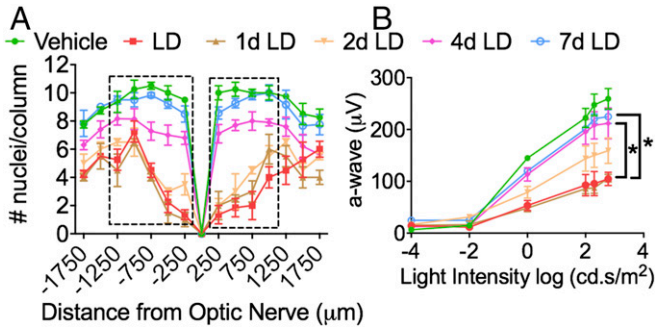

Fig. 1. Metformin prevents retinal degeneration from light damage in a dose-dependent manner. (*A*) Numbers of photoreceptor nuclei of outer nuclear layer (ONL) were counted in histology sections as described in *SI Appendix, Methods and Materials* and plotted versus distance superiorly and inferiorly from the optic nerve head. The dashed boxes indicate regions with statistical differences. (*B*) ERG a-wave amplitudes plotted versus increasing intensities of light stimulus. The legend is the same for both panels. $n = 8$ mice per group. Data were analyzed by two-way ANOVA with Tukey's post hoc test. $^*P < 0.001$. Error bars indicate standard error of the mean (SEM).

To characterize AMPK-subunit expression in the retina, we used publicly available RNA-sequencing data from both humans and mice to analyze the relative expression levels of AMPK isoforms of α-, β-, and γ-subunits in the retina (27, 28). The results suggest that mice express nearly equal levels of the catalytic α1- and α2-subunits but had a strong bias for the β1- and γ1-subunits. Human retinas and RPEs had a strong bias for α1-, β1-, and γ1-subunits (*SI Appendix*, Table S1).

To determine whether the protective activity of metformin required retinal expression of AMPK, we used the Chx10-Cre mouse line to generate mice with a retina-specific knockout of either AMPKα1 or AMPKα2. This conditional knockout strategy has been shown to efficiently remove floxed alleles in 80 to 95% of retinal neurons and Müller glia but not in retinal astrocytes, microglia, vasculature, or the RPE (29). Our strategy successfully knocked out expression of AMPKα1 or α2 in the retinas of these mice (*SI Appendix*, Fig. S4 *A* and *B*). Retinal thickness and scotopic electroretinogram (ERG) in AMPK knockout mice were normal in mice up to 6 wk of age (*SI Appendix*, Fig. S4 *C–E*). To determine whether metformin was still protective in the absence of either subunit, we injected knockout mice with metformin for 7 d before exposing them to light damage. We found that metformin still protected photoreceptor structure and function in AMPKα1 knockouts at a level similar to that of Cre-negative control mice (Fig. 2 *D* and *E* and *SI Appendix*, Fig. S4 *C* and *D*). However, metformin was not able to protect photoreceptors from light damage in mice lacking AMPKα2 (Fig. 2 *D* and *E* and *SI Appendix*, Fig. S4 *C* and *E*). These data indicated that metformin must activate AMPKα2 in the mouse retina to induce protection from light damage, and suggest that this protection is mediated by localized metabolic changes.

Metformin-Altered Gene Expression Suggests Protection Is Mediated by Resistance to Oxidative Damage and Increased Mitochondrial Biogenesis. We used qRT-PCR to measure gene expression for key genes that regulate mitochondrial biogenesis, oxidative defense, and response to DNA damage. For mitochondrial genes, we measured the expression of mitochondrial-encoded cytochrome *c* oxidase subunit II (COX-II), peroxisome proliferator-activated receptor gamma coactivator 1 alpha (PGC-1α), mitochondrial transcription factor A (Tfam), and nuclear respiratory factor 1 (Nrf1). We measured the expression of oxidative defense gene superoxide dismutase 2 (SOD2) and the expression of DNA damage response genes growth arrest and DNA damage-inducible beta (Gadd45b) and poly(ADP ribose) polymerase (PARP-14). The mRNA expression of Gadd45b and PARP-14 went up significantly in retinas from light-damaged mice but not in light-damaged mice that were pretreated with metformin (Fig. 3). Protein levels of PARP-14 also increased with light damage, but not in mice pretreated with metformin (*SI Appendix*, Fig. S5*A*). In addition, the expression of SOD2 was down-regulated with light damage, but not in mice pretreated with metformin (Fig. 3). These results suggest that metformin prevented oxidative DNA damage induced by damaging light. In addition, data show that expression of Nrf1, Tfam, PGC-1α, and COX-II were markedly up-regulated in the metformin-treated light damage group (Fig. 3, MFLD), while

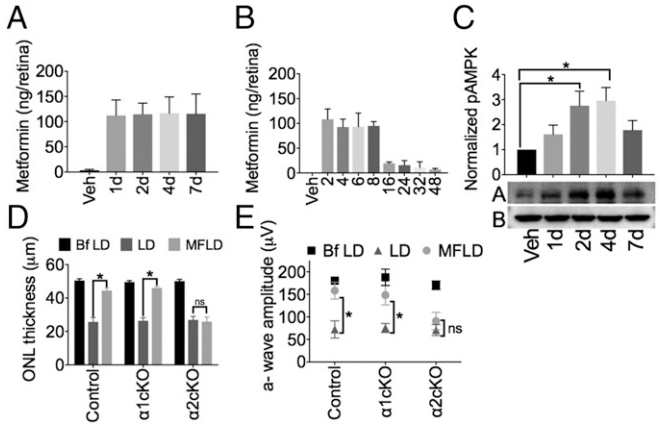

Fig. 2. Metformin-induced protection is mediated by local activation of the AMPK α2-subunit. (*A* and *B*) Metformin concentration in retinal extracts as measured by LC-MS. Veh, PBS control group. (*A*) Metformin concentration in the retina 2 h after each daily dosing for the indicated number of days. (*B*) Metformin concentration in retinal extracts at the indicated time points (numbers on the *x* axis) following a single metformin injection. $n = 6$ per group. (*C*) Western blots of three independent experiments were analyzed by one-way ANOVA with Tukey's post hoc test. $^*P < 0.0035$. Representative Western blots measuring levels of pAMPK in retinal extracts are shown below the graph: A, blot probed with antibodies to pAMPK; B, blot probed with antibodies to β-actin. (*D* and *E*) Cre-negative control mice (Control) and AMPK $\alpha1^{f/f}$ and $\alpha2^{f/f}$ cKO mice were exposed to damaging light following 7 d of pretreatment with metformin (MFLD) or vehicle (LD). (*D*) Retinal volumetric images were collected by OCT, and ONL thicknesses were averaged and plotted along with ONL thickness of mice before light damage (Bf LD). Data were analyzed by two-way ANOVA. $^*P < 0.001$; ns, not significant. (*E*) Scotopic ERG a-wave amplitudes at flash intensities of log 1 (cd·s/cm^2). $n = 5$ mice per group. Data were analyzed by two-way ANOVA. $^*P < 0.001$. Error bars = SD (*A*–*C*), or SEM (*D*, *E*).

expression levels did not change with metformin treatment alone. These data suggested that metformin pretreatment enabled the retina to have a more robust and protective response to damaging light exposure.

To determine whether metformin pretreatment prevented oxidative DNA damage, we stained retinal sections with antibodies to 8-oxo-2′-deoxyguanosine (8-OHdG). In the light damage retinas, photoreceptors had an increase in DNA damage, while pretreatment with metformin prevented light-induced DNA damage (Fig. 4*A*). Repairing oxidative damage requires utilization of NADH and, in light damage retinas, we observed decreased levels of NADH relative to NAD, which is consistent with an oxidative defense response (Fig. 4*B*). Retinal levels of NADH were not reduced with light damage in mice pretreated with metformin (Fig. 4*B*). Together, these results demonstrate that metformin protects photoreceptors by preventing light-induced oxidative stress and DNA damage.

The increased expression of Nrf1, Tfam, PGC-1α, and COX-II suggested a possible increase in mitochondrial biogenesis. To evaluate this possibility, we measured mitochondrial DNA content relative to nuclear DNA content by qPCR. We found that repeated, daily s.c. injections of metformin increased mitochondrial DNA copy number in the retina, with 4 d of metformin injections sufficient to increase mitochondrial DNA content by 1.8-fold (Fig. 4*C*). The increase in mitochondrial DNA content remained unchanged with further treatment and did not decrease after exposure to damaging light. To determine whether increased mitochondrial DNA was associated with increased mitochondrial gene expression, we measured the protein levels of mitochondrial-encoded subunit I of complex IV (COX-I) and nuclear-encoded mitochondrial protein succinate dehydrogenase-A (SDH-A). Protein levels of COX-I were slightly increased in mice treated with metformin before light damage, while levels of SDH-A did not change (*SI Appendix*, Fig. S5 *D*–*F*). These results suggest that metformin increased mitochondrial DNA and increased the expression of mitochondrial-encoded genes COX-I and COX-II but did not alter the gene expression of some nuclear-encoded mitochondrial genes.

Studies have shown that metformin inhibits mitochondrial electron transport chain complex I activity (30). We found that mice given metformin for either 1 or 2 d had a small but reproducible decrease in complex I activity but returned to baseline levels with subsequent daily doses (*SI Appendix*, Fig. S5*B*). Interestingly, light-damaged mice that were pretreated with metformin for 7 d had increased mitochondrial complex I activity (*SI Appendix*, Fig. S5*B*). AMPK is also known to increase ATP through increased glycolysis (31). We measured retinal ATP levels following daily s.c. metformin injections. These data showed that, like AMPK phosphorylation, ATP levels were elevated within 2 h after metformin injection (*SI Appendix*, Fig. S5*C*). The increase in ATP is likely the result of increased glycolysis, since complex I activity was suppressed at the 2-h time point.

Metformin Delays Rod Degeneration and Preserves Cone Photoreceptors in the Rd10 Mouse. To investigate whether metformin can delay degeneration in a mouse model of inherited retinal degeneration, we used *Pde6b*^{rd10} mice, which exhibit highly aggressive rod degeneration with secondary cone loss (32). Degeneration of photoreceptors in Rd10 mice begins around postnatal day 16, with complete rod death by 6 wk of age (Fig. 5 *A* and *B* and *SI Appendix*, Fig. S6*A*) that is followed by cone death around 5 to 6 wk. Daily s.c. injections of metformin into Rd10 mice beginning on postnatal day 13 significantly delayed rod degeneration and preserved rod structure and retinal morphology (Fig. 5 *A* and *B* and *SI Appendix*, Fig. S6*A*). Cones were labeled by immunohistochemistry (IHC) with antibodies to cone arrestin, S opsin, and M opsin in Rd10 retinal sections (*SI Appendix*, Fig. S6*D*) and flat mounts (*SI Appendix*, Fig. S6*E*). Quantification of cones in sections and retinal flat mounts showed that metformin doubled the number of surviving cones in treated Rd10 mice (Fig. 5*C* and *SI Appendix*, Fig. S6*C*). These results suggest that metformin can prolong survival of rods and cones.

To determine whether metformin-induced survival of photoreceptors also preserved photoreceptor function, we analyzed scotopic, photopic, and flicker ERGs in mice treated with and without metformin. Metformin treatment preserved both photopic and flicker ERGs at 6 wk of age (Fig. 5*D* and *SI Appendix*,

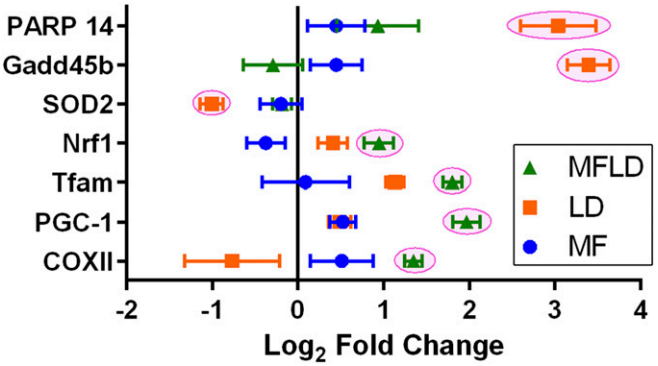

Fig. 3. Gene expression levels of PARP-14, Gadd45b, SOD2, Nrf1, Tfam, PGC-1α, and COX-II were measured by qRT-PCR. Data were normalized to vehicle controls, and $\log_2$ fold change was graphed in box-and-whisker plots. LD, mice light-damaged in the absence of metformin; MF, mice treated with metformin for 7 d without light damage; MFLD, mice light-damaged following 7 d of metformin pretreatment. $n = 3$ mice per group with three independent biological replicates. Data were analyzed by one-way ANOVA and Tukey's post hoc test. Magenta ovals indicate groups with $P < 0.05$.

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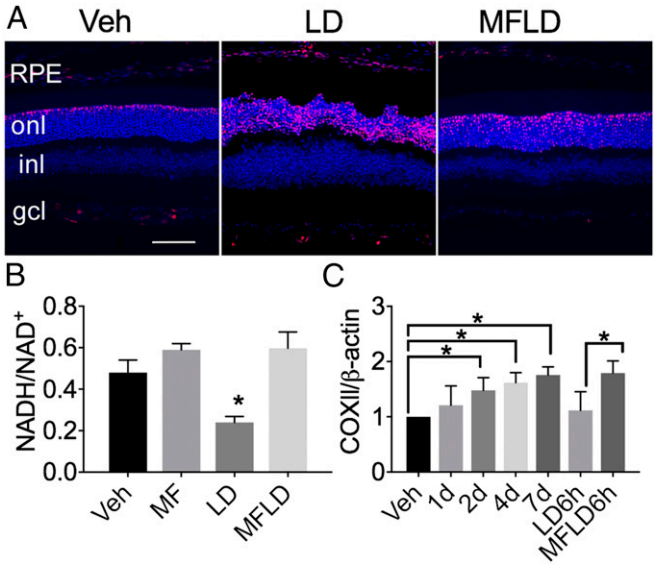

Fig. 4. Metformin prevents light-induced oxidative stress and DNA damage. LD, mice light-damaged in the absence of metformin; MF, mice treated with metformin for 7 d without light damage; MFLD, mice light-damaged following 7 d of metformin pretreatment; Veh, control mice injected with PBS. (*A*) IHC labeling of 8-OHdG in retinal cross-sections to evaluate the DNA oxidative damage. Similar results were seen in six independent replicates. (Scale bar, 100 μm.) (*B*) The ratio of NADH to NAD^+ was measured enzymatically from one retina per group. $n = 3$ biological replicates. $^*P < 0.03$. (*C*) Mitochondrial DNA content was measured in retinas isolated from mice treated with vehicle or metformin for the indicated number of days, mice 6 h after light damage (LD6h), and mice pretreated with metformin for 7 d before light damage (MFLD6h). DNA from mitochondria and the nucleus were measured by qPCR, and the ratio of mitochondrial DNA relative to nuclear DNA is shown. Data were analyzed by one-way ANOVA and Tukey's post hoc test. $n = 4$ to 6 biological replicates per group. $^*P < 0.05$. RPE, retinal pigmented epithelium; onl, outer nuclear layer; inl, inner nuclear layer; gcl, ganglion cell layer. Error bars = SD.

Fig. S7 *A–F*). At 8 wk, metformin-treated mice retained more than 50% of cone activity, while untreated mice were unresponsive to light flashes (Fig. 5*D* and *SI Appendix*, Fig. S7 *A–F*). The Rd10 mutation reduces phototransduction in rods, making it difficult to measure rod activity by scotopic a-wave measurements; however, we could detect preserved rod function in metformin-treated mice by measuring scotopic b waves (*SI Appendix*, Fig. S7 *G–L*). These results suggest that metformin slowed degeneration and preserved structure and function of rods and cones in the Rd10 mouse.

Metformin-Induced Protection in Rd10 Photoreceptors Is Associated with Increased Metabolic Activity. To determine whether the mechanism of protection in Rd10 mice was similar to that of the light-damage model, we measured changes in mitochondrial protein expression, mitochondrial DNA copy number, and ATP levels. Data were similar between Rd10 and light-damaged mice. Metformin treatment did not alter SDH-A expression but did increase expression of COX-I (*SI Appendix*, Fig. S8 *A–C*). Metformin also increased ATP levels and increased mitochondrial DNA copy number by 1.8-fold compared with untreated Rd10 mice (*SI Appendix*, Fig. S8 *D* and *E*). Since it was possible that metformin could induce autophagy (33), we measured the expression of LC3BII, ATG5/12, Beclin, and ATG7. We did not observe an increase in LC3BII or ATG5/12 in light damage and we did not observe an increase in ATG7 or Beclin with metformin treatment in Rd10 mice (*SI Appendix*, Fig. S9). These results suggest that long-term metformin treatment does not stimulate a sustained increase in autophagy.

Metformin Prevents RPE Damage from Sodium Iodate-Induced Injury. While no animal model fully recapitulates the pathologic features of AMD, we used sodium iodate to induce an acute oxidative injury in the RPE that mimics the oxidative stress risk factor of early AMD (34, 35). To determine if metformin could protect other cells in addition to photoreceptors, we used sodium iodate to damage RPE cells in vivo. BALB/c mice injected with sodium iodate at both 30 and 35 mg/kg had damage to the RPE and photoreceptors (Fig. 6 and *SI Appendix*, Fig. S10). However, the RPE and photoreceptors in mice pretreated with metformin before sodium iodate injection were more resistant to damage (Fig. 6 and *SI Appendix*, Fig. S10). RPE flat mounts from these mice were stained with zonula occludens-1 (ZO-1) to quantify protection of the RPE cell structure. Approximately 90% of the RPE structure was preserved in metformin-treated mice following 30 mg/kg sodium iodate injection, and more than 50% was preserved against 35 mg/kg sodium iodate insult (Fig. 6 *A* and *B*). Photoreceptor and RPE function were measured by scotopic ERG a and c waves, respectively. Both were preserved in mice pretreated with metformin before sodium iodate injury (Fig. 6*C* and *SI Appendix*, Fig. S10*B*).

Discussion

In this study, we found that metformin protects photoreceptors from acute light damage, delays inherited retinal degeneration, and protects the RPE from oxidative stress-induced injury. We found that metformin can rapidly cross the blood–retina barrier and activate AMPK signaling in the retina to rapidly alter metabolism, including reduced complex I activity and increased ATP levels. Given these rapid changes, we were surprised to find that 4 d of metformin treatment was required to induce protection of photoreceptors from light damage. This delayed

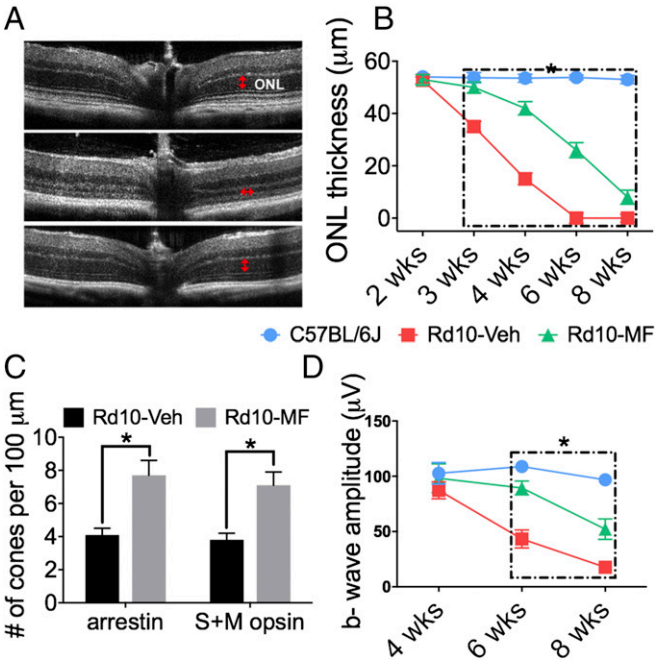

Fig. 5. Metformin delays retinal degeneration in Rd10 mice. (*A*) Representative images of spectral domain optical coherence tomography (SD-OCT) at 4 wk. (*B*) Averaged ONL thicknesses were plotted over time from 2- to 8-wk-old mice. (*C*) The number of cones in each section was counted and plotted as the number of cones per 100 μm of the retina. (*D*) Flicker ERG b-wave amplitudes over time. Error bars indicate SD. $n = 3$ mice per group; experiments were performed with three biological replicates. The same legend was used in *B* and *D*. Data were analyzed by one-way ANOVA with Tukey's post hoc test. $^*P < 0.001$. *, Boxed data points in *B* and *D* were significantly different at each age $P < 0.001$. Error bars = SD (*B*, *C*) or SEM (*D*).

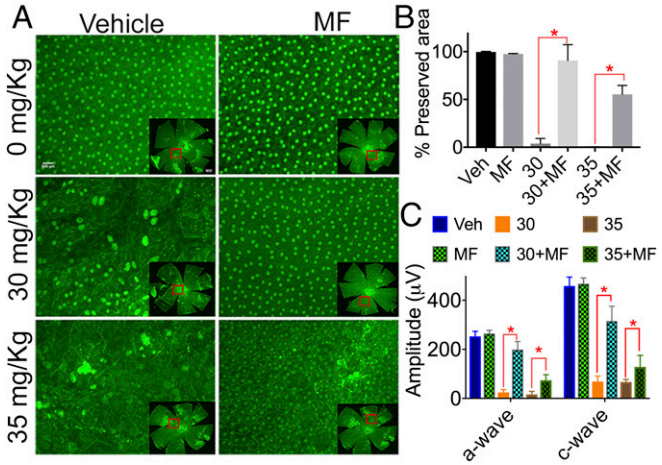

Fig. 6. Metformin protects RPE from sodium iodate-induced damage. (*A*) IHC was used to detect ZO-1 in flat-mounted eyecups to observe RPE damage. Mice were treated with the indicated concentrations of sodium iodate. (Scale bar: 300 μm.) (*B*) The total RPE area and damaged areas were measured in eyecups (five mice per group), and data were plotted as a percentage of total preserved RPE morphology. MF, metformin treatment (30 and 35 are the doses of sodium iodate in mg/kg); Veh, PBS-treated mice. Error bars indicate SD. Data were analyzed by one-way ANOVA with Tukey's post hoc test. $*P < 0.0001$. (*C*) ERG a- and c-wave amplitudes were averaged and plotted from each experimental group ($n = 5$ mice per group). Error bars indicate SEM. Data were analyzed by two-way ANOVA with Tukey's post hoc test. $*P < 0.0001$.

response was not associated with an accumulation of metformin in the retina, since the drug appears to be cleared on a daily basis. Given the time required, it is likely that metformin-induced protection requires changes in gene expression and metabolic reprogramming. Consistent with this hypothesis, protection of photoreceptors was strongly correlated with increased mitochondrial DNA copy number and a more robust expression of mitochondrial genes in response to oxidative stress.

Metformin made photoreceptors and the RPE highly resistant to oxidative stress, which prevented DNA damage and oxidative injury. In the highly aggressive Rd10 retinal degeneration model, metformin slowed rod degeneration and preserved cone photoreceptor numbers and function. Photoreceptors and the RPE have high metabolic demands to maintain photoreceptor outer-segment synthesis and phagocytosis, retinoid recycling, and maintenance of membrane potential and ion flux through Na^+ K^+-ATPases (36). It is, therefore, a logical hypothesis that dysregulated metabolism may be a major contributing factor to retinal disease, and that approaches to enhance energy metabolism will have a therapeutic benefit to retinal diseases such as RP and AMD. The hypothesis has been supported by recent findings. For example, injecting growth factors, including insulin, has been shown to promote survival of cones through activation of AKT and mTOR in $Pde6b^{rd1}$ mice (4). Activating glycolysis by knocking out Sirt6 also protects rods and cones in $Pde6b^{H260Q/H260Q}$ mice (3). Protein or gene delivery of RdCVF into the eye of an *rd1* animal attenuated the loss of cones by increasing glucose uptake for aerobic glycolysis and increasing ATP levels (37). These studies show that enhancing catabolic energy production can rescue photoreceptors in mice with rod degeneration. The increase in ATP production, mitochondrial DNA copy number, NADH/NAD^+ ratios, and COX-I protein expression in our data all suggest that metformin increased retinal metabolic activity, which is likely contributing to protection. In addition to increased metabolism, we found increased expression of SOD2 and reduced oxidative damage. While gene delivery of SOD2 has been shown to protect photoreceptors and the RPE, it is unlikely that the protective mechanism of metformin is simply due to increased oxidative defense, since antioxidant therapies are not effective at delaying degeneration from inherited mutations (38). Since we observed increases in both oxidant defense and markers of metabolism with metformin treatment, we hypothesize that both factors are contributing to metformin-induced protection.

Our data suggest that metformin has the potential to protect photoreceptors and the RPE from a broad spectrum of insults that cause retinal degeneration. However, there are likely to be limitations. In a recent study, metformin was used to protect photoreceptors from a misfolded protein response caused by overexpressing a mutant opsin protein ($Opsin^{P23H}$) in transgenic rats (39). In contrast to our results, the authors found that metformin did not slow rod degeneration in these animals. The study reported that metformin-induced AMPK activation enhanced trafficking of mutant rhodopsin, suggesting that metformin can rescue some aspects of the protein-misfolding responses. The increased trafficking of the mutant opsin led to disorganization of rod outer segments and induced a relatively small (6%) decrease in ONL thickness at 7 wk of age compared with the untreated rat retinas. The authors concluded that the mislocalized opsin in the outer segment might be detrimental. Given these results, it is possible that metformin may not be protective from misfolded protein aggregation. However, our data strongly suggest that metformin can slow degeneration caused by elevated oxidative stress and energy imbalances. Unfortunately, Athanasiou et al. did not study the ability of metformin to protect cones in the transgenic rats.

In our study, mice at 6 to 8 wk of age with single knockouts of α1 or α2 had normal ERG function, including b waves, and retinal morphology. In a previous study, mice with photoreceptor knockouts of AMPK (both α1 and α2) were found to undergo synaptic remodeling similar to advanced aging (40). The results suggested that AMPK activity is necessary for the healthy metabolism of photoreceptors. While this study is consistent with our finding that local activation of AMPK in the retina is important for normal cellular function, we did not observe the same phenotype in young single knockouts as the previous study found with older double knockouts. It is possible that synaptic dysfunction would occur later in life in our single knockouts or would require the loss of both genes. These questions will be addressed in future studies. However, the previous study links the role of reduced AMPK activity and aging. Early AMD has been associated with altered bioenergetics, increased oxidative stress, increased mitochondrial mutations, and decreased antioxidative defense (10, 41). The published results and data presented in this study strongly suggest metformin is a potential drug therapy for AMD and other degenerations associated with age, oxidative stress, and mitochondrial dysfunction. Photoreceptors and the RPE are not the only cells protected by metformin, since a recent retrospective study has shown that metformin use in diabetic patients is associated with a reduction in the risk of retinal ganglion cell death caused by primary open-angle glaucoma (42). Another study has shown that metformin uptake was able to reduce the risk of Parkinson's disease with type 2 diabetes in a Taiwanese population (43). Emerging evidence has shown that metformin also modulates the biology of aging and lifespan (44, 45). Activation of AMPK by metformin to reduce oxidative stress and regulate metabolic homeostasis may be a beneficial therapy to slow retinal cell death and induce broad-spectrum protection in neurodegeneration of the central nervous system.

Materials and Methods

Animals. Five- to 6-wk-old male BALB/cJ mice were obtained from The Jackson Laboratory and acclimated to our animal facility for 2 wk before experiments. *Rd10* mice in the C57BL/6J background were obtained from William Hauswirth at the University of Florida. Chx10-cre mice [Tg(Chx10-EGFP/cre,-ALPP)2Clc*J*] were originally purchased from The Jackson Laboratory (stock number 005105) and crossed into the BALB/cJ strain for more than 10 generations (29). AMPKα1 floxed mice ($Prkaa1^{tm1.1Sjm/J}$) and α2 floxed mice

(Prkaa2$^{tm1.1Sjm/J}$) were purchased from the Jackson Laboratory (stock numbers 014141 and 014142, respectively). They were mated to Chx10-cre mice and backcrossed into the BALB/cJ background for four generations to change strains, and then mated to produce mice of the following genotypes: Chx10-cre^{+};AMPKα1$^{fl/fl}$ or Chx10-cre^{+};AMPKα2$^{fl/fl}$. All mice were reared in 12-h light–12-h dark cycles (lights on at 6 AM) with 60-lx lighting intensities at cage level. Food and water were given ad libitum. Mice were genotyped as described to rule out the confounding mutations *rd1* (46) and *rd8* (47) and for the *Rpe65* genes (48). All animal procedures followed the guidelines of the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research and were approved by the Institutional Animal Care and Use Committee at the University of Florida.

Antibodies. pAMPK antibody (2535S; Cell Signaling), SDH-A and mt-Cox-1 (123545; Abcam), β-actin (8226; Abcam), 8-OHdG (MOG-020P; Genox), rabbit opsin red/green (AB5405; EMD Millipore), goat OPN1SW (N-20) (sc-14363; Santa Cruz), mouse cone arrestin (kind gift of W. Clay Smith, University of Florida), LC3B (2775S; Cell Signaling), ATG5-ATG12 (12994P; Cell Signaling), Beclin (3495P; Cell Signaling), and ATG7 (8558P; Cell Signaling) were used as primary antibodies. Secondary antibodies were Alexa Fluor 488 goat anti-rabbit IgG (A11008; Invitrogen), Alexa Fluor 594 donkey anti-goat IgG (A11058; Invitrogen), and 800CW LI-COR goat anti-rabbit 800 and donkey anti-mouse 680.

Statistics. GraphPad Prism was used for statistical analysis. Differences between two groups were assessed using either paired or unpaired *t* tests, while differences between more than two groups were assessed using analysis of variance (ANOVA) followed by Tukey's post hoc test. A *P* value of less than 0.05 was considered statistically significant.

Other detailed methods are described in *SI Appendix, Methods and Materials*.

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