Evidence of severe mitochondrial oxidative stress and a protective effect of low oxygen in mouse models of inherited photoreceptor degeneration

Dafni Vlachantoni¹, Alexa N. Bramall²,³, Michael P. Murphy⁴, Robert W. Taylor⁵, Xinhua Shu¹, Brian Tulloch¹, Theo Van Veen⁶, Douglass M. Turnbull⁵, Roderick R. McInnes²,³,⁷ and Alan F. Wright¹,*

¹MRC Human Genetics Unit, Institute of Genetics and Molecular Medicine, Edinburgh, UK, ²Program in Developmental Biology, The Research Institute, The Hospital for Sick Children, Toronto, Ontario, Canada, ³Department of Molecular Genetics, University of Toronto, Toronto, Ontario, Canada, ⁴MRC Mitochondrial Biology Unit, Hills Road, Cambridge, UK, ⁵Mitochondrial Research Group, Institute for Ageing and Health, Newcastle University, Newcastle upon Tyne, UK, ⁶Department of Ophthalmology Research, Lund University Hospital, Lund, Sweden and ⁷Lady Davis Research Institute, Jewish General Hospital, McGill University, Montreal, Canada

The role of oxidative stress within photoreceptors (PRs) in inherited photoreceptor degeneration (IPD) is unclear. We investigated this question using four IPD mouse models (Pde6brd1/rd1, Pde6batrd1/atrd1, Rho²⁻/⁻ and Prph2rds/rds) and compared the abundance of reduced glutathione (GSH) and the activity of mitochondrial NADH:ubiquinone oxidoreductase (complex I), which is oxidative stress sensitive, as indirect measures of redox status, in the retinas of wild type and IPD mice. All four IPD mutants had significantly reduced retinal complex I activities (14–29% of wild type) and two showed reduced GSH, at a stage prior to the occurrence of significant cell death, whereas mitochondrial citrate synthase, which is oxidative stress insensitive, was unchanged. We orally administered the mitochondrially targeted antioxidant MitoQ in order to reduce oxidative stress but without any improvement in retinal complex I activity, GSH or rates of PR degeneration. One possible source of oxidative stress in IPDs is oxygen toxicity in the outer retina due to reduced consumption by PR mitochondria. We therefore asked whether a reduction in the ambient O₂ concentration might improve PR survival in Pde6brd1/rd1 retinal explants either directly, by reducing reactive oxygen species formation, or indirectly by a neuroprotective mechanism. Pde6brd1/rd1 retinal explants cultured in 6% O₂ showed 31% less PR death than normoxic explants. We conclude that (i) mitochondrial oxidative stress is a significant early feature of IPDs; (ii) the ineffectiveness of MitoQ may indicate its inability to reduce some mediators of oxidative stress, such as hydrogen peroxide; and (iii) elucidation of the mechanisms by which hypoxia protects mutant PRs may identify novel neuroprotective pathways in the retina.

INTRODUCTION

Inherited photoreceptor degenerations (IPDs) are characterized by rod and/or cone photoreceptor (PR) cell death, variable clinical phenotypes and monogenic inheritance (1). More than 140 genes and 180 loci, influencing many different aspects of PR and RPE function, have been implicated in IPDs to date (http://www.sph.uth.tmc.edu/Retnet/home.htm). This genetic heterogeneity may reflect the increased vulnerability of PR cells to genetic insult, making the mutant PRs abnormally sensitive to a variety of factors, including light exposure, high oxygen tension in the adjacent choroidal vasculature, high polyunsaturated lipid content and a high level of oxidative metabolism (1–3). The predominant mode of cell death in

*To whom correspondence should be addressed. Email: alan.wright@hgu.mrc.ac.uk
†These authors made equal contributions to this work.

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all IPDs is apoptosis, which can be either caspase dependent or caspase independent (4–7).

Oxidative stress is defined as an imbalance between the production of and protection from reactive oxygen species (ROS) (8). About 90% of tissue oxygen is metabolized within mitochondria. Consequently, a widely held view is that mitochondrial oxygen metabolism is the primary source of day-to-day ROS formation, resulting from the partial reduction of molecular oxygen to superoxide and hydrogen peroxide (H₂O₂), the two major species of ROS produced by mitochondria (8). Another potential source of mitochondrial oxidative stress is nitric oxide, which can react with superoxide to form the damaging peroxynitrite radical. Other organelles, such as peroxisomes, and various cytoplasmic enzymes, including NADPH oxidase and xanthine oxidase, also produce ROS and there is debate regarding the relative importance of these systems for ROS formation in different forms of neurodegeneration (9).

Increased levels of oxidative stress, as indicated by oxidative damage to nucleic acids, proteins or lipids, have been associated with a variety of neurodegenerative disorders of the brain as well as with ageing (10–12). A link between oxidative stress and PR degeneration has been proposed (3,13) and supported by both in vitro (14,15) and in vivo (15–17) experiments. However, a direct analysis of oxidative stress within the mitochondrial compartment in IPD models, which is the subject of the present investigation, has not previously been carried out. Perhaps the most robust evidence for a role for oxidative stress has been found in cone PR degeneration (18–20). However, in most IPDs, cones do not express the activity of and protection from reactive oxygen species (ROS) resulting from oxidative stress have been reported in several neurodegenerative disorders, including Alzheimer disease and Parkinson disease (31,32), but this has not been specifically investigated in retinal degeneration models. The same mouse models were also treated in vivo with the mitochondrial antioxidant MitoQ. In addition, Pde6brd1/rd1 retinal explants were either treated with MitoQ or cultured in 6% O₂ to determine their effects on PR cell death. Although MitoQ treatment did not affect mutant PR survival either in vivo or in vitro in IPD models, hypoxia was strongly protective to Pde6brd1/rd1 mutant retinas ex vivo.

RESULTS

Evidence for severe oxidative stress at an early stage in IPD retinas

Oxidative stress can result in the reduced activity of enzymes whose chemical and physical structures make them sensitive to inactivation by ROS. Mitochondrial complex I is particularly prone to inactivation by oxidation, which converts the stable and active [4Fe-4S]° or [4Fe-4S]2+ clusters in these enzymes to unstable [3Fe-4S]2+ clusters, rendering the enzyme inactive and causing release of Fe²⁺ and H₂O₂ (33). These two molecules participate in Haber–Weiss and Fenton reactions, which result in the production of damaging hydroxyl radicals (8). We therefore measured the specific activities of the mitochondrial enzymes complex I, which is oxidative stress sensitive, and citrate synthase, which is oxidative stress insensitive, for evidence of oxidative stress in the retinas of IPD mouse models. The enzymes were assayed spectrophotometrically using post-600g homogenates from both the liver and retinas of the wild-type control and four different mouse retinal degeneration mutants (Pde6brd1/rd1, Pde6brd1/rd1/amrd1, Rh₀⁻/⁻ and Prph2<sup>40d/40</sup>). The Pde6brd1/rd1 and Pde6brd1/rd1/amrd1 mutants were assayed at 1 and 2 weeks of age, respectively, and the Rh₀⁻/⁻ and Prph<sup>40d/40</sup> mutants at 1 month of age. These time points were chosen since in each case the outer nuclear (PR) layer thickness was close to normal (see below). A prior comparison of complex I and citrate synthase activities in the retina, liver and brain of wild-type BALB/C, C57BL/6J, CBA, CD1 and DBA strains showed no difference in enzyme activities, using five males and five females per mouse line (Supplementary Material, Fig. S1), so that age-matched wild-type C57B/6J mice were subsequently used as controls throughout these experiments.

In wild-type retinas, the specific activity of complex I was 0.72 ± 0.17 (SEM) µmol/min/g tissue, but all four IPD specific activities with values ranging from 0.10 ± 0.01 µmol/min/g tissue (Pde6brd1/rd1) to 0.20 ± 0.06 µmol/min/g tissue in...
samples (14–29% of the wild type; all \(P < 0.05; n = 3–6\)) (Fig. 1). In contrast, there was no significant change in the retinal activity of citrate synthase, which ranged from 0.87 ± 0.06 to 1.35 ± 0.38 \(\mu\)mol/min/g tissue in mutant retinas, compared with wild-type activity of 1.10 ± 0.11 \(\mu\)mol/min/g tissue (\(P > 0.05; n = 3–6\)). The specificity of the effect of IPD mutations on retinal complex I versus citrate synthase activities was further demonstrated by the absence of any effect of these mutations on the activities of complex I or citrate synthase in the liver (all \(P < 0.05; n = 3–5\)) (Fig. 1).

To corroborate the evidence of increased oxidative stress in IPD retinas, as indicated by decreased retinal complex I activity, we compared the concentration of reduced GSH in supernatants from the IPD retina and liver with those from wild-type samples (Fig. 1). The GSH concentration of the wild-type retinas was 0.084 ± 0.005 \(\mu\)mol/g protein, while in IPD retina samples it was significantly reduced in both \(Pde6brd1/rd1\) (0.043 ± 0.003; \(P = 0.03\)) and \(Rho^{−/−}\) (0.041 ± 0.002; \(P = 0.02\)) mutants, although it remained at wild-type levels in the \(Pde6brd1/rd1\) (0.075 ± 0.013) and \(Prph2rds/rds\) (0.078 ± 0.005) mutants. In contrast, we found no reduction in the GSH concentration in the liver of the four IPD models versus wild-type liver. The mean GSH concentrations of the IPD liver tissue ranged from 3.58 ± 0.17 to 5.15 ± 0.15 \(\mu\)mol/g protein, similar to the mean wild-type concentration of 4.20 ± 0.15 \(\mu\)mol/g protein (all \(P > 0.05\)).

**Effect of reducing superoxide dismutase 2 activity in retinal degeneration mutants**

We also investigated the role of mitochondrial superoxide dismutase 2 (Sod2) in retinal degeneration by crossing the same retinal degeneration mutants to Sod2 heterozygous knockout mice (\(Sod2^{+/−}\)) in order to see if this produced further increases in oxidative stress (or acceleration of degeneration) in the double mutants. Interestingly, the \(Sod2^{+/−}\) single mutant retinas also showed severe reductions in both complex I activity (19% of wild-type; \(P = 0.048\)) and GSH concentration (28% of wild-type; \(P = 0.004\)), while there was no significant difference in the mitochondrial marker enzyme citrate synthase (Supplementary Material, Tables S1 and S2). Analysis of the outer nuclear layer (ONL) in \(Sod2^{+/−}\) mice showed no evidence of PR degeneration up to 10 months of age. Analysis of complex I and citrate synthase activities in other tissues of \(Sod2^{+/−}\) mice.
showed significant reductions in complex I activities only in the liver \( (P = 0.05) \), heart \( (P = 0.037) \) and brain \( (P = 0.03) \) (Supplementary Material, Table S1). Similarly, GSH concentrations were significantly reduced in the liver \( (P = 0.002) \) and brain \( (P = 0.05) \) but not in the heart (Supplementary Material, Table S2). These results suggest the presence of significant oxidative stress in these mice which is not accompanied by retinal degeneration, perhaps suggesting a difference in the type/origin of the stress.

The double mutants showed no change in rate of retinal degeneration (data not shown), but there were further decreases in complex I activity in the two fast degeneration mutants only—\( Pde6b^{rd1/rd1} \) and \( Pde6b^{rd1/rd1} \) (by 44 and 80% compared with the single mutants, respectively)—although the reduction was only statistically significant in the \( Pde6b^{rd1/rd1}/Sod2^{+/−} \) double mutant \( (P = 0.048) \) (Supplementary Material, Table S1). Similarly, while the \( Pde6b^{rd1/rd1} \) and \( Rho^{+−} \) double mutants did show reductions in retinal GSH compared with the wild type, they were not statistically different from the single mutants (Supplementary Material, Table S2). We therefore found some evidence for increased retinal oxidative stress in the double mutants, but the results were only significant in the \( Pde6b^{rd1/rd1} \) double mutant. The results did, however, provide further validation for the assays as providing reliable indicators of oxidative stress.

**Retinal MitoQ uptake and lack of toxicity in wild-type mice**

To elucidate the role of oxidative stress in IPDs, antioxidant compounds that are targeted to specific subcellular compartments can be applied. MitoQ [mitoquinone or 10-(6′-ubiquinonyl)-decytriphenylphosphonium] is a mitochondrially targeted antioxidant that contains a ubiquinol (coenzyme Q) moiety, whose uptake into cells is achieved by conjugation to the positively charged lipophilic cation, triphenylphosphonium. MitoQ can be administered orally, and cellular uptake of MitoQ is driven initially by the plasma membrane potential \( (−30 \text{ to } −60 \text{ mV inside the cell}) \) and then by the more negative membrane potential across the mitochondrial inner membrane \( (−150 \text{ to } −180 \text{ mV}) \) (34) so that it can accumulate at 100–500-fold higher concentrations in mitochondria compared with plasma (34). Before examining the effect of MitoQ on retinal oxidative stress and cell death in IPDs, we established that MitoQ was taken up by the retina. Smith et al. (35) demonstrated the uptake of MitoQ by a range of tissues, including the heart, brain and liver, but there have been no studies of its retinal uptake. We therefore administered tritium-labelled MitoQ \( ([3H]\text{MitoQ}, 20 \mu M) \) orally to wild-type C57BL/6J mice \( (n = 3) \) for 2 weeks at a joint concentration of labelled plus unlabelled MitoQ of 500 \( \mu M \). We estimated the uptake of \( [3H]\text{MitoQ} \) by the retina, eye minus retina, liver, heart and brain using external standards. Control samples were collected from mice given pure drinking water. We found significant uptake of MitoQ in all tissues assayed \( (P < 0.05) \) (Fig. 2). MitoQ uptake was highest in the heart \( (3.43 \pm 0.62 \text{ SEM}) \) and liver \( (3.19 \pm 1.20 \text{ nmol/g wet weight}) \) with corresponding tissue concentrations of \( >3 \text{ \mu M} \). The steady-state MitoQ concentration in the retina was \( 1.30 \pm 0.28 \text{ nmol/g wet weight} \), which corresponds to a tissue concentration of \( \sim 1.7 \text{ \mu M} \) [assuming a retina water content close to 77\% (36)]. The MitoQ concentration in the brain was similar to that in the retina, at \( 1.08 \pm 0.25 \text{ nmol/g wet weight} \). These findings demonstrate significant MitoQ uptake in mouse heart, liver, brain and retina following oral administration, as reported previously in non-ocular tissues (35). In the present study, the MitoQ tissue concentrations appear to have been sufficient for therapeutic efficacy, since

![Figure 2. Uptake of orally administered \([3H]\text{MitoQ} \) by different tissues. The uptake of \([3H]\text{MitoQ} \) by different tissues was assessed *in vivo*. Mice were administered 500 \( \mu M \) \([3H]\text{MitoQ} \) for 2 weeks and tissue contents were measured by scintillation counting. The control samples were collected from age-matched mice that were administered pure drinking water. The uptake was significant in all the tissues assayed (* \( P < 0.05 \); ** \( P < 0.01 \); all \( n = 3 \)).](https://academic.oup.com/hmg/article-abstract/20/2/322/654274/325)
energized mitochondria show 500–600-fold higher concentrations than those measured in tissue extracts due to the negative membrane potential (35,37–39).

We detected no toxic effects of MitoQ administered in the drinking water at concentrations of 500 μM in wild-type mice, and weight gain, monitored daily for the first month after birth and weekly thereafter, was the same as in wild-type water-only controls. Similarly, we observed no adverse effects of MitoQ administration on the retinas of wild-type mice. Retina samples were collected from 3-month-old wild-type animals (n = 4) administered MitoQ at low (250 μM) or high (500 μM) doses from conception onwards. Neither retinal morphology nor the thickness of the PR ONL was affected by MitoQ administration, and no TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling)-positive cells were detected (data not shown). In addition, the specific activities of the mitochondrial enzymes complex I and citrate synthase in the liver and retina were unchanged in MitoQ-treated compared with untreated wild-type animals (all P > 0.05; n = 3–6, Fig. 1).

MitoQ does not restore retinal complex I activity or GSH concentrations in IPD retinas

To examine the effect of MitoQ administration on oxidative stress in the retinas of IPD mice, we measured the specific activities of complex I and citrate synthase in crude mitochondrial extracts from the retina and liver in MitoQ-treated IPD compared with control-untreated IPD mice (Fig. 1). In wild-type mice, MitoQ had no effect on the complex I or citrate synthase activities in the retina (P > 0.05; n = 3–6) or liver (all P > 0.05; n = 2–5) (Fig. 1). Despite its established role as a mitochondrial antioxidant, we found that MitoQ had no substantial effect on the reduced complex I activity in the retinas of IPD mice. Of the four MitoQ-treated IPD mutants, we observed a significant increase in complex I activity with MitoQ treatment by 3 weeks in Pde6brd1/d1 mice, by 4 weeks in Pde6brd1/rd1 mice, by 3 months in Rhob−/− mutants and by 4 months in Prph2rd2/rd2 retinas (all P > 0.05; n = 5). Similarly, MitoQ had no effect on the percentage of TUNEL-positive cells at any time point in any of the four IPD models (Fig. 3, right column) (all P > 0.05; n = 3–5).

To minimize the possibility that the concentrations of MitoQ attained in the retinas of IPD mice were suboptimal for reducing mitochondrial oxidative stress, thereby accounting for its failure to protect mutant PRs in vivo, we treated retinal explants from Pde6brd1/d1 mice with MitoQ and then compared the survival of PRs in these explants with untreated Pde6brd1/d1 explants. First, we determined whether MitoQ, at concentrations shown to be protective against mitochondrial oxidative stress in other tissues (35,37–39), displayed any evidence of toxicity to the explants. We observed that wild-type explants exposed to 0.1, 1.0 and 5 μM MitoQ from PN10 to PN17 maintained normal morphology and PR numbers (Fig. 4A). Explants treated with decyltriphenylphosphonium (DecylTPP), which has the same hydrophobicity as MitoQ but lacks the ubiquinol moiety, served as controls (Fig. 4A). We found that 5 μM MitoQ had no effect on PR death from PN10 to PN17 degeneration rate in Pde6brd1/d1 explants (all P > 0.05; n = 6); both MitoQ-treated and -untreated Pde6brd1/d1 explants had on average four rows of PR nuclei at PN17 (Fig. 4B).

Hypoxia reduces PR death in Pde6brd1/d1 retinal explants

to evaluate further the role of mitochondrial oxidative stress in the pathogenesis of PR death in IPDs, we asked whether a reduction in the ambient O2 concentration would improve PR survival in Pde6brd1/d1 retinal explants. The exposure of mice to reduced ambient O2 levels has been shown to reduce PR death from light damage in intact mice (26,27), but the effect of hypoxia on the pathogenesis of PR death in IPDs has not been examined. The use of retinal explants allowed us to isolate the effects of hypoxia per se from the broad range of systemic changes that hypoxia would induce in mice raised in lowered ambient O2. The effect of lowering the oxygen concentration below 21% on ROS formation is complex (25). Initially, it causes a linear reduction in the formation of ROS such as superoxide by complex I, but at very low oxygen concentrations (1–3%) there is a paradoxical increase in ROS production, probably mediated by complex III (25,40). At mild hypoxia levels, such as 6% oxygen, there may be a dual benefit in the context of IPD. First, it should mitigate the effect of under-utilization of oxygen by PR mitochondria in the Rho−/− and Prph2rd2/rd2 mutants which lack outer segments and hence show reduced energetic demand due to ion transport in the inner segment. At a later stage, all of the mutants are expected to show reduced oxygen utilization due to reduced PR numbers. The lowered oxygen concentration should therefore reduce ROS production, at the same time as causing induction of the oxygen-sensitive HIF-1 pathway, which can lead to neuroprotection of PRs, at least in the context of light damage (26,27). We therefore maintained wild-type control C57BL/6J and Pde6brd1/d1 retinal explants from PN7 to PN9 in incubators at close to normal ambient oxygen (17% O2), and then switched the test explants to hypoxic (6% O2) chambers from PN10 to
Figure 3. Photoreceptor degeneration in retinal degeneration mutants after administration of MitoQ. ONL analysis (A, C, E, G) and TUNEL assays (B, D, F, H) were performed on paraffin-embedded eye sections from Pde6brd1/rd1 (A, B), Pde6batrd1/atrd1 (C, D), Rho2/2 (E, F) and Prph2rds/rds (G, H) mutants administered MitoQ. A comparison was made between wild-type (blue), retinal degeneration mutants (green) and retinal degeneration mutants administered MitoQ (black). ONL analysis (ONL, solid lines; INL, dotted lines) revealed that, irrespective of antioxidant treatment, photoreceptor degeneration was complete by 3 weeks, 4 weeks, 3 months and 4 months of age in the Pde6brd1/rd1, Pde6batrd1/atrd1, Rho2/2 and Prph2rds/rds mutants, respectively. MitoQ administration had no effect on the number of apoptotic cells detected by TUNEL assays (*P < 0.05, †P < 0.01, ‡P < 0.001 compared with the wild type). INL, inner nuclear layer; ONL, outer nuclear layer.
PN17, at which time PR survival was examined. Wild-type retinas maintained in 6% O2 until PN17 showed no morphological changes or abnormalities in PR numbers, compared with wild-type retinas maintained in 17% O2 (Fig. 5A). In contrast, we found that PR survival increased by 31% in Pde6brd1/rd1 explants maintained in 6% O2 compared with 17% O2, from a mean of 3.8 ± 0.25 (SEM) rows of PRs to a mean of 5 ± 0.16 (SEM) rows, respectively (n = 5; P = 0.004) (Fig. 5A).

To obtain preliminary evidence that the effect of hypoxia on its downstream targets is comparable in wild-type and Pde6brd1/rd1 explants exposed to 6% O2 from PN10 to PN17, we used immunoblotting of retinal explant homogenates to examine the expression of VEGF, an important target of HIF-1, a major regulator of hypoxia-induced transcriptional responses. We also compared the expression of the p42/44 MAPK (ERK1/2) and activated ERK1/2 (p-ERK1/2) in wild-type and Pde6brd1/rd1 explants exposed to 6% O2 from PN10 to PN17, since VEGF protects axotomized retinal ganglion cells from death via an ERK1/2/Akt pathway, and the VEGF-mediated survival of serum-deprived neuronal cells is dependent on ERK1/2 activation (41). We found that VEGF expression increased 2.9-fold and 4.7-fold, respectively, in wild-type and Pde6brd1/rd1 explants exposed to 6% O2 (Fig. 5B), but there was no significant difference in VEGF expression between wild-type and Pde6brd1/rd1 explants (Fig. 5B). Similarly, the expression of p-ERK1/2 was upregulated in hypoxic samples; 1.6-fold in wild-type retinas and 4.4-fold in Pde6brd1/rd1 explants exposed to 6% O2.
pERK1/2 was down-regulated 1.6-fold in normoxic Pde6brd1/rd1 explants compared with the wild type (Fig. 5B). ERK1/2 expression was not significantly different between wild-type and Pde6brd1/rd1 explants, under either normoxic or hypoxic conditions. These findings suggest that IPD retinas respond normally to hypoxia by up-regulating the expression of VEGF, which may lead to the activation of ERK1/2.

**DISCUSSION**

Mitochondrial oxidative stress is a significant feature of IPDs

Our findings suggest that substantial mitochondrial oxidative stress is a common if not general characteristic of IPDs. The 71–86% reductions in activity of mitochondrial complex I that we observed in the retinas of four mouse models of IPDs do not appear to reflect non-specific abnormalities in retinal mitochondria resulting from a degenerating retina, because the activity of the oxidative stress-insensitive mitochondrial enzyme, citrate synthase, was not altered in any of the IPD models. There are two main possibilities to explain the reduced complex I activity, first, oxidation of iron-sulphur clusters in complex I by superoxide to form unstable clusters, promoting the formation of damaging hydroxyl radicals (8,33). Alternatively, H₂O₂ can cause reversible glutathionylation of complex I subunits, especially when the mitochondrial GSH pool is oxidized, which causes a vicious cycle of further increases in superoxide and H₂O₂ formation (42).
The redox status of the retina and liver was also assessed by measuring the concentration of the major cellular antioxidant, reduced GSH. Only 10–20% of cellular GSH is present within mitochondria, the majority being cytoplasmic (43). It scavenges a wide range of ROS and efficiently controls H₂O₂ concentrations in the cell by working with cellular peroxides. The ratio of reduced to oxidized GSH is usually >10:1 in cytoplasm and mitochondria so that measuring reduced GSH in mouse retinas, in which 80% of cells are rod PRs (44), provides a good measure of both mitochondrial and cytoplasmic redox status. It is depleted at an early stage in neurodegenerative disorders such as Parkinson’s disease, contributing to the susceptibility of substantia nigra cells to subsequent oxidation (45). Low retinal GSH concentrations have not previously been reported in retinal degenerations, but in the present study retinal GSH was reduced in Pde6b<sup>rd1/rd1</sup> and Rho<sup>-/-</sup> retinas by 49 and 51%, respectively, indicating severe oxidative stress. Signs of generalized oxidative stress have previously been reported in Pde6b<sup>rd1/rd1</sup> retinas based on increased hydroethidine staining of superoxide radicals in P114 retinas (46) and indirectly by increased expression of the oxidative stress markers ceruloplasmin and clusterin, in both Pde6b<sup>rd1/rd1</sup> and Prph2<sup>2ds/2ds</sup> mutants (47). However, this is the first time that oxidative stress within the mitochondrial compartment has been specifically examined in a range of different retinal degeneration mutants.

The IPD mutants analysed have distinct genetic defects, so it was unexpected that they should each indicate the presence of severe oxidative stress, in both mitochondrial and cellular compartments, at a stage when significant PR cell loss has not yet occurred. The Pde6b<sup>rd1/rd1</sup> and Pde6b<sup>rd1/rd1</sup> mutants both have mutations affecting rod cGMP phosphodiesterase activity, with the rd1 null allele (48) being the more severe of the two (49). Both alleles are thought to increase intracellular cGMP and Ca²⁺ levels in rods (50) which can trigger PR apoptosis (51). Increased intracellular and mitochondrial calcium concentrations are well established although poorly understood causes of both increased ROS formation and cell death (52). The underlying cause of oxidative stress in the Rho<sup>-/-</sup> null mutant is less clear. The most obvious possibility relates to the absence of outer segments secondary to deficiency of rhodopsin, which normally constitutes 85–90% of outer segment protein mass. Similarly, in the Prph2<sup>2ds/2ds</sup> mutant, absence of peripherin 2 (Prph2) protein from PR outer segments results in their failure to form or to be maintained (53). Oxidative stress in both of these mutants could result from exposure of PRs to increased oxygen tension in the outer retina, due to reduced demand for ATP for ion pumping in the inner segments. The high choroidal blood flow, which is 30 times the retinal blood flow, helps to avoid hypoxia in normal dark-adapted retinas caused by the high oxidative activity of inner segment mitochondria (54). However, when ion fluxes in the outer segment are reduced by mutation, the high oxygen tension of the outer retina may then become a liability (55). This possibility is supported by oxygen electrode measurements during retinal degeneration (22–24,55,56). A small increase in oxygen tension in PR inner segment and nuclear layers could have a disproportionate effect on mitochondrial ROS formation since superoxide formation is a second-order reaction that is directly proportional to both oxygen concentration and the proportion of mitochondrial electron carriers in a suitable redox state (25).

The presence of oxidative stress prior to the IPD retinas undergoing significant PR loss on the one hand suggests that changes in these two components (complex I, GSH), which are essential for maintaining redox balance, may have a direct and causal role in PR cell death. On the other hand, the presence of similar reductions in both complex I and GSH in Sod2<sup>+/−</sup> mice, which did not show evidence of PR degeneration, strongly argues against this. There are many different forms of oxidative stress and the increases in superoxide concentration expected to result from a 50% reduction in Sod2 activity may not be the critical species of oxidant associated with an increased probability of PR apoptosis in IPDs (1,2). Alternatively, oxidative stress may not be causally associated with IPD at all.

**Why did MitoQ fail to correct the low complex I activity in mutant retinas?**

Oral MitoQ has been safely administered to mice at the same dose (500 μM) for up to 1 month (35,57) and in the present study was administered for up to 6 months. The animals’ weight and food intake were monitored and no adverse health effects were observed. The absence of MitoQ effects on liver enzymes (complex I, citrate synthase) is also consistent with a lack of significant MitoQ toxicity. Smith et al. (35) previously showed the uptake of orally administered MitoQ in a variety of non-retinal tissues but, to confirm its uptake by the retina, 3H-labelled MitoQ was delivered orally and shown to be taken up by the retina and other tissues at concentrations sufficient to exert antioxidant effects (35,37–39), although it was impossible to estimate intra-mitochondrial concentrations due to membrane potential changes on tissue extraction.

MitoQ has been tested in a wide range of *in vitro* and *in vivo* models and has been shown to protect against several forms of oxidative damage (34). For example, oral MitoQ (500 μM) reduces heart dysfunction, cell death and mitochondrial damage in ischaemia–reperfusion injury (34,38). In the present studies, it produced no evident reduction either in oxidative stress (complex I, GSH) or in PR cell death. MitoQ associates most strongly with the matrix surface and intra-membranous portions of mitochondrial inner membranes, suggesting that it is most effective against lipid peroxidation.

It is thought to act as an antioxidant by being oxidized to the ubiquinone form and recycled to the active ubiquinol form by complex II (34,58). It can also react with superoxide radicals, although it was unable to decrease superoxide formation in a rotenone-treated fibroblast model. However, MitoQ shows negligible reactivity towards H₂O₂, which might explain its inability to influence oxidative stress in the IPD models (58). H₂O₂ is present at substantially higher intracellular concentrations than superoxide [10⁻⁷ M compared with 10⁻¹⁰ M (59)], has a 10-fold longer half-life and is able to diffuse across membranes (59). Although the hydroperoxyl radical is relatively unreactive, it readily interacts with free iron to form damaging hydroxyl radicals by the Fenton reaction (43) and can cause reversible glutathionylation of...
complex I subunits, as discussed above. One possibility is therefore that MitoQ failed to prevent the consequences of increased H$_2$O$_2$ formation within mitochondria in both in vivo and ex vivo explant models, leading either to increased pro-apoptotic signalling at low H$_2$O$_2$ concentrations (1,2) or to direct damage to macromolecules at higher concentrations.

What possible sources of H$_2$O$_2$ could escape quenching by MitoQ? In vitro measurements suggest that mitochondrial membranes are quantitatively the most important physiological source of superoxide (60), which is rapidly converted to H$_2$O$_2$ by superoxide dismutase (61). The lack of PR degeneration in Sod2$^{+/--}$ mice despite substantial evidence of oxidative stress might result from a reduction in H$_2$O$_2$ formation due to Sod2 haploinsufficiency. An alternative source of H$_2$O$_2$ could be p66$^{	ext{SIC}}$, which translocates into the mitochondrial inter-membrane space in response to a wide range of cellular stresses, catalysing electron transfer from reduced cytochrome c to molecular oxygen to form H$_2$O$_2$ (62). p66$^{	ext{SIC}}$ is estimated to account for one-third of the intracellular H$_2$O$_2$ pool (59).

**Hypoxia rescue: what is the underlying mechanism?**

Exposure of IPD explant retinas to a reduced oxygen concentration could have the dual benefits of lowering oxygen free radical formation, attributed to the relative hyperoxia of the outer retina, and of increasing local release of neuroprotective agents such as VEGF (hypoxic preconditioning). Hypoxia was shown to enhance PR survival in a model of light-induced PR degeneration (27), with maximum rescue at oxygen concentrations of 10% or less, suggesting that 6% oxygen might rescue PRs in genetic models of retinal degeneration, whereas 1–3% oxygen could promote severe oxidative stress (25). Mouse Pde6br$^{rd1}$retinal explants were found to show a 31% rescue of degeneration when cultured in a 6% O$_2$ hypoxic chamber. Although systemic but not intraocular EPO can prolong PR survival in Prph2$^{rd1}$ retinas (63), there is no functional vasculature in the retinal explant system, making it improbable that the observed rescue of Pde6br$^{rd1}$ retinal explants is due to EPO. Hypoxia can therefore protect PRs irrespective of secondary effects involving retinal vessels.

Since VEGF up-regulation is a response to retinal hypoxia (64), increased VEGF levels in both wild-type and degenerating retinas exposed to 6% O$_2$ were verified by immunoblotting. Müller-cell-derived VEGF may signal via VEGFR2 receptors in PRs (64) since VEGF has been shown to be directly protective to PRs (30). The evidence indicating a PR survival function for VEGF and the strong up-regulation of VEGF in hypoxic retinas suggests that VEGF is a possible candidate for the protective effect of 6% O$_2$ in the Pde6br$^{rd1}$/rd1 explant system.

PR protection in light-damaged retinas has also been shown to occur through MAPK signalling (65) while VEGF protects axotomized retinal ganglion cells from death via an ERK1/2/Akt pathway (66). VEGF also protects stressed neuronal cultures via activation of ERK1/2 (41). Although ERK1/2 was not differentially expressed in hypoxic retinas, p-ERK1/2 was up-regulated in both wild-type and degenerating retinas cultured in 6% O$_2$, and may therefore be another component of the neuroprotective response to hypoxia. Although p-ERK1/2 expression was lower in wild-type than in Pde6br$^{rd1}$/rd1 explants under normoxic conditions, this could be a consequence of the reduced number of PRs in Pde6br$^{rd1}$/rd1 retinas at PN17 (three to four rows in Pde6br$^{rd1}$/rd1 explants versus seven to eight rows in C57BL/6J explants).

The extent of hypoxia in the explant retinas is not easy to determine, since it depends on cellular respiratory activity, oxygen concentration gradients and diffusion rates across membranes as well as intracellular location. Tissue pO$_2$ levels are expected to be substantially lower than normal in explants lacking a choroidal blood supply. Severe hypoxia (extracellular pO$_2$ of 1.5%) results in a paradoxical increase in ROS, which can stimulate HIF-1 activation (25). However, an additional benefit in the 6% oxygen explant model in reducing the rate of Pde6br$^{rd1}$/rd1 degeneration may result from the reduction of tissue oxygen, which in some forms of retinal degeneration can increase from 1% in PR inner segments to 5% or more (23) as the retina degenerates, with an expected linear increase in mitochondrial superoxide and/or H$_2$O$_2$ production by the respiratory chain and redox enzymes such as p66$^{	ext{SIC}}$ (67).

Oxidative stress has been implicated in many different forms of neurodegeneration. The present study extends this to different genetic types of retinal degeneration and suggests that aberrant mitochondrial electron transport activity associated with increased ROS formation may be a common response in these disorders. The precise mechanisms by which oxidative stress is increased in the different models remain to be elucidated but may include both shared and distinct pathways. However, the failure either to correct intra-mitochondrial oxidative stress or to retard rod PR degeneration with MitoQ suggests that lipid peroxidation is unlikely to be a major mechanism, while increased H$_2$O$_2$ remains a possibility. The MitoQ result may be informative in pointing towards new approaches, such as mitochondrially targeted peroxidases (68). In contrast, hypoxic exposure increased the survival of Pde6br$^{rd1}$/rd1 explant retinas, either directly from a lowering of tissue oxygen or indirectly from local neuroprotection due to release of growth factors such as VEGF. Identifying the molecules responsible for this hypoxia-induced PR protection may elucidate important neuroprotective pathways in the retina, which could be exploited in the development of clinical therapies.

**MATERIALS AND METHODS**

**Animals**

All studies were performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and under the guidance of the Medical Research Council in Responsibility in the Use of Animals for Medical Research (July 1993) and UK Home Office Project Licence 60/3124 or the guidelines for animal maintenance at the Hospital for Sick Children. Mice were fed ad libitum and kept under standard lighting with a 12 h light:dark cycle. The Pde6br$^{rd1}$/rd1 and Pde6br$^{rd1}$/rd1 mice were provided by Professor Ian Jackson (MRC Human Genetics Unit, Edinburgh) and maintained on a C3H background (Jackson...
Laboratory, Bar Harbor, ME, USA). Rho<sup>−/−</sup> mice were on a C57BL/6J background and were made available by Professor Peter Humphries (Trinity College Dublin, Ireland). The Prph2<sup>rds/rds</sup> mice were maintained on a CBA background and were obtained from Professor R. Ali (University College London, UK).

Ear-clip DNA was prepared by the incubation of the tissue in 100 μl of 25 mM NaOH/0.2 M EDTA and heated for 20 min at 95°C using a PCR block. The incubation buffer was neutralized by addition of 100 μl of 40 mM Tris–HCl pH 4.5 and the samples were finally mixed by vortexing and stored at 4°C. The samples were genotyped as described before for Prph2<sup>rds/rds</sup> (69), Rho<sup>−/−</sup> (70), Pde6b<sup>rd1/rd1</sup> and Pde6b<sup>ar/d</sup> (49,71).

**Measuring rates of PR degeneration**

Animals were killed at ages ranging from 1 week to 6 months, and the eyes were removed and fixed in 4% paraformaldehyde (PFA) solution for up to 20 h, dehydrated and embedded in paraffin. For histology and ONL analysis, sections were prepared essentially as previously described (72). Briefly, eyes were enucleated by using sharp tweezers and were placed in a 6% O<sub>2</sub> enclosed chamber (Biospherix, Lacona, NY, USA) at PN10 and the O<sub>2</sub> level maintained by attaching the chamber to a pressurized nitrogen tank (Praxair, Danbury, CT, USA). Media changes for hypoxic culturing were performed in a pre-equilibrated 6% O<sub>2</sub> hood (Biospherix, Lacona, NY, USA). The entire media were replaced for the first two changes and then half the media thereafter, and all explants were cultured until PN17. Retinal explants were fixed for 2 h with 4% PFA and 10 μM ice-cold 3% perchloric acid. The homogenized tissue was centrifuged at 9000 g for 10 min at 4°C and the supernatant retained for GSH measurements. GSH concentration was measured by the production of nitroblue tetrazolium, which was monitored spectrophotometrically at 412 nm at 25°C for 5 min. The concentration of GSH was determined by comparison with a standard curve generated using appropriate concentrations of purified reduced GSH (Sigma-Aldrich, UK).

**MitoQ administration and uptake measurement**

MS010 (MitoQ) (Antipodean Pharmaceuticals, New Zealand) was administered to the animals orally through the drinking water at a concentration of 500 μM. The water bottles were covered with tin foil to keep the solution in the dark. Fresh dilutions were made and administered every 3 days. For uptake measurements, tritium-labelled MitoQ ([3H]MitoQ, 0.743 mCi/ml) (Antipodean Pharmaceuticals) was administered to wild-type animals orally through their drinking water for a period of 10 days. [3H]MitoQ (0.0743 μCi) was added to 500 μM dilutions of unlabelled MitoQ in water. The tritium content of the samples of interest was measured in liquid scintillant (BDH) using an LS 6500 Multi-Purpose scintillation counter (Beckman Counter™). Samples of known protein content were mixed, in 5 ml scintillation vials (LabLogic, Sheffield, UK), with an equal volume of the scintillation fluid and the quenching program was set for 5 min.

For retinal explants, 0.1, 1 and 5 μM MitoQ and DecylTTP dissolved in ethanol were added to media at PN10. Media were replenished with fresh compound every 48 h. Since no toxicity was observed at 5 μM, Pde6b<sup>rd1/rd1</sup> explants were incubated with 5 μM MitoQ and DecylTTP and fixed at PN17 for the examination of the PR ONL.

**Biochemical assays**

Mitochondrial-enriched (post-600g) homogenates were prepared essentially as previously described (72). Briefly, retinas were collected on ice, washed in isolated medium (120 mM KCl, 20 mM HEPES, pH 7.2, 5 mM MgCl<sub>2</sub>, 1 mM EGTA, 5 mg/ml BSA), drained on a filter paper and weighed in plastic Eppendorf tubes. Using a Dounce homogenizer, the tissue was homogenized on ice in 5× weight/volume isolation medium and the homogenate was centrifuged at 600g for 10 min at 4°C. The supernatant was collected and re-centrifuged under the same conditions. The second supernatant was used for enzyme measurements. A volume of 1–10 μl of homogenate (0.2–2.0 mg of tissue) was used for each enzyme assay, and activity measurements were expressed in μmol/min/g tissue. Citrate synthase activity was measured by following the formation of 5-thio-2-nitrobenzoate ion at 412 nm with 425 nm as the reference wavelength (72). Complex I oxidoreductase activity and was measured by following the production of nitroblue tetrazolium, which was monitored spectrophotometrically at 412 nm at 25°C for 5 min. The concentration of GSH was determined by comparison with a standard curve generated using appropriate concentrations of purified reduced GSH (Sigma-Aldrich, UK).

**Measurement of reduced GSH content**

For sample preparation, the washed tissue was homogenized with a Dounce homogenizer on ice in 5× weight/volume
sections cut in OCT compound medium (Tissue-Tek, Miles, Elkhart, IN, USA) using a Leica cryostat for staining and PR counting or suspended in tissue lysis buffer for subsequent immunoblotting. For quantification, the number of PR rows in the ONL was counted at the same distance from the optic nerve. Percentage rescue was determined as (number of rows of photoreceptors in condition 1 − number of rows of photoreceptors in normal)/number of rows of photoreceptors in normal.

**Retinal protein isolation and immunoblot analysis**

Tissue from retinal explants was scraped from the surface of the membrane and placed in a microfuge tube containing 200 μl of tissue lysis buffer: 10 mM Tris–HCl, 1% SDS, 1 mM sodium orthovanadate, 2 mM EDTA and 1× protease inhibitors (Roche, Laval, Quebec, Canada). Samples were vortexed and the lysate passed through a 23 gauge needle 20× followed by incubation at 60°C for 20 min and centrifugation at 21 000 × g for 20 min. The supernatant was then isolated and the protein concentration quantified using the Bio-Rad protein assay (Biorad, Mississauga, Ontario, Canada). Eighty micrograms of protein from retinal explants were combined with loading buffer, vortexed briefly, boiled for 5 min and electrophoresed on a 10% SDS-polyacrylamide gel. The resulting proteins were transferred to Hybond-C extra membrane (Amersham) in normal. The membrane was blocked in 5% non-fat milk in TBS-T and probed with a range of primary antibodies at the indicated dilutions: phospho-ERK1/2 (Cell Signaling), 1:500 VEGFA (R&D Systems, Minneapolis, MN), 1:100 ERK1/2 (Cell Signaling, Danvers, MA, USA), 1:100 b-tubulin 1:1000 (Cell Signaling), 1:500 VEGFA (R&D Systems, Burlington, Ontario, Canada) and b-tubulin 1:1000 (Cell Signaling). Anti-rabbit or anti-goat conjugated horse-radish peroxidase secondary antibody was then incubated with the blot for detection by enhanced chemiluminescence and exposure to film.

**SUPPLEMENTARY MATERIAL**

Supplementary Material is available at HMG online.

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