Retinal metabolic state of the proline-23-histidine rat model of retinitis pigmentosa

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Acosta ML, Shin Y, Ready S, Fletcher EL, Christie DL, Kalloniatis M. Retinal metabolic state of the proline-23-histidine rat model of retinitis pigmentosa. Am J Physiol Cell Physiol 298: C764–C774, 2010. First published December 23, 2009; doi:10.1152/ajpcell.00253.2009.—We determined the metabolic changes that precede cell death in the dystrophic proline-23-histidine (P23H) line 3 (P23H-3) rat retina compared with the normal Sprague-Dawley (SD) rat retina. Metabolite levels and metabolic enzymes were analyzed early in development and during the early stages of degeneration in the P23H-3 retina. Control and degenerating retinas showed an age-dependent change in metabolite levels and enzymatic activity, particularly around the time when phototransduction was activated. However, lactate dehydrogenase (LDH) activity was significantly higher in P23H-3 than SD retina before the onset of photoreceptor death. The creatine/phosphocreatine system did not contribute to the increase in ATP, because phosphocreatine levels, creatine kinase, and expression of the creatine transporter remained constant. However, Na+/K+-ATPase and Mg2+-Ca2+-ATPase activities were increased in the developing P23H-3 retina. Therefore, photoreceptor apoptosis in the P23H-3 retina occurs in an environment of increased LDH, ATPase activity, and higher-than-normal ATP levels. We tested the effect of metabolic challenge to the retina by inhibiting monocarboxylate transport with α-cyano-4-hydroxycinnamic acid or systemically administering the phosphodiesterase inhibitor sildenafil. Secondary to monocarboxylate transport inhibition, the P23H-3 retina did not demonstrate alterations in metabolic activity. However, administration of sildenafil significantly reduced LDH activity in the P23H-3 retina and increased the number of terminal deoxynucleotidyl transferase biotin-dUPT nick end-labeled photoreceptor cells. Photoreceptor cells with a rhodopsin mutation display an increased demand for the execution of apoptotic and nonapoptotic pathways, which have several energy-dependent reactions, thus altering energy demand (17, 23, 55–57, 59, 62, 63, 86, 107, 109). The current understanding is that ATP is necessary only for the early stages of apoptosis (17, 55).

To investigate the metabolic activity that precedes degeneration, we used the proline-23-histidine (P23H) line 3 (P23H-3) rat model of retinal dystrophy. The P23H-3 rat contains a rhodopsin-mutant transgene created from the human rhodopsin mutation, leading to a slow rate of photoreceptor degeneration (54). In the P23H rat, photoreceptor cell death appears to result from aggregation of the mutant rhodopsin form that is targeted for degradation by the ubiquitin proteasome system (49, 85). In this rat model, although the peak in apoptosis is observed between postnatal days 21 (P21) and 25 (P25) in an environment of high oxygen metabolism, numerous photoreceptor cells, mainly cones, survive into adulthood (103). The slow rate of degeneration is largely sustained by upregulation of protective mechanisms (103). Nevertheless, light exposure increases apoptosis in the P23H rat beyond that observed in other strains, an effect that may be the result of accelerated oxidative stress (75). This strain thus provides a model for analysis of the metabolic changes that precede photoreceptor death (103, 106) and infer whether high oxidative phosphorylation and overactivation of ATP-producing pathways, gener-
ally speaking, a metabolic overload, are the triggers of photoreceptor death. To test this, our first experimental question was as follows: Does the P23H-3 rat retina display metabolic overload? We addressed this question as follows. 1) We measured the activity of lactate dehydrogenase (LDH), a key enzyme in retinal energy production and a sensitive indicator of retinal metabolic function (3, 4, 37–39, 100, 106). 2) We measured metabolite levels and key metabolic enzymes to address whether metabolic demand was met by ATP production. ATP levels play an important role in biasing cell death pathways toward apoptotic and nonapoptotic paths (17, 26, 27, 55, 56). Inasmuch as ATP is consumed by Na\(^+\)-K\(^+\)-ATPase activity, which fuels cation extrusion as part of the photoreceptor dark current (6), we reasoned that increased ATP may be related to its consumption by the Na\(^+\)-K\(^+\)-ATPase channels. Alternatively, inasmuch as the Na\(^+\)-K\(^+\)-ATPase is also involved in the modulation of Na\(^+\) and K\(^+\) flux that causes cell shrinkage in apoptosis (10, 70, 76, 99, 105), its activity may reflect activation of the death pathway. We also tested the contribution of the creatine/phosphocreatine system, inasmuch as high concentrations of creatine kinase (CK) in photoreceptors (97), in addition to the high level of creatine transporter (CRT) expression (5), are consistent with a role of creatine as an energy supplier in the retina.

Our second question was as follows: How does the P23H-3 rat retina respond to metabolic challenge? Animal models of retinal dystrophy are extremely susceptible to light exposure (19, 78, 98, 106), leading us to hypothesize that a metabolic challenge would potentially alter short- and long-term metabolic activity in the P23H rat retina. We addressed this issue as follows. 1) We tested the effect of monocarboxylate transporter (MCT) inhibition using α-cyano-4-hydroxycinnamic acid (4-CIN) (13, 14, 45). 2) We tested the response of the retina to inhibition of phosphodiesterase, which may result in the maintained opening of photoreceptor cation channels (8), with a subsequent demand to increase cation extrusion and, thus, an increased metabolic demand (29, 80).

**MATERIALS AND METHODS**

**Animal manipulation.** All experiments were performed according to procedures approved by the University of Auckland Animal Ethics Committee and in accordance with the Association for Research in Vision and Ophthalmic Statement for the Use of Animals in Ophthalmic and Vision Research. Sprague-Dawley (SD) rat retina was used as a control to the P23H-3 rat retina. The P23H rat carries an autosomal-dominant form of human rhodopsin mutation, causing retinitis pigmentosa (58, 69). We employed heterozygous P23H-3 rats, obtained by crossing homozygous P23H-3 males with SD females, to reflect the genetic make-up found in the human population. The P23H-3 rat has a normal rhodopsin distribution in the photoreceptors (40), and 30% of photoreceptors have degenerated by 60 days of age (69). LDH activity was monitored over an extensive age range (69). LDH activity was monitored over an extensive age range (69). LDH activity was monitored over an extensive age range (69).

**Tissue processing.** After the rats were anesthetized, the eyes were dissected from the orbit, and the anterior part of the eye was removed by cutting 1 mm posterior to the limbus using dissection scissors and forceps. The retinal pigmented epithelium, choroid, and sclera were separated, and only the retina was processed. The vitreous fluid was drained from the retinal samples prepared for biochemical assays, and the retinal tissue was carefully scraped from the eyecup. Any blood coagula or surface blood on the isolated retina was gently removed with tweezers and absorbed in tissue paper. Retina samples were processed in three ways: 1) they were quickly snap-frozen; 2) they were stored at −80°C until subsequent extraction of metabolites with perchloric acid; or 3) they were immediately homogenized in 0.9% saline for measurement of lactate levels, CK, or ATPase activity. Values were normalized to protein content in each sample. Alternately, samples were immediately fixed in 4% (wt/vol) paraformaldehyde-0.01% (vol/vol) glutaraldehyde in 0.1 M phosphate buffer, pH 7.4, for 30 min. After fixation, the eyecups were washed in 0.1 M phosphate buffer and cryoprotected in graded sucrose solutions up to 30% (wt/vol). A cryostat (model CM3050 S, Leica, Heidelberg, Germany) was used to section the tissue in the vertical plane at a thickness of 16 μm. The retinae were sectioned. Samples were collected onto Superfrost Plus glycerol-coated slides, which were stored at −20°C until they were processed for immunocytochemistry.

**Extraction of metabolites.** Metabolites were extracted by homogenization of the retina in 0.5 M perchloric acid with a glass-Teflon homogenizer. The samples were kept on ice for 5 min while they were periodically vortexed; then they were processed in a refrigerated centrifuge (model 5415R, Eppendorf, Hamburg, Germany) at 2,500 g for 5 min. After they were centrifuged, the samples were neutralized with 2 M KHCO\(_3\) and kept on ice for another 5 min while they were periodically vortexed. After a 15-min final centrifugation, the supernatant was collected and used for the metabolic assays. The pellet was resuspended and used for the bichromatic acid (BCA) protein assay (Pierce, Rockford, IL).

**ATP and phosphocreatine assays.** ATP concentration in the retina was assayed by addition of a known amount of homogenized sample to a solution containing 50 mM Tris buffer (pH 7.4), 32 mM glucose, 0.4 mM NADP\(^+\), 12 μM hexokinase, 5 μM glucose-6-phosphate dehydrogenase, and 0.03 mM bovine serum albumin. The fluorescence of the reagent was measured with a fluorometer (Turner Biosystems Modulus, Sunnyvale, CA) within 30 min of sample preparation. ATP concentration in the samples was determined relative to an ATP standard curve. For the phosphocreatine assay, 1 U of CK and 0.65 mM ADP were added to each of the samples, which were then mixed by inversion. The reactions were incubated at room tempera-
ture in the dark, and values were recorded after 60 min of incubation. The amount of ATP produced after addition of CK was subtracted from the values obtained in the first reaction (47). ATP and phospho-

creatin values are plotted as millimoles per gram of protein.

**Measurement of lactate.** Lactate concentration in the retina was determined using a lactate assay kit (catalog no. K607-100, BioVision Research Products). Left and right retinas from each animal were pooled and homogenized in 0.9% saline and centrifuged at 2,800 g for 6 min, and the supernatant was diluted to 1:7 with saline. Then 50 µl of both samples and the lactate standards were added in duplicate to a 96-well microplate. An equal volume of a reaction mix containing the lactate assay buffer, lactate probe, and enzyme mix was added to each of the wells. The plate was incubated at 37°C for 30 min in the lactate assay buffer, lactate probe, and enzyme mix was added to each of the wells. The plate was incubated at 37°C for 30 min in darkness, and absorbance was read in a microplate reader at 565-nm wavelength. Protein content was taken into account, and the average amount of lactate at each age is plotted as millimoles per gram of protein.

**CK assay.** For determination of CK activity, left and right retinas were pooled, homogenized in 0.9% (vol/vol) saline, and briefly centrifuged at 2,800 g. The supernatant was diluted to 1:7 in saline and added to Thermo Trace CK-NAC reagent, which contains 100 mM Tris buffer, 31.5 mM creatine phosphate, 5.3 mM AMP, 2.2 mM NADP, 2.1 mM EDTA, 10.3 µM PiP1-diadenosine 5′-pentaphosphate, 10.5 mM Mg2+, 2.7 mM ADP, 21 mM d-glucose, 21 mM N-acetyl-l-cysteine (NAC), 3,000 U/l hexokinase, and 2,000 U/l glucose-6-phosphate dehydrogenase. The change in NADH absorbance over time was measured six times for each retina with use of a spectrophotometer (model UV-2501PC, Shimadzu). Protein content was determined using a BCA assay kit. Average CK activity was calculated and is expressed in micromoles per minute per milligram of protein.

**Biochemical (LDH and ATPase) assays.** LDH activity was assayed using a commercially available kit (Trace, Noble Park, Victoria, Australia), as previously described (3, 4). Total ATPase, Na+-K+-ATPase, and Mg2+-Ca2+-ATPase activities were determined on the basis of a modification of a protocol described by Else and co-workers (28), as previously described (3, 4). For all assays, protein content was determined using the BCA protein assay, as previously described (28).

**Agmatine permeation.** Agmatine (AGB) is a suitable marker of permeability of cationic channels in the vertebrate retina (71). Adult P23H-3 retinas were dissected, mounted on filter paper, and incubated in physiological medium containing AGB for assessment of the cation channel activity of the photoreceptors (52, 71, 72) following a procedure previously reported (3). After the incubation step, the retina was fixed, and AGB-positive cells were immunocytochemically detected and counted in retinal tissue collected from saline-injected (n = 5) and sildenafil-treated (n = 6) P23H-3 retinas. The animals were injected at P16 and P59 and processed for AGB incubation 1 day after treatment. Values were averaged from six randomly selected ONL areas each. AGB-labeled cells were normalized to unit area and are reported as AGB cells per square millimeter of ONL of retina.

**Immunocytochemistry.** The protocol for detection of AGB gating employs indirect immunocytochemistry using a rabbit polyclonal AGB antibody (71, 73), as previously described (2, 106). Immunocytochemistry was also used for detection of the expression pattern of the CK using a rabbit anti-CK antibody (5, 18). Mouse anti-calretinin (1:1,000 dilution; BD Transduction) was employed as a reference marker of inner retina cells. The characteristics of the anti-CK antibody have been fully described by Acosta et al. (5), and the calretinin antibody has been fully characterized in the mouse (1) and rat (48) retina. The immunocytochemical protocol includes 1 h of blocking in 6% normal goat serum, 1% (vol/vol) bovine serum albumin, and 0.5% (vol/vol) Triton X-100, followed by incubation overnight in antibodies diluted in 3% (vol/vol) normal goat serum, 1% (vol/vol) bovine serum albumin, and 0.5% (vol/vol) Triton X-100. The labeling was detected using goat anti-mouse or goat anti-rabbit secondary antibodies conjugated to Alexa Fluor 488 or Alexa Fluor 594 (Molecular Probes, Eugene, OR). The immunolabeling was visualized using confocal microscopy (model TCS 4D, Leica). Micrographs correspond to the average intensity projection of a 7-µm stack of images from a central area of the retina. Images of control and P23H-3 retinas were processed equivalently by adjustment of brightness and contrast using Adobe Photoshop software (Mountain View, CA).

**Apoptotic assay.** Cell death was detected using a fluorescein-based kit (Roche Diagnostics, Mannheim, Germany). We previously used the TUNEL method as a marker of apoptosis (106). A fluorescent microscope with FITC filters and a ×40 objective lens was employed, and the retinas were photographed with a Leica DC 500 camera (Leica Microsystems, Heerburg, Germany). The number of TUNEL-positive photoreceptors in sildenafil-treated P23H-3 rats (n = 6) was compared with that in age-matched control saline-injected P23H-3 rats (n = 5). TUNEL in saline- or sildenafil-injected SD rats at P16 and P59 was assessed 1 wk after injection (n = 5 each age group). TUNEL in 4-CIN-injected P23H-3 retinas (n = 5) was compared with that in contralateral saline-injected eyes. TUNEL-positive photoreceptor cells were counted in three randomly selected ONL areas within 4 mm2 of the optic nerve. Linear distance of each sample was ~1,200 µm. Our sampling was carried out in areas where the photoreceptors were predominantly aligned along their long axis, thus avoiding potential sampling artifacts associated with oblique sections. To confirm this, we measured the ONL thickness of our samples and found no significant differences between control (saline-injected) and sildenafil-treated samples of P23H-3 rat retina (47.2 ± 4.3 vs. 44.5 ± 1.3 µm, P = 0.3 by Student’s t-test). We display the results as the number of TUNEL-positive cells per millimeter of linear ONL retina.

**Inhibition of MCTs.** Inhibition of MCTs was achieved by using 4-CIN (Sigma-Aldrich, St. Louis, MO) at a concentration that inhibits pyruvate and lactate transport (13, 14). 4-CIN was dissolved in 0.5 M NaOH, and pH was brought to 7.4 using 0.5 M HCl. The drug was administered intravitreally. With the assumption that average vitreous chamber volume is 40 µl for the rat (24) and <1 µl is lost during the injection, the final concentration of 4-CIN inside the vitreous chamber was calculated to be 10 mM. An aliquot of 4 µl of 4-CIN or saline (control) was delivered into the vitreous chamber by insertion of a 30-gauge needle at a 45° angle behind the limbus in the superior retina. Young (P17) rats were also subjected to the intracocular injection procedure, with doses and injection volume recalculated for the smaller eye size. Retinas were analyzed 40 min after 4-CIN treatment, given that the largest functional changes in the electrotoretinogram are observed at this time (13). Anatomic analysis showed that the saline and 4-CIN injections resulted in areas of retinal detachment and hemorrhages similar to those reported by Hancock et al. (46). Intravitreal injections cause a break in the blood-retina barrier, and the increase in metabolite levels in SD and P23H-3 saline-injected retinas (see Fig. 5) is likely the result of anatomic and hypotonic changes. To analyze the effect of 4-CIN independent of the injection damage, we compared paired control and 4-CIN retinas incubated in a physiologic medium for 40 min. The long-term effect of 4-CIN was tested by injection of 10 mM 4-CIN into the vitreous chamber each week for wk and measurement of LDH activity 1 wk after the last injection. Apoptotic assays were conducted in P23H-3 retinas collected 1 day after injection of 10 mM 4-CIN at P59.

**Inhibition of phosphodiesterase.** Sildenafil (Pfizer Pharmaceuticals, New York, NY) is the active ingredient of Viagra tablets. The tablets were macerated using a mortar and pestle and dissolved in sterile physiological saline to a concentration of 2.6 mg/ml in a manner identical to that reported by Behn and Potter (8). The solution was warmed to 37°C, and animals received intraperitoneal injections via a 1-ml syringe with a 30-gauge needle. The injection regimen in young (P16) rats involved five injections over the day to achieve a maximal dose of 10 mg/kg. Adult (P59) male rats were injected with a total dose of 4 mg·kg⁻¹·day⁻¹ on 5–12 occasions (60–120 µl per injection for adult rats). Only male rats were used, inasmuch as clearance
rates of sildenafil differ between male and female rats. The multiple injections noted earlier would maintain an overall higher systemic concentration of the drug. The 10 mg/kg dose is ~7 times the equivalent dose-by-weight comparison for a 70-kg human, and the 4 mg/kg doses are 2.5 times the maximum dose (8). Toxicology studies conducted by Pfizer indicate that mortality in adult rats occurs at doses of 500 mg/kg (http://www.docstoc.com/docs/12409146/5898023pdf), which is well above our 1-day treatment with 4 mg/kg. Rats in all experimental groups were allowed to recover for 24 h or 7 days before the biochemical assays. Apoptotic assays were conducted in tissue collected 2 wk after injection.

**Statistics.** All data sets were statistically analyzed using two-way ANOVA with post hoc paired-sample t-test for age groups (SPSS version 16, SPSS, Chicago, IL). Student’s t-test was used for paired comparisons. \( P \leq 0.05 \) was considered to be statistically significant. Values are means ± SE.

**RESULTS**

**LDH activity in SD and P23H-3 retinas.** In control SD rat retina, LDH activity increases as a function of age, reaching an asymptote at approximately P30 (Fig. 1A), when retinal function is similar to that of an adult. Given that the expression of apoptotic markers in the P23H-3 retina peaks at ~3 wk of age (103), we wanted to determine whether the LDH activity ratio was different between SD and P23H-3 rat retinas before and during the early stages of photoreceptor degeneration. We therefore plotted LDH activity as a ratio of LDH activity in P23H-3 retinas to LDH activity in SD retinas (Fig. 1B) to highlight this interaction. Each datum point is the ratio of LDH activity of an individual P23H-3 retina to mean LDH activity of SD retinas at the corresponding age. When collapsed across the two rat groups, age has a significant effect on the LDH ratio (\( P < 0.0001 \) by ANOVA), with no significant difference between the two groups (\( P = 0.47 \) by ANOVA). However, a significant interaction (\( P < 0.0001 \) by ANOVA) was found, indicative of altered LDH activity for the two rat strains. Overall, there was an increase in the amount of LDH activity up to P21, followed by a reduction at P30 and thereafter.

The significant interaction and known biological changes allow us to undertake age-specific statistical analysis, examining the ages before (P1–P21) and during (P30–P200) degeneration. At P1–P21, LDH activity was higher in the P23H-3 than SD retinas (\( P < 0.01 \) by ANOVA) and then was significantly reduced at P30–P200 (\( P < 0.001 \) by ANOVA). At P62, no significant differences were found in LDH activity between SD and P23H-3 retinas (\( P = 0.23 \) by t-test), despite the loss of a considerable number of photoreceptors (~30% of the ONL (69)). This suggests that the LDH activity within the remaining photoreceptors is likely to be elevated, given that the ONL is the major contributor to overall LDH activity (3, 9). Given the significant increase in the LDH activity ratio at early ages (Fig. 1B), we conclude that the P23H-3 retina is highly metabolically active before and during the early stages of retinal degeneration. Similar to our previous studies, we identified reduced LDH activity at late stages of degeneration, highlighting the preponderant location of LDH in photoreceptor cells (3, 4). We therefore focused our studies on metabolite levels, metabolic enzymes, and metabolic challenge before and during the early stages of retinal degeneration.

**ATP levels in SD and P23H-3 rat retinas.** To investigate energy availability in the normal and degenerating retina, we measured ATP concentration as a function of development. Throughout development, ATP levels were significantly higher in P23H-3 than SD retinas (Fig. 2A; \( P < 0.01 \) by ANOVA). Age also had a significant effect (\( P < 0.0001 \) by ANOVA), with no significant interaction (\( P = 0.32 \) by ANOVA), implying that ATP levels are, on average, significantly higher before and during the early stages of degeneration. Overall, significantly higher ATP levels were observed in P23H-3 retinas at P6 (13.5%), P12 (18%), P17 (28%), P25 (6%), and P60 (16%).

**Phosphocreatine levels in SD and P23H-3 rat retinas.** To determine the contribution of the creatine/phosphocreatine system to the metabolic demand of the developing and degenerating retina, we measured the mean concentration of phosphocreatine in normal SD and P23H-3 retinas as a function of age (Fig. 2B). Phosphocreatine levels fluctuated throughout development in SD and P23H-3 retinas, although a significant effect of age was identified (\( P < 0.0001 \) by ANOVA), but no significant difference was found between the two rat strains, when all the ages were analyzed (\( P = 0.52 \) by ANOVA). A significant interaction was found (\( P < 0.0001 \) by ANOVA), allowing us to use statistical analysis to determine whether each rat strain behaved differently before degeneration (P6–P17; \( P = 0.1 \) by ANOVA) and during the early stages of retinal degeneration (P25–P60; \( P < 0.02 \) by ANOVA). We concluded that phosphocreatine levels do not differ significantly between P23H-3 and SD retinas before the onset of degeneration, although they differed at the peak of apoptosis in...
the P23H-3 retina and declined during the early stages of photoreceptor degeneration (P60).

**Lactate content in SD and P23H-3 rat retinas.** The amount of lactate was measured at five key ages during development (Fig. 2C). Statistical analysis showed that age was a significant factor (P < 0.0001 by ANOVA), with neither significant differences between the two strains nor a significant interaction (P = 0.93 and P = 0.18, respectively, by ANOVA). We conclude that lactate levels are not different between the two strains at any stage during development and degeneration.

**CK activity in SD and P23H-3 rat retinas.** Phosphorylation of creatine is determined by CK activity. We measured CK enzyme activity as a function of development and found that age significantly affected CK activity (P < 0.001 by ANOVA), although neither a significant difference between rat strains nor a significant interaction (P = 0.71 and P = 0.29, respectively, by ANOVA) was observed. Overall, no significant differences were seen in CK activity between SD and P23H-3 rat retinas (Fig. 3A). The results emphasize that constant phosphocreatine levels are not related to its utilization, because CK activity is comparable to the activity in control retina.

**Expression of CRT in SD and P23H-3 retinas.** We wanted to determine whether the small changes in retinal phosphocreatine levels during early stages of degeneration (Fig. 2B) were accompanied by changes in CRT expression (89). We performed colocalization studies using a CRT antiserum and a marker of amacrine and ganglion cells, used here to visualize the inner retina. The pattern of CRT labeling in the rat retina was similar to that in the mouse retina (5). Control and P23H-3 retinas showed a very similar pattern of CRT labeling. In the P23H-3 retina at P60, the thickness of the ONL was reduced. Although CRT expression was not quantified in P23H-3 retinas, it was not visibly different from that in control retinas (Fig. 3, B and C).

**ATPase activity in SD and P23H-3 rat retinas.** The availability of ATP throughout the initial stages of degeneration suggests that energy-dependent cell death pathways can be used. We next wanted to determine whether the high metabolic state of cells in the P23H-3 retina signified a potential susceptibility to metabolic challenge. To determine whether high ATP levels were related to retinal function, we measured total ATPase activity in SD and P23H-3 rat retinas.
ATPase activity (Fig. 4A), Na⁺-K⁺-ATPase activity (Fig. 4B), and Mg²⁺-Ca²⁺-ATPase activity (Fig. 4C) in P23H-3 and SD rat retinas. Total ATPase activity was similar in SD and P23H-3 retinas throughout development, with no significant difference between the rat strains (P = 0.07 by ANOVA) and no significant interaction (P = 0.18 by ANOVA). Age was a significant factor in total ATPase activity (P < 0.0001 by ANOVA). Although total ATPase activity was not different for the two rat strains, Na⁺-K⁺-ATPase activity was significantly elevated in P23H-3 rat retinas (P < 0.006 by ANOVA), and age had a significant influence on Na⁺-K⁺-ATPase activity (P < 0.0001 by ANOVA). Age was a significant factor, and a significant interaction (P = 0.34 by ANOVA), and thus we conclude that Na⁺-K⁺-ATPase activity was significantly different throughout development (P < 0.0001 by ANOVA). Mg²⁺-Ca²⁺-ATPase activity accounted for most of the ATPase activity of the retina and was significantly different before and during the early stages of photoreceptor degeneration in the P23H-3 rat retina. Mg²⁺-Ca²⁺-ATPase activity was not significantly different between the rat strains when all the ages were analyzed (P = 0.1 by ANOVA). Age was a significant factor, and a significant interaction (P < 0.003 by ANOVA) was present, suggesting differences between the SD and P23H-3 retinas. The significant interaction and known biological changes allowed us to examine the ages before (P6–P12–P21) and during (P30–P60) degeneration. At P6–P21, Mg²⁺-Ca²⁺-ATPase activity was higher in P23H-3 than SD retinas (P < 0.05 by ANOVA), with no significant reduction in Mg²⁺-Ca²⁺-ATPase activity at P30–P60 (P = 0.06 by ANOVA).

The availability of ATP in P23H-3 rats throughout the initial stages of degeneration suggests that energy-dependent cell death pathways can be used. Given the elevated LDH activity and increased Na⁺-K⁺-ATPase activity in the P23H-3 rat retina early in development and degeneration, we next wanted to determine whether the high metabolic state of cells in the P23H-3 retina signified a potential susceptibility to metabolic challenge.

Effect of 4-CIN on short- and long-term metabolism in SD and P23H-3 rat retinas. Lactate is a valuable substrate for the production of energy that, in the retina, is associated with active communication between neurons and Müller cells (81, 91). To determine whether ATP and phosphocreatine are altered secondary to short-term lactate transport inhibition in control and P23H-3 retinas, we applied 4-CIN, a general inhibitor of MCTs (45). The effect of intraocular injection of 4-CIN was analyzed 40 min after treatment. Significantly greater changes in metabolite levels were observed after injection of saline, suggesting that the procedure causes transient metabolic changes (Fig. 5, A and B) in young (P17) and older (P60) SD and P23H-3 retinas. However, there was no statistically significant difference in the levels of ATP or phosphocreatine in P23H-3 compared with SD retinas, suggesting that the retinas were not susceptible to metabolic insult over and above that induced by the injection procedure (Fig. 5, A and B). The dystrophic and normal retina responded in a similar way shortly after metabolic challenge, by maintaining ATP and phosphocreatine levels.

To discern the effect of 4-CIN independent of the injection procedure, we incubated P23H-3 and SD retinas in a physiological buffer containing 4-CIN and analyzed LDH activity of the retina after 40 min of incubation. Figure 5C shows no changes in the enzymatic activity of LDH after incubation in 4-CIN. We also injected P23H-3 eyes and analyzed LDH activity 1 wk after two weekly injections (3 wk after the 1st injection) and observed no significant changes between saline- and 4-CIN-injected eyes (Fig. 5C). Quantification of TUNEL-positive photoreceptor cells per unit area (Fig. 5D) 1 day after treatment of P23H-3 rats at P59 revealed no differences between saline- and 4-CIN-injected retinas (P = 0.9 by t-test). Overall, the P23H-3 retina displayed resilience to direct metabolic inhibition of MCT.

Short- and long-term metabolism in phosphodiesterase-inhibited retinas. The resilience to a metabolic challenge (4-CIN experiment) indicates the ability of the retina to adapt to changes in metabolic substrate availability. We wanted, however, to challenge the P23H-3 rat retina using a specific photoreceptor enzyme inhibitor that likely results in elevated cation entry into photoreceptors. We applied sildenafil, a general inhibitor of phosphodiesterase enzymes (20), which is the active ingredient of Viagra, and analyzed the effect on LDH activity in normal and P23H-3 rat retinas. Dysfunction of phosphodiesterase causes accumulation of cGMP in rd1 mice, resulting in photoreceptor apoptosis early in development (30, 64–66, 82). LDH activity, as a key indicator of the metabolic demand of the retina, together with its strategic location in the photoreceptors (4), allowed us to assess whether metabolic demand is increased or reduced by metabolic downregulation or because of photoreceptor loss.
There was no difference in LDH activity between the retinas of noninjected rats and those that had received an intraperitoneal injection of saline (Fig. 6A). LDH activity of the SD retina at P60 was not affected by intraperitoneal injection of sildenafil; however, in the P23H-3 rat, LDH activity was significantly reduced (8.18 ± 0.24 and 7.29 ± 0.24 mg/kg, respectively, P < 0.001 by Student’s t-test). To reduce phosphodiesterase activity in the younger P23H-3 retinas, we applied a 10 mg/kg dose, which reduced LDH activity by 8.5% 1 day after intraperitoneal administration (7.44 ± 0.20 and 6.81 ± 0.20 mg/kg·min⁻¹·mg protein⁻¹ for saline and sildenafil, respectively, P < 0.05 by t-test). However, at P16, LDH activity was significantly higher in retinas of sildenafil- than saline-injected SD rats (6.47 ± 0.16 vs. 7.04 ± 0.16 mg/kg·min⁻¹·mg protein⁻¹, P < 0.001 by t-test). SD rats were not affected in the long term. At 1 wk after intraperitoneal injection, the reduction in LDH activity was still observed in P23H-3 rats (Fig. 6B). At P66, P23H-3 rats showed a 15% reduction in LDH activity (8.93 ± 0.54 and 7.64 ± 0.54 mg/kg·min⁻¹·mg protein⁻¹ for saline and sildenafil, respectively, P < 0.05 by t-test) compared with controls. The results suggest that a reduction in LDH activity is a marker of the impending cell death; thus we tested photoreceptor cation overload and expression of cell death markers in P23H-3 rat retina.

**Cell death markers in P23H-3 rat retina.** We compared the TUNEL pattern in saline- and sildenafil-treated P23H-3 rats and observed the greatest number of positive cells in the ONL region (Fig. 6, C and D). However, in sildenafil-treated animals, TUNEL-positive cells were also observed occasionally in the inner nuclear layer (Fig. 6D, arrowhead). TUNEL photoreceptor cells were quantified in tissue collected 2 wk after sildenafil injection, and the result was compared with the number of TUNEL photoreceptor cells in saline-injected age-matched P23H-3 rats. We observed 41 ± 12.6 and 26 ± 9.8 cells/mm retina in sildenafil-treated (4 mg/kg) and saline-injected adult P23H-3 rats, respectively (P < 0.05 by Student’s t-test; Fig. 6E). In SD rats, injection of sildenafil at P16 (10 mg/kg) and P59 (4 mg/kg) did not result in increased TUNEL, even 1 wk after treatment (Fig. 6F).

**Cation gating in P23H-3 rat retina.** We then wanted to determine whether the systemically administered phosphodiesterase inhibitor altered cation entry into photoreceptors, as assessed using AGB gating and labeling. If the increased TUNEL was due to increased cation entry, we would expect to find an increased number of labeled photoreceptors as soon as the day after sildenafil injection in the P23H-3 rat retina. AGB-labeled photoreceptor cells were counted in saline- and sildenafil-injected young (P17) and early-degenerating (P60) P23H-3 retinas (Fig. 6, G–I). There were no differences in AGB-labeled photoreceptor cells between young and adult retinas (P = 0.6 by ANOVA), and treatment with sildenafil did not significantly change the number of AGB-labeled cells at either age (P > 0.5 by Student’s t-test).

**DISCUSSION**

We have investigated the metabolic demand, energy production, and availability, and energy requirements of the degenerating retina. We found elevated LDH activity and high ATP levels in the developing P23H-3 retina, suggesting that the retina is working in an environment of higher-than-normal metabolism. It appears that the P23H-3 retina is working under chronic stress (103), and we demonstrated that degeneration in the P23H-3 retina is occurring in an environment of high ATP levels and high Na⁺-K⁺-ATPase and Mg²⁺-Ca²⁺-ATPase activity, despite a normal high-energy phosphate transport and normal lactate levels. Inhibition of cGMP-phosphodiesterase resulted in reduced LDH activity in the P23H-3 rat, a reduction that has been shown to be indicative of photoreceptor loss in other models (3, 9). We also confirm an increase in the number of TUNEL-positive photoreceptors in the P23H-3 rat retina exposed to sildenafil. The results from the sildenafil experiments in the P23H-3 retina suggest that 1 day of exposure to presumed higher cGMP levels due to phosphodiesterase inhib-
bition accelerates photoreceptor death in a metabolically overloaded retina.

What causes metabolic overload in P23H-3 retina? The P23H-3 retina functions under a chronic high-stress environment, where there is a progressive but slow rate of photoreceptor degeneration, upregulation of stress-inducible proteins, and chronic hyperoxia in the outer retina (103, 104). The mutation of rhodopsin in the P23H-3 rat has not been linked to rhodopsin sorting (40); however, in transgenic mice expressing the human P23H mutation, overexpression of rhodopsin and accumulation in the outer plexiform layer have been reported (74, 83). In fact, cell culture studies also support the idea that the mutant P23H rhodopsin leads to aggresome accumulation; nevertheless, the dynamic of opsin synthesis and recycling in inner and outer segments cannot be absolutely explained with undifferentiated cell lines. Yet human donor tissue shows shortened rods and cones in the outer segment and relocalization of rhodopsin to the inner segments, cell bodies, and synapses (50). Saito et al. (84) recently reported that altered dynamics of P23H rhodopsin phosphorylation result in upregulation of energy production. The results suggest that the P23H-3 retina is producing enough ATP to compensate for the delayed photoresponse recovery proposed by Saito et al., and essentially the profile of the P23H-3 retina resembles a cellular metabolic overload scenario similar to that of the rd/rd and rds/rds retinas (3, 63).

We highlighted that upregulation of the phosphocreatine system is not responsible for the higher ATP levels in the P23H-3 retina. It is likely that the Pasteur effect, “compensation of ATP production by glycolysis when mitochondrial activity is inhibited” [see Winkler et al. (101)], is sufficient to provide ATP if glucose is still available (103). To highlight this effect, we used a concentration of 4-CIN high enough to block mitochondrial and neuron-glia MCT. ATP levels were main-
tained after this retinal monocarboxylate inhibition. In the brain, an active MCT between neurons and glia is important for normal brain function (79). In contrast, the retina produces lactate in neurons and glia (67, 90, 96), and there is support for the notion that carbon skeletons provided by amino acids could be an alternative substrate for ATP production (13, 14, 51, 101, 102, 108). Our results indicate that MCT inhibition at the plasma membrane or mitochondrial membrane is not as detrimental to retinal function as is hypoglycemia, hypoxia (88), or short periods of light damage in the P23H-3 retina (106). The lack of a difference in TUNEL-positive cells in 4-CIN-treated retinas suggests that the retina displays metabolic resilience. In fact, 4-CIN treatment does not act as a cytotoxin unless metabolites are depleted (15, 43, 44). Our results suggest that lactate levels are high throughout development of the SD and P23H retina (Fig. 2), and this contributes to protection of the retina from the effect of 4-CIN. Nevertheless, 4-CIN alters the electroretinogram b-wave in SD rats, indicating mild metabolic changes with recovery of retinal function (13, 14).

Phosphodiesterase inhibition affects the metabolically overloaded P23H-3 retina. The fundamental outcome from our studies is that even a retina that is metabolically overloaded can maintain its function until a threshold level for cell death is reached. When we applied sildenafil to the P23H-3 rat model, we were able to modify retinal LDH activity, which suggests a downregulation of aerobic metabolism and/or accelerated photoreceptor death, reminiscent of that noted in other retinal dystrophy models (RCS and rd/rd retina) (3, 9, 87). Inhibition of phosphodiesterase affects the already compromised cellular environment of the P23H-3 retina (high oxygen tension and high ATP levels) but not that of the normal SD retina. This differential response may explain why human and animal studies showed only small transient changes in the electrophotogram or human visual psychophysical function secondary to sildenafil administration (8, 53, 68). Chronic long-term use of sildenafil in normal rats did not demonstrate anatomic changes (95) or increase in TUNEL labeling with the doses used in this study. In fact, inhibition of phosphodiesterases (87) causes calcium entry, which is an established activator of cell death pathways (33, 60, 61, 80, 86). Also, cation entry causes energy substrate depletion due to activation of extrusion mechanisms via calcium-associated ATPases and the energy requirements of Na⁺/K⁺-ATPase to drive calcium exchangers (41). Despite the increase in TUNEL-positive photoreceptor cells and reduced LDH activity, we did not observe any differences in AGB labeling in the photoreceptors. This indicates that no long-term cation entry changes in photoreceptors destined to die. We previously showed that AGB labeling and TUNEL do not necessarily overlap in the light-exposed P23H retina (106), which suggests the existence of multiple cell death pathways. Our results confirm that the effect of sildenafil mediates apoptosis, presumably through a mechanism similar to the rd/rd mutation (30, 87) or the degenerating PDE6 dog retina (93). We do not reject the possibility that other mechanisms of cell death are activated secondary to elevated cGMP levels, such as increased activity of cGMP-protein kinase in the photoreceptors (35, 77) secondary to sildenafil inhibition of phosphodiesterase. In addition to characterizing metabolic demand in the developing normal and dystrophic retina, we have highlighted altered metabolic activity in the dystrophic retina and susceptibility to alterations in cyclic nucleotide levels in an animal model of a human rhodopsin mutation.

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DISCLOSURES

No conflicts of interest are declared by the author(s).

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