The Effect of Calcium Channel Blocker Diltiazem on Photoreceptor Degeneration in the Rhodopsin Pro23His Rat

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PURPOSE. To determine whether the calcium channel blocker cis-diltiazem promotes photoreceptor survival in rats with the Pro23His rhodopsin mutation.

METHODS. Heterozygous Pro23His rhodopsin line 1 rats (n = 11) were treated daily, according to a protocol applied successfully in rd mice, with cis-diltiazem hydrochloride increased incrementally from 21 to 54 mg/kg in a divided dose (8 AM and 8 PM) administered by intraperitoneal (IP) injection for 21 days, beginning on days of age 10 through 12. Saline-treated line 1 rats (n = 6) received IP injections of an equal volume of 0.9% saline. Analysis on day 35 of age included dark-adapted corneal electroretinogram (ERG) b- and a-waves recorded from threshold to 0.63 log candelas-seconds [cd-s/m²], saturated a-waves elicited with a 2.1 log cd-s/m² flash, and morphometry of the outer nuclear layer (ONL) and rod outer segments (ROS).

RESULTS. ONL width and cell counts of diltiazem-treated and saline-treated animals at 35 days were reduced to 64%–68% of 15-day-old untreated Pro23H line 1 retinas. No photoreceptor rescue was found by measuring ONL width (P = 0.84), cell count (P = 0.42), or ROS length (P = 0.85). Functional assays by ERG b-wave threshold (P = 0.57), b-wave maximum amplitude (P = 0.46), and saturated a-wave amplitude (P = 0.59) also showed no rescue.

CONCLUSIONS. cis-Diltiazem did not rescue photoreceptors of Pro23His rhodopsin mutation line 1 rats treated according to the protocol used in rd mouse. (Invest Ophthalmol Vis Sci. 2000;41:2697–2701)

Frosson et al. recently demonstrated that a calcium-channel blocker cis-diltiazem provides some degree of photoreceptor rescue in the retinal degeneration (rd) mouse, which has a mutation in the gene encoding the β-subunit of rod cyclic guanosine monophosphate (cGMP) phosphodiesterase. Mutations in this gene are also found in some human subjects with retinitis pigmentosa (RP), an inherited degeneration of rod and, subsequently, cone photoreceptors that ultimately causes blindness. The rd mouse has elevated levels of cGMP, and it is hypothesized that this results in elevated intracellular calcium due to the continuous influx of Ca²⁺ through cGMP-gated and L-type voltage-gated channels, causing cell death. Thus, cis-diltiazem, which preferentially blocks L-type voltage-gated channels, was administered to rd mice under the premise that it would reduce the influx of calcium, lower intracellular levels, and stabilize and rescue rod photoreceptors.

Rod photoreceptor degeneration is the end stage of all forms of RP and is the result of many different genetic mutations. Among the most common are mutations in the rhodopsin gene including Pro23His (P23H). The P23H rhodopsin mutation causes retinal diseases by a dominant negative mechanism, a single mutant allele causing autosomal dominant disease. The precise mechanism by which P23H causes disease is uncertain, but it may involve abnormal disc morphogenesis. In human adRP from the P23H mutation, activation of the phototransduction cascade is reduced, as is recovery from activation. Recovery from activation is slowed in the P23H mouse model also. It is well known that calcium is involved in recovery of the photoreceptor, and slowed recovery could therefore result from abnormal Ca²⁺ ion movement or levels. In addition, cell death occurs through the process of apoptosis in the P23H mouse in which Ca²⁺ ions may play a significant role.

To determine whether a Ca²⁺ channel blocker would retard degeneration in this model, we applied the methods of the previous studies of diltiazem in the rd mouse and scaled the dosage appropriately for body weight. No rescue of rod photoreceptors was found by histologic cell count or by functional assay with the electroretinogram.

METHODS

Animals

These studies were conducted in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and protocols reviewed by the Unit of Laboratory Animal Medicine at the University of Michigan. Rats used in this
study were heterozygous P23H rats generated by mating homozygous rhodopsin P23H line 1 rats (Matthew M. LaVail, University of California, San Francisco, CA) against albino Sprague-Dawley (SD) rats (Harlan, Indianapolis, IN). The transgenic rat model with P23H mutant rhodopsin was developed for use in photoreceptor rescue studies. It carries a mutant P23H mouse opsin gene in addition to the wild-type alleles. Among the several lines of the P23H rat, line 1 has the most rapid loss of photoreceptors, but none is as rapid as that found in the rd mouse. There is no previously published information on the level of degeneration in line 1 at the ages used in this study. Steinberg et al. reported photoreceptor cell loss of 70% to 90% by 2 months of age. Our own unpublished observations show a loss of approximately 54% of photoreceptor cells by 4 weeks of age compared with age-matched controls. Also by 4 weeks of age, the dark-adapted b-wave is reduced to 50% of that in SD control animals, and the dark-adapted a-wave also by 4 weeks of age, the dark-adapted b-wave is reduced to 24% in P23H line 1 rats (Machida et al., unpublished data, 2000), indicating an opportunity to see functional preservation by rod electroretinogram (ERG) recordings in this study.

All rats were bred, born, and reared in our laboratory on a 12-12 hour light-dark cycle of fluorescent white 5-lux light at cage level. Animals were weaned at 21 days and were fed a high-fat breeding chow (Formulab; PMI Feed, Richmond, IN) ad libitum. Pups from two litters were used.

Treatment and Dose Regimen
d-Diltiazem hydrochloride (D2521; Sigma, St. Louis, MO) was dissolved in 0.9% saline, sterilized through a 0.20-μm filter (Corning, Corning, NY) and one half the total daily dose was administered by intraperitoneal injection twice daily (8 AM and 8 PM). The treatment regimen was identical with that used in the rd mouse study in which dose was scaled for body weight, and in this case, by weight of the rats. Also, as in the rd mouse study, the dosage was escalated over the first few days and reached a final dosage of 54 mg/kg per day from postnatal day 17 onward.

Two litters were treated, each for 21 days. Litter 1 began on postnatal day 12, (average weight 40 g), and had four diltiazem- (two male, two female) and two saline-treated (male) rats. Litter 2 began on postnatal day 10, (average weight, 31 g), with seven diltiazem- (three male, four female) and four saline-treated (one male, three female) rats. Diltiazem-treated animals (n = 11) received incrementally increasing doses to avoid toxicity, beginning at 21 mg/kg per day and increasing to 33 mg/kg on day 13 and 54 mg/kg from day 17 onward. Control-treated rats (n = 6) received IP injections of filter-sterilized 0.9% saline. Rats were weighed each day to calculate the dose. Average weight at termination was 150 g for litter 1 and 115 g for litter 2.

These doses are far higher than those used to treat human cardiac disease, in which an oral dose of less than 1 mg/kg is administered four times daily. In 5-week-old SD rats (140 g) we found lethality with single IP doses of 100 to 300 mg/kg. This is intermediate between the median lethal dose (LD50) of 38 mg/kg by the intravenous route and 535 mg/kg by subcutaneous injection in rat. The dose schedule in this study is higher than clinical doses of the drug and nontoxic levels in rat.

ERG Recording
ERGs were recorded beginning 56 hours after the final injection to allow for partial clearance of diltiazem. Rats were dark adapted for 12 hours and prepared under dim red light. Animals were anesthetized with a loading dose mixture of xylazine (13 mg/kg, intramuscular injection) and ketamine (86 mg/kg, intramuscularly) and maintained with subcutaneous infusion of the anesthesia mixture by pump. Pupils were dilated with topical 0.1% atropine and 0.1% phenylephrine HCl. The animals were held steady with a bite bar and nose clamp and placed on a heating pad to maintain body temperature. Scotopic ERGs were recorded simultaneously from both eyes using gold wire loops on the cornea, with 1% tetracaine topical anesthesia and methylcellulose to maintain corneal hydration. A gold reference electrode was positioned on the sclera 1 mm from the temporal limbus, and the ground electrode was clipped to the ear. Responses were amplified at 5000 gain from 0.1 to 1000 Hz and digitized at a 10-kHz rate. Scotopic intensity response functions were obtained from threshold to a maximum of 0.63 log candela-seconds (cd-s)/m² (PS33 Stimulator; Grass Instrument, Quincy, MA) in a ganzfeld bowl and attenu-
ated with neutral-density filters. Saturated a-waves were elicited by bright photostrobe flashes of 2.1 log cd-s/m² (model 283; Vivitar, Santa Monica, CA) and computer averaged, with stimulus intervals of 3 to 120 seconds, depending on stimulus intensity. Threshold criterion amplitude was 50 mV for both the scotopic a- and b-waves. a-Waves were measured from the baseline to trough, and b-waves were measured from the baseline or from the a-wave trough.

**Histology**

Rats were killed with a sodium pentobarbital overdose after ERG recordings. Eyes were removed, marked for orientation, and kept overnight at 4°C in fixative of 2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M cacodylate buffer. Eyes were trimmed and postfixed in 1% osmium for 1 hour. Epon-embedded tissue was sectioned at 1 m along the vertical meridian through the optic nerve and stained with toluidine blue for light microscopy. Outer nuclear layer (ONL) thickness in column cell counts and rod outer segment (ROS) length were measured every 400 m in each retinal half beginning 200 m from the optic nerve head and averaged across the entire retinal section, excluding the measurements nearest the ora serrata. ROS length was not measured in areas of artifactitious retinal separation that occurred after death with lens removal for tissue processing. Data were compared with the two-tailed Student’s t-test in age-matched animals.

**RESULTS**

**Histology**

Figure 1 shows retinal histology in P23H line 1 rats at the beginning and end of treatment. Untreated P23H line 1 rats at age 15 days had an average ONL width of 8.9 ± 0.49 cells and an ROS length of 17.1 ± 0.59 m (Table 1). The ONL width had thinned to approximately six cells, a 35% decrease, at 35 days in both the diltiazem-treated and saline-treated groups, which is consistent with our previous unreported observations on line 1 animals at 4 weeks of age. Comparison of the diltiazem- and saline-treated groups showed no significant difference in ONL width in cell count (P = 0.42), linear measurement (P = 0.84), or ROS length (P = 0.85).

**Electroretinogram**

Figure 2 shows dark-adapted ERG waveforms from a diltiazem-treated and a saline-treated line 1 rat at 35 days of age. Stimulus flash intensity is indicated on the left for both series of traces.

![Figure 2](image)

**TABLE 1.** Effect on Photoreceptor Morphology of Daily Treatment of P23H Line 1 Rats with d-cis-Diltiazem

<table>
<thead>
<tr>
<th></th>
<th>ONL Cell Count</th>
<th>ONL Width (μm)</th>
<th>ROS Length (μm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>15-Day-old untreated (n = 5)</td>
<td>8.9 ± 0.49</td>
<td>49.3 ± 3.0</td>
<td>17.1 ± 0.59</td>
</tr>
<tr>
<td>Diltiazem-treated (n = 11) at 35 days of age</td>
<td>5.7 ± 0.18</td>
<td>33.4 ± 1.43</td>
<td>15.7 ± 1.69</td>
</tr>
<tr>
<td>Saline-treated (n = 6) at 35 days of age</td>
<td>5.8 ± 0.50</td>
<td>33.6 ± 2.18</td>
<td>15.5 ± 1.46</td>
</tr>
<tr>
<td>P</td>
<td>0.42</td>
<td>0.84</td>
<td>0.85</td>
</tr>
</tbody>
</table>

Values are means ± SD; P is by two-tailed Student’s t-test.

![Figure 3](image)

**FIGURE 2.** ERG waveforms from a diltiazem-treated (left) and a saline-treated (right) P23H line 1 rat at 35 days of age. Stimulus flash intensity is indicated on the left for both series of traces.

**FIGURE 3.** Dark-adapted ERG a-wave and b-wave response functions across a 6-log-unit range of stimulus intensity. The SE bars of the response overlap each other at each intensity.

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amplitudes are shown in Table 2 and indicate no significant difference in any measure between the diltiazem and saline groups. Saturated a-wave amplitude in P23H rats reflects the product of ROS length \times ONL cells (Machida et al., unpublished data). That there was no difference between groups again indicates that diltiazem had no impact on preserving rod function.

**DISCUSSION**

\(d\)-cis-Diltiazem conveyed no evident protection against photoreceptor degeneration in the P23H line 1 rat. No preservation was seen histologically, by measurement of ROS length and ONL cell width or by functional assay with the dark-adapted ERG.

We performed this study to learn whether the protection conveyed by diltiazem in the \(rd\) mouse with the \(\beta\)-PDE mutation could be extended to other genetic forms of retinal degeneration—specifically, to disease caused by the P23H rhodopsin mutation that is present in approximately 12% of patients with autosomal dominant retinitis pigmentosa (adRP) in the United States.

The calcium-channel blocker \(d\)-cis-diltiazem has been used in the \(rd\) mouse with the rationale that this animal model of photoreceptor degeneration causes an abnormal increase in the cGMP concentration that is correlated with rod cell death to a level that can become toxic to normal photoreceptors. Although the mechanism of protection by \(d\)-cis-diltiazem in the \(rd\) mouse is not fully understood, a modulation of calcium levels by this drug was suggested. Intracellular calcium levels have not been directly imputed in the mechanism of disease leading to cell death in the P23H rat or mouse. However, VPP mice with the P23H rhodopsin mutation and patients harboring P23H have delayed recovery after exposure to bleaching light. Further, VPP mouse studies showed delayed photoreceptor recovery by double-flash measurements that suggested an abnormality in the biochemical reactions that underlie recovery, possibly involving delays in the kinetics of rhodopsin phosphorylation or in the binding and action of arrestin. These mechanisms as well as guanylyl cyclase and cGMP phosphodiesterase activity in the ROS, which are also involved in recovery of the photoreceptors, are modulated by calcium levels. Therefore, it is possible that the P23H rhodopsin mutation results in abnormal intracellular calcium levels. Because intracellular calcium levels are known to influence cell survival and death, it is conceivable that manipulating calcium levels with a calcium-channel blocker such as \(d\)-cis-diltiazem would affect the course of retinal degeneration in the rodent model. The results of the present study, however, provide no support for either a protective or accelerating effect on cell death by diltiazem in the P23H rhodopsin mutation rat.

The \(rd\) mouse study with \(d\)-cis-diltiazem demonstrated a modest protective effect on cone photoreceptor survival. The \(rd\) mouse has poorly functional and nearly nonfunctional rods at a very young age, which forces the ERG end point to focus on cone responses. By contrast, the present study of P23H line 1 rat used rod function and rod cell counts to assay for protection. The cone ERG in the P23H line 1 rats remains normal at 4 weeks of age and, consequently, could not be used as an end point for this protection study.

**SUMMARY**

We found no protective effect in the rat P23H rhodopsin mutation model using a treatment protocol identical with that used previously in the \(rd\) mouse, altered only to scale for body weight. Intracellular calcium levels have not been measured in the P23H rat model, but evidence of abnormal recovery from bleaching and of slowed photoreceptor response suggests that intracellular calcium may be altered. Consequently, studies of diltiazem in this rat model may be warranted, with the use of other dose regimens and at different stages of degeneration.

**Acknowledgments**

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**References**