Systemic Administration of Phenyl-N-tert-Butylnitrone
Protects the Retina from Light Damage

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PURPOSE. This study was conducted to test the hypothesis that phenyl-N-tert-butylnitrone (PBN), a spin-trapping agent known to cross the blood–brain barrier and protect the brain from ischemia–reperfusion injury, is incorporated into the retina after intraperitoneal injection and protects photoreceptor cells from the damaging effects of constant light.

METHODS. Albino rats were injected intraperitoneally with PBN (aqueous solution) or water, or were not injected, and then were placed in constant light (2700 lux) for 24 hours. The incorporation of PBN into the retina was determined by high-performance liquid chromatography. Electroretinograms (ERGs) were recorded before light treatment and 1 and 15 days after the cessation of exposure to constant light. Eyes were taken for histology at each time point and outer nuclear layer (ONL) thickness determined.

RESULTS. PBN was incorporated into the retina after intraperitoneal injection. Both control (water-injected and uninjected) groups exposed to constant light maintained only 28% of ONL thickness and 20% of retinal function, compared with the unexposed control group. In contrast, the PBN-treated animals maintained 80% of ONL thickness and exhibited 87% of retinal function.

CONCLUSIONS. PBN protects the albino rat retina from the damaging effects of constant light stress. That light-induced and hereditary retinal degenerations share certain morphologic hallmarks and follow a similar apoptotic mechanism of degeneration raises the possibility of pharmacologic therapy for hereditary and environmentally induced neurodegenerative disorders.

A variety of genetic and environmental factors cause death of retinal photoreceptor cells and degeneration of the retina, with subsequent loss of vision. Surgical or laser therapies are available only in specific cases of age-related macular degeneration or diabetic retinopathy and do not prevent further loss of vision. During the past decade, several therapeutic approaches have been tried to treat retinal degeneration. In animals, gene therapy,1 retinal transplantation,2 intravitreal administration of growth factors3–5 and a melatonin antagonist,6 and systemic administration of antioxidants7–9 and α2-adrenergic agonists10 have been tested. Recently, the calcium channel blocker d-cis-diltiazem was found to slow the rate of retinal degeneration in the rd mouse,11 but not in the rat with a P23H rhodopsin mutation.12 Although these treatments have had some success in animals, most of them require invasive intraocular procedures and some have significant systemic side effects. The only medical therapy currently available to slow the progression of retinitis pigmentosa in humans is vitamin A supplementation.13 No effective medical therapy is available for treating age-related macular degeneration, the leading cause of blindness in people more than 50 years of age.

Phenyl-N-tert-butylnitrone (PBN) was originally developed as a spin-trapping agent in biologic systems because it reacts with free radicals, mainly hydroxyl radicals, to generate a more stable molecule that can be quantified. It was first used to trap trichloromethyl radicals in an in vitro metabolic reaction.14,15 However, PBN was found to have cytoprotective properties in a number of experimental paradigms, including reduction in the mortality associated with endotoxin shock,16–21 neuroprotection in ischemia–reperfusion and aging models22–25 amelioration of the central nervous system damage associated with the human immunodeficiency virus envelope protein gp 120,24 and prevention of streptozotocin-induced diabetes in mice.25 More recently, PBN has been shown to have a variety of pharmacologic effects, which we describe in the Discussion section.

We used the light-damage model of retinal degeneration to investigate the role of PBN as a neuroprotective agent in the retina. Albino rats were given intraperitoneal injections of PBN and exposed to bright light for 24 hours, and their retinal status was evaluated by histology and electroretinography. Our results show that PBN administered systemically crosses the blood–retinal barrier and protects the retina from light damage.

MATERIALS AND METHODS

Materials

PBN was synthesized by Yashige Kotake (Oklahoma Medical Research Foundation, Oklahoma City). Phenacetin was from Sigma (St. Louis, MO). High-performance liquid chromatography (HPLC) solvents were from Fisher Scientific (Fairlawn, NJ).

Animals

Adult male albino Wistar rats (weight, 150–200 g) were purchased from Harlan Sprague–Dawley (Indianapolis, IN). All animals were kept for at least 2 weeks in dim cyclic light (12 hours on; 12 hours off; 5–10 lux). They were fed laboratory chow ad libitum and had free access to water. Twelve rats were used in the PBN pharmacokinetic experiment; 60 rats were used for electroretinography (ERG) and histology. The animal care strictly conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and the University of Oklahoma Health Sciences Center (OUHSC) Guidelines for Animals in Research. All protocols were reviewed and approved by the Institutional Animal Care and Use Committees of the OUHSC and the Dean A. McGee Eye Institute.

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PBN Pharmacokinetics in the Retina

Rats were given intraperitoneal (IP) injections of PBN (50 mg/kg body weight with a solution of 25 mg PBN/ml water), and retinas were collected and frozen in liquid nitrogen 0.5, 1.5, 5, and 6 hours later. Three animals were used for each time point. An internal standard (10 µl phenacetin at 0.1792 mg/ml in acetonitrile-water; 60:40 vol/vol) was added to each sample in 1.0 ml deionized water before homogenization. An aliquot of the homogenate was taken for protein assay, and the remainder was extracted three times with 4, 2, and 2 ml of chloroform. The extracts were pooled, washed with 1 ml water, and centrifuged at 3600g for 4 minutes. After evaporation of the chloroform under nitrogen, 200 µl of acetonitrile/water (60:40 vol/vol) was added, and 100 µl was injected on the HPLC column. HPLC was conducted with a commercial system (Supelcosil LC-18 column; 25 cm × 4.6 mm; Supelco, Bellefonte, PA) with a mobile phase of acetonitrile-water (60:40 vol/vol) at a flow rate of 1.0 ml/min, and the wavelength for detection was 289 nm.

Protein Assay

Protein concentration was determined using bovine serum albumin as a standard (Pierce, Rockford, IL), according to the manufacturer’s protocol.

Light-Damage Paradigm

Rats were divided into two groups: One was exposed to damaging light and the other with exposure to normal dim light served as a control. Within each group, three subgroups were either untreated, treated with vehicle (water), or treated with an aqueous solution of PBN. Treatment consisted of 5 IP injections of water or PBN in water (50 mg/kg body weight), the first administered 30 minutes before light exposure and the remainder every 6 hours thereafter until the end of light exposure. Light exposure continued for 24 hours in a box with white, reflecting surfaces, equipped with three fluorescent tubes (cool white, 34 W). The illuminance measured at the position of the rat’s eyes (3 cm above the cage floor) was set at 2700 lux. Exposure under these conditions was found to cause a predictable but incomplete loss of photoreceptor cells. During exposure, the rats had free access to food and water. After exposure, the animals were placed in darkness for 24 hours and then returned to the dim cyclic light conditions. Animals in the control groups were maintained on their normal dim light cycle.

Electroretinography

Rats were dark adapted overnight and prepared under dim red light for the ERG study. They were anesthetized with intramuscular injections of ketamine (120 mg/kg body weight) and xylazine (6 mg/kg body weight). One drop of 1% tropicamide was applied to the cornea to dilate the pupil, and one drop of 0.5% proparacaine HCl was applied for local anesthesia. The white light stimulus used to evoke ERGs was delivered in 10-msec pulses by an integrating sphere (Labsphere, North Sutton, NH), with a 60-sec interval between flashes. These conditions have been shown to be sufficient to prevent light adaptation to the light flashes.2 ERGs were recorded with gold electrodes at 21 intensities presented in ascending order, beginning below threshold, to get the b-wave sensitivity curves. A computer running commercial software (Origin 6.0; Microcal, Northampton, MA) was used to fit the data of each rat, giving the saturated b-wave amplitude (B\text{max}).

ERGs were recorded before treatment and light exposure, and at day (D)1 and D15. ERGs of unexposed rats (treated or untreated) were recorded in parallel.

Histology

In each of the six groups, 10 rats were killed for light microscopic evaluation of retinal structure. Immediately after death, eyes were excised, placed in fixative (4% paraformaldehyde, 2% trichloroacetic acid, 20% isopropl alcohol, 2% aqueous zinc chloride, and 72% dis-
tilled water), and embedded in paraffin. Sections of 5 µm were cut along the vertical meridian through the optic nerve. The thickness of the outer nuclear layer (ONL) was measured at 0.5-mm distances from the optic nerve to the inferior and superior ora serrata.26 The area under the curves was integrated using the statistical analysis program (Origin 6.0; Microcal).

Statistical Analysis

Results are plotted as mean ± SD. Significant differences across groups were assessed using an unpaired t-test for the ERG data and for the histologic data, with a level of significance set at P = 0.05. Significant differences in a defined group before and after exposure were assessed using a paired t-test for the ERG data with a level of significance set at P = 0.05.

RESULTS

PBN Level in the Retina after Intraperitoneal Injection

After intraperitoneal injection, the concentration of PBN in the retina decreased exponentially from (3.58 ± 0.55) × 10^{-4} µg PBN/µg retinal protein at 0.5 hours (the earliest measurement) to (0.264 ± 0.065) × 10^{-4} µg PBN/µg protein at 6 hours (Fig. 1). The half-life of PBN in the retina was approximately 2 hours, which is similar to values reported for other tissues, such as liver, lung, brain, and heart.26 Five injections of PBN every 6 hours did not lead to the accumulation of PBN in the retina (results not shown).

Retinal Function Preservation by PBN

Electroretinograms were recorded from 10 rats from each of six groups (see Fig. 2) before treatment and at D1 and D15 after light exposure. The b-wave sensitivity curves for the three control groups that were not exposed to constant light were not different (Figs. 2A, 2C, 2E). However, in the groups that were not injected (Fig. 2B) or injected with water only (Fig. 2D), 24 hours of exposure to 2700-lux light caused a large reduction in retinal function, evidenced by collapse of the sensitivity curves. In both of these exposed groups, B\text{max} was reduced to less than 20% of control values at D1 (P < 0.001), and no recovery or further degradation was observed at D15 (Figs. 2B, 2D). There was no significant difference in B\text{max} between these two exposed groups. In contrast, the retinal function of animals treated with PBN and exposed to the damaging light was preserved to a large extent (Fig. 2F). B\text{max} was 65% of control

FIGURE 1. PBN concentration in the retina at various times after intraperitoneal administration. The points were determined experimentally and the line drawn using a computer program (Excel; Microsoft, Redmond, WA). The results are expressed as a mean ± SD (n = 3 for each time point).

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values at D1 (P < 0.001) and 87% at D15 (P < 0.02). Comparison of the $B_{\text{max}}$ of the PBN treated animals with that of the water-injected or uninjected animals at D15 showed that PBN treatment significantly preserved photoreceptor function (P < 0.001 for each group). Treatment with PBN did not cause any change in the retinal sensitivity curves in the unexposed group (compare Fig. 2E with Figs. 2A, 2C), showing that PBN was not toxic to the retina.

**DISCUSSION**

This study shows that PBN administered systemically can be incorporated into the retina, where it protects the morphologic and functional properties of the retina against the damaging effects of constant light exposure. In the light-exposed PBN group, $B_{\text{max}}$ and ONL thickness were significantly more than in the two exposed control groups. Under the conditions of the study, treatment with PBN resulted in preservation of 87% of retinal function and 80% of the ONL area, compared with PBN-treated but unexposed animals. Untreated animals retained only 20% and 28% of retinal function and ONL area, respectively.

**Photoreceptor Structure Preservation by PBN**

Figures 3 and 4 show the morphologic and morphometric analyses of each experimental group, respectively. The histology sections are from the same region in the superior central retina. In the unexposed animals, there was no difference in retinal morphology among the three groups (Figs. 3A, 3B, 3C). The thickness of the ONL, a measure of photoreceptor cell number, was the same in the three groups in all regions of the retina (Fig. 4A). However, exposure to 2700-lux light for 24 hours caused extensive damage to the retinas of the animals that received no treatment or only water. Photomicrographs of the superior central retinas of these animals taken at D1 (Figs. 3D, 3E) show massive degeneration, with many photoreceptor nuclei missing and many other pyknotic nuclei. By D15 (Figs. 3G, 3H), the debris had been cleared, and only one row of nuclei remained in the ONL, indicating extensive loss of photoreceptor cells. The extent of the damage is evidenced by the large loss of ONL thickness at D15 (Fig. 4B), with the superior central retina being the most affected.

In contrast, when the rats were treated with PBN during light exposure, the ONL was preserved (Figs. 3F, 3I). Only a small decrease in the ONL thickness was observed in the superior central region of the retina at D15 (Fig. 4B).

Integration of the area under the ONL thickness curves at D15 produced virtually identical values for untreated and water-treated exposed groups, with an average of 28% preservation of ONL area, compared with that of unexposed control retinas (Fig. 5). By contrast, PBN-treated exposed animals had an 80% preservation of ONL area. Thus, systemic administration of PBN afforded significant protection against light damage compared with untreated or water-treated control retinas ($P < 0.0001$).
An interesting observation in the PBN-treated group was that $B_{\text{max}}$ was lower at D1 than at D15 (65% versus 87%, $P < 0.02$) compared with control values, which was not the case in either the water-injected or uninjected animals. Part of the loss of function is due to the loss of photoreceptor cells. However, light stress also leads to outer segment disorganization and shortening, which could contribute to the loss of function as well. In the PBN-treated animals, there was a significant recovery of function between D1 and D15, which suggests that the light-induced degenerative process was interrupted by PBN. The surviving photoreceptor recovered their integrity (a turnover period for the renewal of rod outer segments is approximately 10 days), and therefore the reduction of $B_{\text{max}}$ at D15 was caused solely by photoreceptor cell death. Therefore, light exposure in the PBN-treated animal induced a transient disorganization of the outer segment photoreceptor (decreasing retinal sensitivity temporarily) that did not progress further to cell death.

The mechanism by which PBN protects the retina is not known. Nevertheless, PBN has well-defined free radical-trapping capabilities, and, given the role of oxidant stress in retinal light damage, it seems logical to propose some antioxidant function for PBN. By scavenging free radicals, PBN can act early in the degenerative cascade of events and prevent cell death.

PBN has also been shown to have several pharmacologic effects, such as preventing the induction of inducible nitric oxide synthetase (iNOS), inhibiting the activation of the transcription factor NF-$\kappa$B, inhibiting the expression of multiple cytokine genes, activating transcription factors, inhibiting expression of multiple apoptosis-associated genes, and downregulating the mitogen-activated protein kinase (MAPK) pathway. All these PBN effects could be secondary to its radical scavenging properties. However, PBN alone has recently been shown to reduce basal protein phosphorylation and to upregulate the expression of heat shock proteins such as hsp27. Therefore, the protection provided by PBN may be from a cumulative effect of its multiple pharmacologic activities.

During the past few years, several medical therapeutic approaches to inherited and light-induced retinal degenerations have had some success. Growth factors such as basic fibroblast growth factor (bFGF), ciliary neurotrophic factor (CNTF), and brain-derived neurotrophic factor (BDNF) have slowed the rate of degeneration, but have the disadvantage of requiring intravitreal administration because the peptides do not cross the blood–retinal barrier. Systemic administration of $\alpha$-cis-diltiazem, a calcium channel blocker used to treat hypertension, slowed the degeneration in the rd mouse but was ineffective in the P23H rhodopsin mutant rat. Because oxidant stress has been implicated in inherited retinal degenerations and age-related macular degeneration, drugs such as PBN may be good candidates as potential therapeutic agents. PBN is efficient, bioavailable, stable, soluble, and nontoxic at the dose used in this study. Although more information is needed on its metabolism, its ability to cross the blood–retinal and blood–brain barriers makes it potentially effective in the treatment of neurodegenerative diseases.

In conclusion, we have shown for the first time that PBN administered systemically enters the retina and efficiently protects the retina from light damage. Whether the protective effect is through scavenging free radicals or through some other mechanism is currently under study in our laboratory, as is the effect of PBN on the survival of photoreceptors in animals with inherited retinal degeneration.

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![Figure 4](image-url)

**Figure 4.** ONL thickness along the vertical meridian: (A) unexposed groups, (B) groups exposed and examined at D15. (□), Uninjected; (⊙), water injected; (●), PBN injected. The results are expressed as mean ONL thickness ± SD ($n = 10$ for each group).

![Figure 5](image-url)

**Figure 5.** Relative area units under the curves from the ONL thickness measurements. The results are expressed as a mean area units ± SD ($n = 10$ for each group).
References


