Delay of Photoreceptor Cell Degeneration in *rd* Mice by Systemically Administered Phenyl-N-tert-butylnitrone

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Purpose: To study the effect of systemic administration of phenyl-N-*tert*-butylnitrone (PBN) on the degeneration of photoreceptor cells in *rd* mice.
Methods: PBN was injected intraperitoneally into FVB/*rd* mice on postnatal days (P) 5 to 14 (group A), and P10 to 18 (group B). At days P14, 16, 18, 20 and 27, morphological changes and apoptosis were analyzed by staining with hematoxylin and eosin or DAPI. The effect of PBN on apoptosis was analyzed in retinal pigment epithelial (RPE) cells by the measurement of caspase-3 activity.
Results: In control and group B mice, the outer nuclear layer (ONL) of the retina was composed of 8-10 rows at P12, and rapidly decreased to one row at P18. In group A mice, the ONL was preserved with 5-7 rows at P18, and decreased to one row at P22. PBN inhibited caspase-3 activity in cultured RPE cells.
Conclusions: PBN delayed, but did not block, the degeneration of photoreceptor cells in *rd* mice. PBN may exert its inhibitory effect during the early phase of photoreceptor cell degeneration. *Korean Journal of Ophthalmology 19(4):288-292, 2005*

Key Words: Blood retinal barrier, Caspase-3, Delay, Phenyl-N-*tert*-butylnitrone, *rd* mice

Hereditary retinal degeneration is a disease caused by a wide range of factors,[1] many of which cause retinal cells to die as the result of activation of a genetic pathway that leads to apoptotic death.[2-4]

During the past decade, various procedures have been tested in the treatment of hereditary retinal degeneration including transplantation of photoreceptors or retinal pigment epithelial (RPE) cells,[5-8] growth factor injection,[9-11] and gene therapy.[12-14] In addition, other modalities may help to rescue retinal degeneration, for example antioxidants,[15] diet,[16] melatonin antagonists,[17] α_2-adrenergic agonists,[18] calcium channel blockers,[19] and immunologic treatment.[20]

Phenyl-N-*tert*-butylnitrone (PBN) is a nitrone-based compound that acts as a free radical scavenger. PBN has been shown to have many beneficial effects, including protection from ischemic brain injury,[21,22] bacterial meningitis,[23] endotoxin shock,[24,25] drug-induced diabetogenesis,[26] and choline-deficient hepatocarcinogenesis.[27] It was also reported that PBN has a protective effect against retinal photic injury,[28] although the mechanism of retinal protection is not known.

Since PBN can cross the blood-brain and the blood-retinal barriers,[28-30] it can be administered systemically. This is a significant advantage over agents previously used in animal models that had to be administered by the invasive technique of intravitreal injection due to the inability to cross the blood-retinal barrier.

The aim of this study is to determine whether systemic treatment with PBN can delay or block retinal degeneration using *rd* mice as a model for human retinitis pigmentosa.[31]

Materials and Methods

1. Maintenance and injection of animals

Mice were housed and treated in accordance with the guidelines of the NIH and the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Five-week-old male/female FVB/*rd* mice were obtained from the Jackson laboratory (Maine, U.S.A.) and were bred in-house. Mother and pups were kept under a 12-hour light-dark cycle at 23℃ with food and water ad libitum. PBN (50 μg/g body weight in a solution of 25 μg PBN/μl normal saline) was

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injected intraperitoneally on postnatal days (P) 5 to 14 for group A, and P10 to 18 for group B. At days P12, 14, 16, 18, 20, 22 and 27, five pups from each group were sacrificed with an intraperitoneal injection of phenobarbital (60 mg/kg).

2. Retinal PBN concentration

Mice were sacrificed 1, 2, 3 and 6 hours after intraperitoneal PBN administration. Four animals were used for each time point. Both eyes were quickly removed and the retinas were dissected and frozen in liquid nitrogen. Retinal concentration of PBN was measured as described previously.[28]

Before homogenization, an internal standard (10 µl phenacetin at 0.1792 mg/ml in acetonitrile-normal saline; 60:40 vol/vol) was added to each sample in 1 ml distilled water. The extracts were pooled, washed with 1 ml normal saline, and centrifuged at 3600 g for 4 minutes. After evaporation of the chloroform under nitrogen, 200 µl acetonitrile-normal saline (60:40 vol/vol) was added, and 100 (l injected onto the high performance liquid chromatographic (HPLC) column. HPLC was performed using a commercial system (supelcosil LC-18 column; 25C cm×4.6 mm; supelco, Bellefonte, PA, USA) with a mobile phase of acetonitrile-normal saline (60:40 vol/vol) at a flow rate of 1 ml/min, and a detection wavelength of 289 nm.

3. Tissue preparation for histology and DAPI staining

Both eyes of each animal were quickly removed and fixed by immersion in Carnoy's solution for 2 hours at room temperature. Each eye was dehydrated in a graded ethanol series and embedded in paraffin using standard techniques. Paraffin-embedded eyes were sectioned at 4 µm and mounted on slides coated with bovine serum albumin for hematoxylin and eosin staining. Only sagittal sections parallel to the superior-inferior axis of the eye and including the optic nerve were collected.

4. Histology

Sections were deparaffinized, rehydrated, stained with hematoxylin and eosin according to normal histologic procedures, and examined by light microscopy (Leica, Wetzlar, Germany). To quantify the spatiotemporal progression of photoreceptor degeneration, the number of photoreceptor nuclei in three adjacent columns of the outer nuclear layer (OLN) was counted in the retina 500 µm superior from the optic nerve. Nuclei in three adjacent columns were counted, and the mean and standard deviations calculated using Excel (Microsoft, Redmond, WA, USA).

5. DAPI staining

Sections were deparaffinized, rehydrated and treated with 10 µg/mL DAPI (Sigma). After 5 min of incubation in the dark, slides were washed three times with washing buffer and the stained tissue visualized by fluorescence microscopy (Leica, Wetzlar, Germany).

6. RPE cell culture and caspase-3 activity assay

ARPE cells were maintained in Dulbecco's Modified Eagle's medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 µg/ml streptomycin at 37℃ in a humidified 5% CO_2 atmosphere.

Cells (2.5×10^5) were treated with 120 µM H_2O_2 in the presence or absence of 1-mM PBN for 24 hours prior to lysis with cell lysis buffer (caspase-3 activity assay kit, Roche, NJ, USA). Samples were incubated on ice for 1 min and centrifuged at 1800 g for 15 min at 4℃. Caspase-3 activity in the supernatant was measured in a spectrofluorimeter using Ac-DEVD-AFC as a substrate according to the manufacturer's instructions.

7. Data analysis

Data are expressed as the mean±S.D. unless otherwise indicated. Student's t-test was performed for two-group comparisons. Values were considered statistically significant at $p<0.05$.

Results

1. Retinal PBN concentration

The retinol concentration of PBN was measured using HPLC at various time points following injection to determine whether PBN could enter the sensory retina. Considerable levels of PBN were found in the sensory retina 1 hour after intraperitoneal injection. The PBN concentration decreased exponentially with increasing time and did not show any significant changes after 3 hours (Fig. 1). These data demonstrate that PBN can cross the blood retinal barrier into

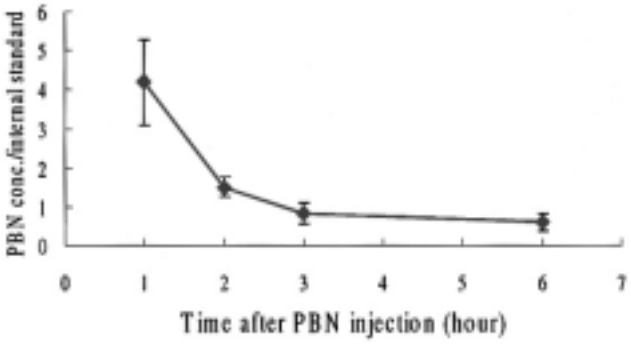

Fig. 1. PBN concentration in sensory retina after intraperitoneal injection. The concentration of PBN in the sensory retina was measured 1, 2, 3, and 6 hours after intraperitoneal injection using HPLC.

the sensory retina.

2. ONL preservation following intraperitoneal injection of PBN

The number of cells in the ONL was determined at various times after birth for three groups: control (no PBN), group A (PBN administered at days P5-P14) and group B (PBN administered at days P10 - P18). The rate of cell loss in group A was slower than that in the control group and group B. In the control group, abrupt cell loss in the ONL occurred from days P14 to P16 (Fig. 2). At day P14 there were 7 to 10 rows of cells, decreasing to one or two rows at P16 with one row remaining after P16. The mean number of rows at each time point were: P12, 15.2±0.84; P14, 7.8±1.3; P16, 1.4±0.55; P18, 1±0; P20, 0.8±0.45; P27, 0.8±0.45. In group A, 10 to 12 rows of cells were preserved up to day P16. At P18, 6 to 9 rows remained, decreasing to one row by P20 (P12: 16.6±0.89; P14: 14.4±1.14; P16: 10.8±0.84; P18: 7.4±1.14; P20: 1±0; P27: 0.8±0.45). In group B the pattern of cell loss was similar to that of the control group (P12: 15.6±0.55; P14: 8.2±0.84; P16: 3.2±1.09; P18: 1.6 ±0.55; P20: 0.8±0.45; P27: 0.8±0.45). Therefore, in group A the rate of cell loss was significantly reduced from P14 to P18, compared to control and group B mice ($p<0.05$). However, since cells are ultimately lost in all three groups, this represents a delay in cell loss, and not a complete block in degeneration.

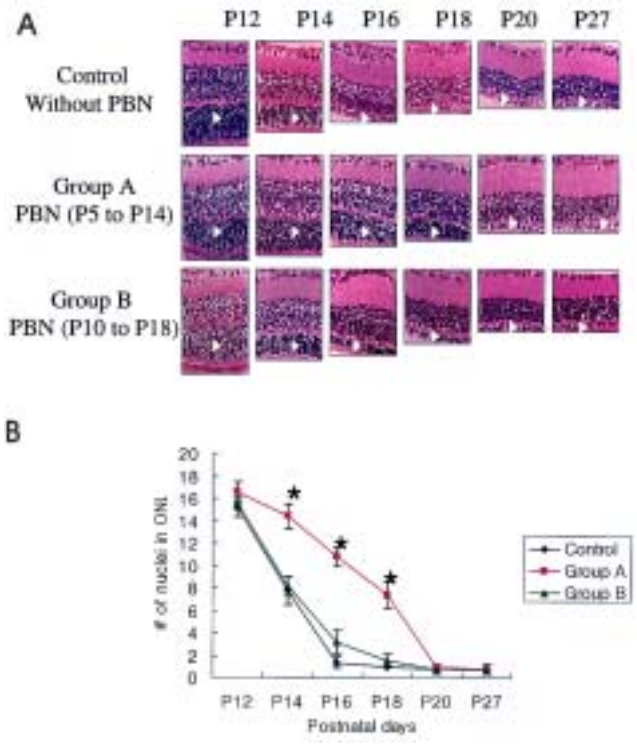

Fig. 2. ONL preservation following Intraperitoneal Injection of PBN. (A) PBN injection from P5 to P14 (group A) delayed the loss of cells in the ONL. H & E staining (×400) (P: postnatal day; White arrow: ONL); (B) The number of photoreceptor nuclei per column in the ONL at days P12, 14, 16, 18, 20 and 27 was as follows; (Control/ Group A/ Group B): P12, 15.2±0.84/16.6 ±0.89/15.6±0.55; P14, 7.8±1.3/14.4±1.14/8.2±0.84; P16, 1.4 ±0.55/10.8±0.84/3.2±1.09; P18, 1±0/7.4±1.14/1.6±0.55; P20, 0.8±0.45/1±0/0.8±0.45; P27, 0.8±0.45/0.8±0.45/0.8±0.45. In group A, cell loss was significantly blocked by PBN from P14 to 18, compared to control and group B (*$p<0.05$)

3. DAPI staining

Apoptosis in the ONL was detected by DAPI staining to visualize the morphological changes in chromatin. Many strong fluorescent spots, indicating apoptotic bodies, were observed primarily in the ONL. These increased as degeneration progressed, and declined thereafter as the ONL was lost. As reported above, some rows of cells were maintained in the ONL of group A at day P18. DAPI staining revealed that these cells were undergoing apoptosis (Fig. 3).

4. Caspase-3 activity inhibition by PBN in RPE cell culture

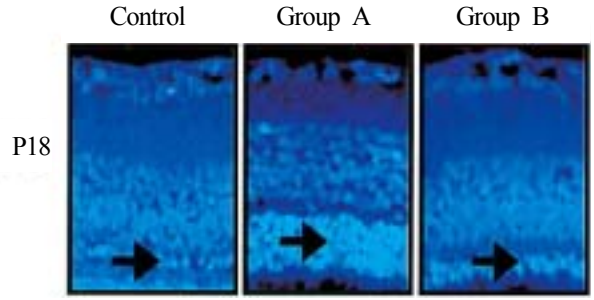

Fig. 3. DAPI staining. Strong fluorescent spots, indicating apoptotic bodies, were observed primarily in the ONL. Apoptosis is occurring in the rows of OLN cells maintained in group A at day P18. (×400 (P: postnatal day; Black arrow: ONL).

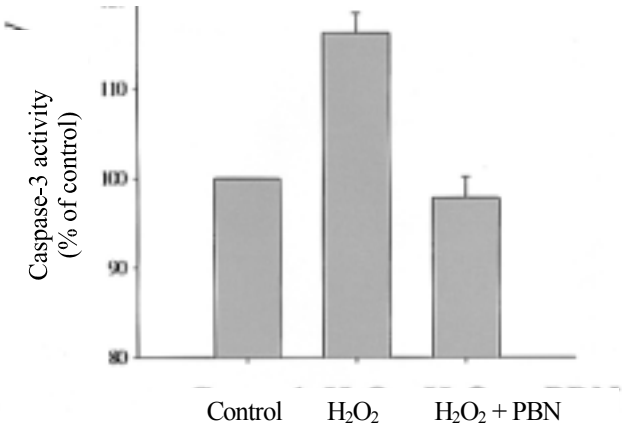

Fig. 4. Caspase-3 activity inhibition by PBN in ARPE cells were treated with 120 μM H_2O_2 for 24 hrs in the presence or absence of 1 mM PBN. PBN inhibited the increase in caspase-3 activity that occurs in response to H_2O_2.

In the absence of PBN, treatment of RPE cells with 120 μM H_2O_2 resulted in an increase in caspase-3 activity, indicating activation or release of caspase-3 in response to H_2O_2. This increase in caspase-3 activity was inhibited by PBN (Fig. 4).

Discussion

PBN has a variety of pharmacological effects, which have been attributed to free radical trapping and scavenging.[32-34] The anti-apoptotic activities of PBN are closely associated with its role as a scavenger for free radicals.[35-38] Since retinal degeneration is often associated with apoptotic cell death, the anti-apoptotic action of PBN may delay or block the process of retinal degeneration.

The present study demonstrates that systemically administered PBN crosses the blood-retinal barrier. Over the past decades there have been many trials for the treatment of hereditary retinal degeneration. However, the blood-retinal barrier has presented a serious limitation to many therapeutic approaches, necessitating the use of invasive techniques such as intravitreal injection. The ability to administer PBN systemically is a great advantage.

In *rd* mice, degeneration of retinal rod photoreceptor cells begins at about day P8, and peaks at P16 when few photoreceptors remain. Eventually no photoreceptors are left. The activation of caspase-3 during photoreceptor degeneration is observed,[39] and the caspase-3 inhibitor is transiently effective in delaying retinal degeneration in C3H mice carrying the *rd* gene.[40] Retinal degeneration is preceded by accumulation of cyclic GMP in the retina and is correlated with deficient activity of the rod photoreceptor cGMP-phosphodiesterase. However, it is not clear how a genetic defect in the β subunit of the phosphodiesterase enzyme triggers apoptosis. A calcium-channel blocker that acts at cyclic GMP-gated channels rescued photoreceptors and preserved visual function in *rd* mice,[19] and calcium channel antagonists suppress the expression of procaspase-3.[41] Furthermore, PBN potentiates the activation of Erk and Src kinases by H_2O_2. This potentiation requires extracellular calcium and appears to be dependent on voltage sensitive calcium channels.[42]

To determine when PBN is active during retinal degeneration of *rd* mice, we administered PBN in the early phase (before the appearance of retinal degeneration) or late phase (after the appearance of retinal degeneration). The data show that PBN delays retinal degeneration in *rd* mice when administered in the early phase (P5 to P14), but not in the late phase. The anti-apoptotic action of PBN may be associated with inhibition of caspase-3 activity. Caspase-3 is not critical for rod photoreceptor development, and does not play a significant role in mediating pathologic rod death in *rd*-1 mice.[43]

Recent studies using P23H and S334ter rhodopsin transgenic rats reported that PBN protects against light damage, but is not effective in preventing inherited retinal degeneration.[44] The mechanisms by which cell death is initiated in inherited degeneration are different from those in light damage, although apoptosis is the final common cause of cell death in both cases. In addition, the effect of PBN on the mutant rats may be different from that on *rd* mice. *rd* mice carry a mutation on a gene encoding cyclic GMP phosphodiesterase and have fast rod degeneration. S334ter rats express a rhodopsin protein lacking the 15 C-terminal amino acids, and P23H rats express a rhodopsin protein with a histidine substitution at proline 23; both have slow rod degeneration with initially normal cone function. It is assumed that the underlying mechanisms that cause retinal degeneration, and the effect of PBN on them, are different between the mutant mice and rats. Although apoptosis is the final common pathway, there is considerable divergence in the upstream events that lead to cell death.

In conclusion, systemically administered PBN effectively functions at the sensory retina. It can delay, but not block, photoreceptor cell degeneration in *rd* mice when administered in the early phase of degeneration. This delay is associated with inhibition of caspase-3 activity.

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