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ScienceDirect
Experimental Eye Research 83 (2006) 1252–1259
EXPERIMENTAL EYE RESEARCH
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Synthesis and characterization of a novel class of reducing agents that are highly neuroprotective for retinal ganglion cells

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Received 13 February 2006; accepted in revised form 11 July 2006
Available online 24 August 2006

Abstract

Retinal ganglion cells (RGCs) undergo apoptosis after axonal injury, in part regulated by an intracellular superoxide anion burst, for which the target(s) are unknown. Shifting the RGC redox state towards reduction and preventing sulfhydryl oxidation is neuroprotective *in vitro* and *in vivo*, implying that one or more sulfhydryls on one or more critical proteins may be involved. We synthesized novel borane-protected analogues of the reductant tris(2-carboxyethyl)phosphine (TCEP) with the intent of increasing cell permeability and improving chemical stability, and tested their ability to increase RGC survival *in vitro*. Retinal ganglion cells of postnatal day 2–4 Long–Evans rats were retrogradely labeled with 4′,6-diamidino-2-phenylindole (DAPI). At postnatal days 11–13 the animals were sacrificed, the retinas enzymatically dissociated and plated on poly-L-lysine-coated 96-well flat-bottomed tissue culture plates for 72 h in Neurobasal-A, B27 supplement lacking antioxidants, and TCEP, bis(3-propionic acid methyl ester)phenylphosphine borane complex (PB1), (3-propionic acid methyl ester)diphenylphosphine borane complex (PB2), or three commercially available phosphines. Viable DAPI-positive RGCs were identified by calcein-AM staining. At 72 h, PB1 was effective at rescuing acutely axotomized RGCs at concentrations from 1 nM to 100 μM. RGC survival with 1 nM PB1 was 174 ± 12% of control ($p = 0.002$). Another compound, PB2, rescued RGCs at 10 pM (177 ± 24%; $p = 0.006$) and 10 nM (251 ± 34%; $p = 0.004$) at 72 h. A PAMPA assay demonstrated that PB1 and PB2 were substantially more permeable than TCEP. These data demonstrate that modified reductants are effective RGC neuroprotectants at picomolar-nanomolar concentrations. We propose that these novel molecules may act by inhibiting the sulfhydryl oxidation effect of an intracellular superoxide burst.

Keywords: retinal ganglion cells; neuroprotection; optic nerve; oxidation-reduction; redox; intracellular signaling; tris(2-carboxyethyl)phosphine

1. Introduction

Death of retinal ganglion cells (RGCs) is the final common pathway of all optic neuropathies, in most cases following injury to the RGC axon. The mechanisms by which axonal injury signals RGC death is not definitely known, but may include neurotrophic factor deprivation (Mansour-Robaey et al., 1994), physiological excitotoxicity or pathological glutamate levels (Yoles et al., 1997), free radical production (Cui and Harvey, 1995), accumulation of excess anterogradely transported macromolecules (McKinnon et al., 2002), and induction of p38 MAP kinase (Kikuchi et al., 2000).

Our previous work demonstrated that reactive oxygen species (ROS), specifically superoxide anion, is a parallel signaling pathway for RGC death after axonal injury (Geiger et al., 2002; Kortuem et al., 2000; Levin et al., 1996; Lieven et al., 2006). Specific ROS scavengers and hypoxia reduce the death

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doi:10.1016/j.exer.2006.07.002

of cultured neonatal RGCs after axotomy, and RGC survival is dependent on their redox state, with greatest survival observed under mildly reducing conditions (Castagne and Clarke, 1996; Castagne et al., 1999; Geiger et al., 2002). There is an asynchronous rise in RGC superoxide after axotomy (Lieven et al., 2006), and axotomy itself can increase the superoxide generated by an exogenous oxidative stress (Nguyen et al., 2003).

If superoxide serves as an intracellular signal for axonal injury, then how could this signal be transduced? Protein targets for ROS-mediated signal transduction often have a redox sensitive cysteine sulfhydryl at the active site, involve interactions between ROS modulatory proteins, or involve covalent protein modifications (e.g. *S*-nitrosylation with NO^+, *S*-nitration with peroxynitrite, or glutathiolation with reduced glutathione). ROS modulate protein function via altering redox states and effectively modify adjacent cysteine sulfhydryls. This oxidative cross-linking results in a disulfide bond that produces conformational changes to the active site (Carugo et al., 2003; Park and Raines, 2001).

If sulfhydryl oxidation accounts for RGC death after axonal injury, then it is not surprising that reducing agents inhibit RGC death, as we have previously shown. Studies with tris(2-carboxyethyl)phosphine (TCEP) demonstrated that blocking sulfhydryl oxidation prevents RGC death after acute axotomy *in vitro* (Geiger et al., 2002) and optic nerve crush in rat models (Swanson et al., 2005), indicating that disulfide reduction is important in neuroprotection of RGCs. Compounds that protect the protein conformation by reducing disulfide bonds created via ROS would stabilize the protein moiety and presumably interfere with the signaling of cell death. We therefore modified phosphines to be highly potent intracellular reducing agents, and evaluated them as potential pharmaceutical agents for neuroprotection in axonal injury. We found that two compounds protected RGCs from axonal injury at picomolar-nanomolar concentrations.

2. Materials and methods

2.1. Animals

All experiments were performed in accordance with Association for Research in Vision and Ophthalmology (ARVO), institutional, federal, and state guidelines regarding animal research.

2.2. Materials

Cell culture reagents were obtained from GIBCO (Grand Island, NY). The retrograde fluorescent tracer 4′,6-diamidino-2-phenylindole (DAPI), the fluorescent viability agent calcein-AM, and the cell-permeable ethidium derivative dihydroethidium (HEt) were obtained from Molecular Probes (Eugene, OR). Papain was obtained from Worthington Biochemical (Freehold, NJ). The triphenylphosphines 2-(diphenylphosphino)benzoic acid (2DPBA), 4-(diphenylphosphino)benzoic acid (4DPBA), and 3,3′,3″-phos-phinidyne-tris(benzene-sulfonic acid) trisodium salt (3BSA) were obtained from Aldrich Chemical. The novel borane-protected phosphines bis(3-propionic acid methyl ester)phenylphosphine borane complex (PB1) and (3-propionic acid methyl ester)diphenylphosphine borane complex (PB2) were synthesized as described below. PB1, PB2, 4DPBA and 2DPBA were dissolved in ethanol while 3BSA was dissolved in water to a desired concentration of 100 mM. Unless noted, all other reagents were obtained from Sigma Chemical (St. Louis, MO).

2.3. RGC labeling and culture

RGCs were labeled and cultured according to our published procedures (Lieven et al., 2003, 2006; Nguyen et al., 2003; Vrabec et al., 2003). Briefly, RGCs were retrogradely labeled by stereotactic injection with 5 mM of the fluorescent tracer DAPI dissolved in dimethylsulfoxide into both superior colliculi of hypothermic anesthetized postnatal day 2–4 Long–Evans rats. DAPI binds to nuclear DNA and fluoresces under UV light. At postnatal days 11–13 the animals were sacrificed by decapitation, the eyes enucleated, and all retinas dissected in Hank's balanced salt solution (HBSS). After two incubations in enzyme solution containing papain (3.7 U/ml) and L-cystein (0.2 mg/ml) each for 30 min at 37 °C, the retinas were gently triturated with a Pasteur pipette and plated on poly-L-lysine-coated 96-well flat-bottomed tissue culture plates (0.32 cm^2 surface area/well) at a density of approximately 2000 cells/mm^2. The cells were cultured for 72 h in Neurobasal-A, B27 supplement lacking antioxidants in a humidified 5% CO_2 incubator at 37 °C.

2.4. Retinal ganglion cell identification and counting

RGCs were identified by the presence of DAPI, which appears blue when viewed with appropriate filters under epifluorescence. Cell viability was determined by metabolism of calcein-AM to calcein, which produces a green fluorescence when viewed with fluorescein filters. Viable RGCs were identified if they co-stained with DAPI and calcein (Fig. 1). Cells were incubated in a 1 μM solution of calcein-AM in phosphate-buffered saline (PBS) for 30 min, after which the medium was replaced with fresh PBS. RGC viability was

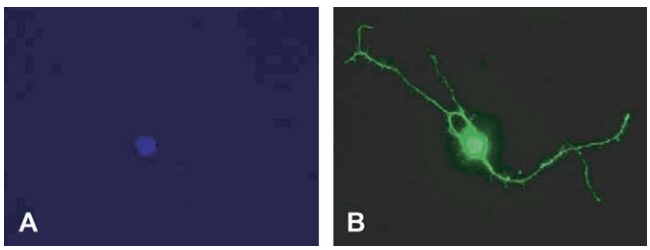

Fig. 1. RGC identification and viability assessment. (A) Ganglion cells were identified in mixed retinal cultures by distinct DAPI staining of the nucleus. (B) Cells staining strongly with calcein-AM, which is cleaved to fluorescent calcein by intracellular esterases, were considered viable.

assessed at 72 h days. The total well surface area was counted, and each condition was plated in duplicate or triplicate.

2.5. Synthesis of PB1 and PB2

Chemicals and solvents were from Aldrich Chemical (Milwaukee, WI). Reactions were monitored by thin-layer chromatography and visualized by ultraviolet light or staining with I_2. NMR spectra were obtained with a Bruker AC-300 or Varian Inova-600 spectrometer. ^{31}P NMR spectra were proton-decoupled and referenced against an external standard of deuterated phosphoric acid. Mass spectra were obtained with electrospray ionization (ESI).

2.6. Synthesis of bis(3-propionic acid methyl ester) phenylphos-phine borane complex (PB1) (Fig. 2)

2.6.1. bis(3-Propionic acid methyl ester) phenylphosphine (*1*) (Rampal et al., 1981)

Phenylphosphine (10 g, 90 mmol) was dissolved in acetonitrile (10 ml, degassed) in a flame-dried round bottom flask under Ar(g). Potassium hydroxide (1.0 N, 1.0 ml) was added to this mixture, and the resulting solution was cooled to 0 °C. Methyl acrylate (16.2 ml, 180 mmol) was added at a rate that maintained the reaction temperature below 35 °C. Upon complete addition of methyl acrylate, the reaction was heated at 50 °C for 8 h. The reaction mixture was then washed with brine (2 × 10 ml). The organic layer was dried over $MgSO_4$(s), filtered, and concentrated in vacuo. The residue was purified by distillation with the desired product distilling at 160–170 °C (0.5 mmHg). Phosphine **1** was isolated as a clear liquid (20.7 g, 73 mmol, 81% yield).

2.6.2. Spectral data

^{1}H NMR (300 MHz, $CDCl_3$:CD_3OD) δ 7.54–7.48 (m, 2H), 7.37–7.30 (m, 3H), 3.62 (s, 6H), 2.46–2.23 (m, 4H), 2.10–2.03 (m, 4H) ppm; ^{13}C NMR (75 MHz, THF-d_6) δ 173.25 (d, $J = 12.9$ Hz), 133.51 (d, $J = 15.5$ Hz), 132.28 (d, $J = 19.4$ Hz), 129.22, 128.43 (d, $J = 7.2$ Hz), 51.47, 30.22 (d, $J = 16.9$ Hz), 22.63 (d, $J = 11.9$ Hz) ppm; ^{31}P NMR (121 MHz, $CDCl_3$:CD_3OD) δ −23.06 ppm; MS (ESI) *m/z* 305.0905 ($MNa^+[C_{14}H_{19}O_4PNa^+] = 305.0919$).

2.6.3. PB1

Phosphine **1** (20.7 g, 73 mmol) was dissolved in dry THF in a flame-dried round bottom flask under an argon atmosphere. This solution was cooled to 0 °C and borane-THF (1.0 M in THF, 80.6 ml, 80.6 mmol) was added slowly. The reaction was stirred at 0 °C for 45 min and then at room temperature for an additional 1.5 h. The solvent was removed under reduced pressure, and the residue was purified by flash chromatography (silica gel, 80% v/v methylene chloride in hexanes). Phosphine-borane complex **1** (PB1) was isolated as a clear oil (7.6 g, 25.6 mmol, 35% yield).

2.6.4. Spectral data

^{1}H NMR (300 MHz, $CDCl_3$:CD_3OD) δ 7.78–7.70 (m, 2H), 7.57–7.47 (m, 3H), 3.64 (s, 6H), 2.70–2.56 (m, 2H), 2.41–2.19 (m, 6H), 0.68 (m, 3H) ppm; ^{13}C NMR (75 MHz, THF-d_6) δ 172.29 (d, $J = 19.1$ Hz), 131.88 (d, $J = 13.5$ Hz), 131.84, 128.93 (d, $J = 12.3$ Hz), 126.11 (d, $J = 61.4$ Hz), 51.83, 27.37, 20.71 (d, $J = 45.4$ Hz) ppm; ^{31}P NMR (121 MHz, $CDCl_3$:CD_3OD) δ 17.34 (d, $J = 70.4$ Hz) ppm; MS (ESI) *m/z* 318.1292 ($MNa^+[C_{14}H_{22}BO_4PNa^+] = 318.1283$).

2.7. Synthesis of (3-propionic acid methyl ester)diphenylphosphine borane complex (PB2) (Fig. 3)

2.7.1. PB2 (Imamoto et al., 1985)

Borane-diphenylphosphine complex (0.190 g, 1.0 mmol) was dissolved in methanol (8 ml) in a flame-dried round bottom flask under Ar(g) at room temperature. Potassium hydroxide (0.0028 g, 0.05 mmol) was added to this mixture, followed by the drop-wise addition of methyl acrylate (0.108 ml, 1.2 mmol). The reaction mixture was allowed to stir at room temperature for 6 h, after which the methanol was removed en vacuo. The white residue was taken up in dichloromethane (10 ml) and washed with 0.5 N HCl (1 × 5 ml) and brine (1 × 5 ml). The aqueous layers were washed with dichloromethane (10 ml), and the combined organic layers were dried over $MgSO_4$(s), filtered, and concentrated in vacuo. The residue was purified by flash chromatography (silica gel, 30% v/v ethyl acetate in hexanes). Phosphine **2** was isolated as a pale yellow oil (0.219 g, 0.76 mmol, 76% yield).

2.7.2. Spectral data

^{1}H NMR (300 MHz, $CDCl_3$) δ 7.72–7.65 (m, 5H), 7.51–7.45 (m, 5H), 3.64 (s, 3H), 2.55 (m, 4H), 0.96 (m, 3H) ppm; ^{13}C NMR (75 MHz, $CDCl_3$) δ 132.37 (d, $J = 9.20$ Hz), 131.67, 129.17 (d, $J = 10.1$ Hz), 128.84, 52.24, 28.01, 21.15 (d, $J = 39.5$ Hz) ppm; ^{31}P NMR (121 MHz, $CDCl_3$) δ 16.26 (d, $J = 59.0$ Hz); MS (ESI) *m/z* 309.1190 ($MNa^+[C_{16}H_{20}BO_2PNa^+] = 309.1192$).

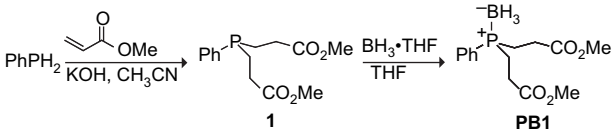

Fig. 2. Synthesis of bis(3-propionic acid methyl ester)phenylphosphine borane complex (PB1).

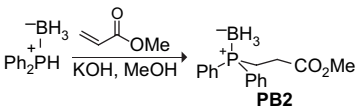

Fig. 3. Synthesis of (3-propionic acid methyl ester)diphenylphosphine borane complex (PB2).

2.8. PAMPA assay for cell permeability

To assess membrane permeability of PB1 and PB2 with respect to the other studied phosphino compounds, a microplate-based parallel artificial membrane permeability assay (PAMPA) was used (Millipore; Billerica, MA). Briefly, 5 µl of a lecithin solution (2% w/v in dodecane) was applied to each well in a MultiScreen filter plate. Compounds were dissolved to the desired concentrations in 150 µl phosphate-buffered saline containing 5% DMSO and 20% D_2O, added to the filter plate. The filter plate was then placed over a PTFE microplate containing 300 µl of buffer, so that buffer in the PTFE micro plate was in contact with the lecithin/dodecane membrane. The plates were incubated at room temperature for 19 h to allow the compounds to cross the membrane, and then fractions were collected from both the MultiScreen (donor) plate and the PTFE (acceptor) plate. Concentrations were assessed by UV spectrometry for PB1 ($\lambda = 265.5$ nm), PB2 (266 nm), 2DPBA (285 nm), 4DPBA (285 nm), 3BSA (275 nm), and HEt (269 nm). TCEP concentration was assessed by ^{31}P NMR. Permeability was calculated according to Wohnsland and Faller (2001).

2.9. Statistical analysis

All RGC viability calculations were normalized to the control (no treatment) condition by dividing the mean number of living RGCs in an experimental condition by the mean in controls. Dose–response curves were analyzed with ANOVA for linear trend using the software package R (http://www.r-project.org/). Two-sample comparisons were by unpaired *t*-test. Differences were considered significant at $p < 0.05$.

3. Results

3.1. Novel reducing agents PB1 and PB2 protect RGCs in vitro at very low concentrations

Our previous studies demonstrated that (1) the reducing agent TCEP potently prevented RGC death *in vitro* to a degree equivalent to the combined effect of brain-derived neurotrophic factor (BDNF) and ciliary neurotrophic factor (CNTF) (Geiger et al., 2002) and (2) TCEP injected intravitreally into adult rats inhibited RGC death after optic nerve crush (Swanson et al., 2005). The concentrations required for RGC neuroprotection with this molecule were on the order of 100 µM, which is unlikely to be pharmacologically available via typical (e.g. topical or transscleral) routes. The TCEP molecule, being highly polar, does not cross cell membranes well, and the extracellular stability of the compound is low.

This low efficacy was also demonstrated with other phosphines. We tested the effects of three commercially available triphenylphosphines *in vitro* using postnatal day 11–13 RGC cultures, a time after which development of RGC death has already completed in the rat (Perry et al., 1983), and measuring viability at 72 h. All comparisons are to vehicle-treated parallel cultures. Cells rapidly die as a result of axotomy at the cell body, with 32 ± 7% alive at 72 h compared to 24 h (data not

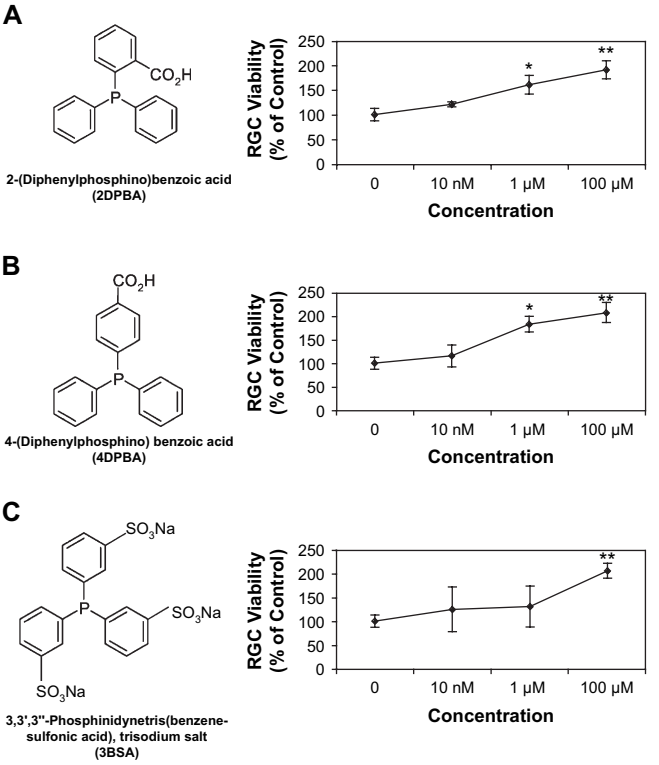

Fig. 4. Neuroprotection by three tri-phenylated phosphino compounds. Mixed retinal cultures were incubated in Neurobasal A and B27 supplement without antioxidants for 72 h and viability of DAPI-labeled RGCs assessed with calcein-AM. (A) Dose–response analysis of 2DPBA. (B) Dose–response analysis of 4DPBA. (C) Of the three tri-phenylated phosphines, 3BSA required the highest concentration (100 µM) to increase RGC survival after acute dissociation. An asterisk indicates $p < 0.05$ and a double asterisk indicates $p < 0.01$. All data points were counted in duplicate, and experiments were conducted using four retinas per experiment.

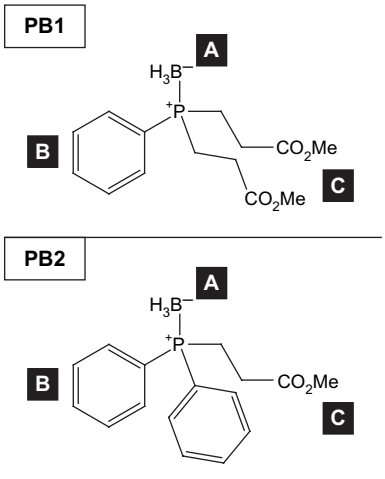

Fig. 5. Structure of the novel reductants PB1 and PB2. (A) The borane protects the phosphine from oxidation, and thus stabilizes the molecule. (B) The phenyl group is nonpolar (which is likely to increase the molecule's cell permeability), delocalizes the electron pair of the phosphino group by resonance, and provides minimal steric hindrance. (C) The methyl esters are likely to be cleaved by cytosolic esterases, resulting in an anionic molecule that is unlikely to exit the cytosol.

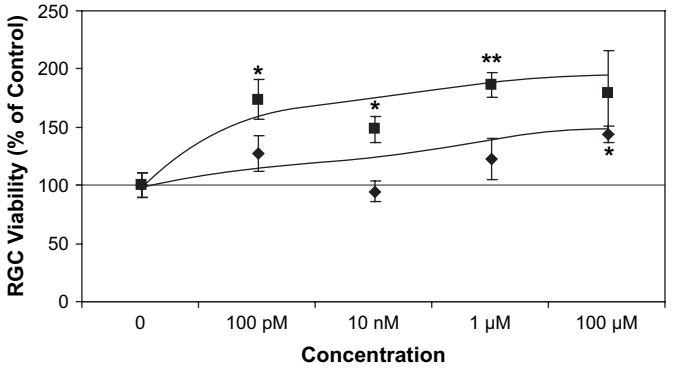

Fig. 6. PB1 is neuroprotective at lower concentrations than TCEP. A dose–response curve of TCEP (diamonds) and PB1 (squares) was determined at 72 h *in vitro*. TCEP rescued RGCs at 100 μM, whereas PB1 was highly effective at picomolar concentrations. Two-way ANOVA demonstrated significant dose ($p = 0.0009$) and drug ($p = 0.0003$) effects, with PB1 being more effective than TCEP. An asterisk indicates $p < 0.05$, and a double-asterisk indicates $p < 0.01$ for comparisons between control and drug conditions. Experiments ($n = 3$) were plated in duplicate.

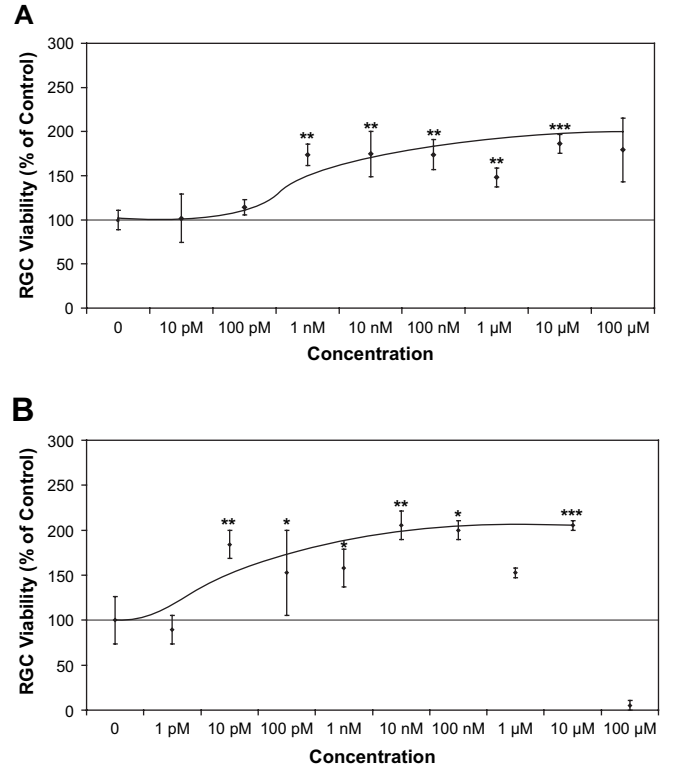

Fig. 7. PB1 and PB2 are highly neuroprotective for axotomized RGCs at nanomolar and picomolar concentrations. RGCs were cultured for 72 h in defined medium, following which DAPI-positive RGCs were identified and calcein-AM staining used to assess viability. Dose–response curves of PB1 and PB2 ranging from 1 pM to 100 μM were assessed at 72 h. (A) PB1 was neuroprotective for RGCs at ≥1 nM ($p = 0.030$ by ANOVA for linear trend). (B) PB2 inhibited was neuroprotective at ≥10 pM ($p = 0.047$ by ANOVA for linear trend). All data points were in duplicate or triplicate, and all experiments were performed 2–5 times, using four retinas per experiment. An asterisk indicates $p < 0.05$, a double asterisk indicates $p < 0.01$, and a triple asterisk indicates $p < 0.001$.

shown). Increased RGC survival was seen with 2DPBA at concentrations of 1 μM (160 ± 19%; $p = 0.042$) (Fig. 4). Similarly, increased survival was seen with 4DPBA at 1 μM (183 ± 19%; $p = 0.015$). A concentration of 100 μM 3BSA was needed to see significantly increased survival (206 ± 6%; $p = 0.0023$). Although the reduction potential of these phosphines prevents disulfide bond formation, 2DPBA, 4DPBA, and 3BSA are not effective at low concentrations, since the phosphino group is left unprotected and can react with extracellular molecules before crossing the cell membrane.

Our goal was to synthesize analogues of TCEP that could be effective at lower concentrations. Our approach was to protect the phosphino group from reactivity in the extracellular environment, facilitate transmembrane diffusion, and install a functional group that could be cleaved once intracellular, thus maintaining a suitable intracellular concentration. PB1 and PB2 are novel phosphines that have these attributes (Fig. 5). These two molecules were synthesized and characterized by NMR spectroscopy and mass spectrometry.

We studied the TCEP analogue PB1 in RGC cultures at 72 h after dissociation. PB1 showed greater RGC protection at lower concentrations than did TCEP (Fig. 6), with significant dose ($p = 0.0009$) and drug ($p = 0.0003$) effects by two-way ANOVA. Dose–response curves for PB1 and PB2 were generated over the range of 1 pM to 100 μM, and relative RGC survival (compared to control) was assayed at 72 h after dissociation (Fig. 7). PB1 increased RGC viability at concentrations as low as 1 nM (174 ± 12%; $p = 0.002$) at 72 h and PB2 increased RGC viability at concentrations as low as

Table 1
Membrane permeability of phosphino compounds

Compound	Log P_e
PB1	−5.58 ± 0.02
PB2	−5.61 ± 0.08
2DPBA	−5.25 ± 0.01
4DPBA	−4.96 ± 0.04
3BSA	−6.71 ± 0.33
TCEP	<−7.19*
HEt	−5.41 ± 0.03

$$\log P_e = \log\left\{C\cdot - \ln\left(1 - \frac{[\text{drug}]_{\text{acceptor}}}{[\text{drug}]_{\text{equilibrium}}}\right)\right\}$$

$$\text{where } C = \left(\frac{V_D \cdot V_A}{(V_D + V_A)\text{Area}\cdot\text{Time}}\right)$$

Compounds were screened with a cell-free parallel artificial membrane permeability assay (PAMPA). The log P_e, a measure of permeability, was calculated by the equations of Wohnsland and Faller (2001). Hydroethidium (HEt), a cell-permeable ethidium derivative, was used as a highly permeable control, and its log P_e is provided as a reference. TCEP did not reach measurable levels in the acceptor buffer chamber for the majority of trials, indicating a very low permeability through the dodecane membrane. The value provided should be considered an upper limit to the permeability of TCEP, as this was calculated from the only trial in which TCEP reached a measurable level. PB1, bis(3-propionic acid methyl ester)phenylphosphine borane complex; PB2, (3-propionic acid methyl ester)diphenylphosphine borane complex; 2DPBA, 2-(diphenylphosphino)benzoic acid; 4DPBA, 4-(diphenylphosphino)benzoic acid; 3BSA, 3,3′,3′′-phos-phinidyne-tris(benzene-sulfonic acid) trisodium salt; TCEP, tris(2-carboxyethyl)phosphine; HEt, hydroethidium.

10 pM (177 ± 14%; $p = 0.006$), all compared to untreated cultures. The 100 μM concentration of PB2 was toxic.

We used a PAMPA assay to assess the ability of the all the studied compounds to diffuse across a lipid bilayer, simulating transmembrane diffusion into a cell. PB1 and PB2 were markedly more permeable than TCEP (Table 1).

4. Discussion

The borane-protected TCEP analogues PB1 and PB2 were neuroprotective for RGCs *in vitro* at nanomolar and picomolar concentrations, respectively. They were also more neuroprotective for a given concentration than the parent compound, TCEP. The nonprotected triphenylphosphines, 2DPBA, 4DPBA and 3BSA, were less effective at rescuing RGCs from acute dissociation. By modifying TCEP so that (1) its phosphino group was protected from reaction in the extracellular milieu; and (2) its pendant carboxyethyl groups were either replaced with a nonpolar phenyl group or converted to esters that could be cleaved by endogenous esterases within the RGC, we markedly increased the potency of these compounds. Passage of PB1 and PB2 across the cell membrane and cleavage of their protective groups produce highly effective reducing compounds. Because deprotected PB1 and PB2 are polar and highly charged, they will be confined within the RGCs. There, the phosphines can protect biomolecules from oxidation by ROS during optic nerve injury.

Injury to the RGC axon is an initiating event for apoptosis (Fig. 8). After axotomy, a rise in superoxide occurs, which is

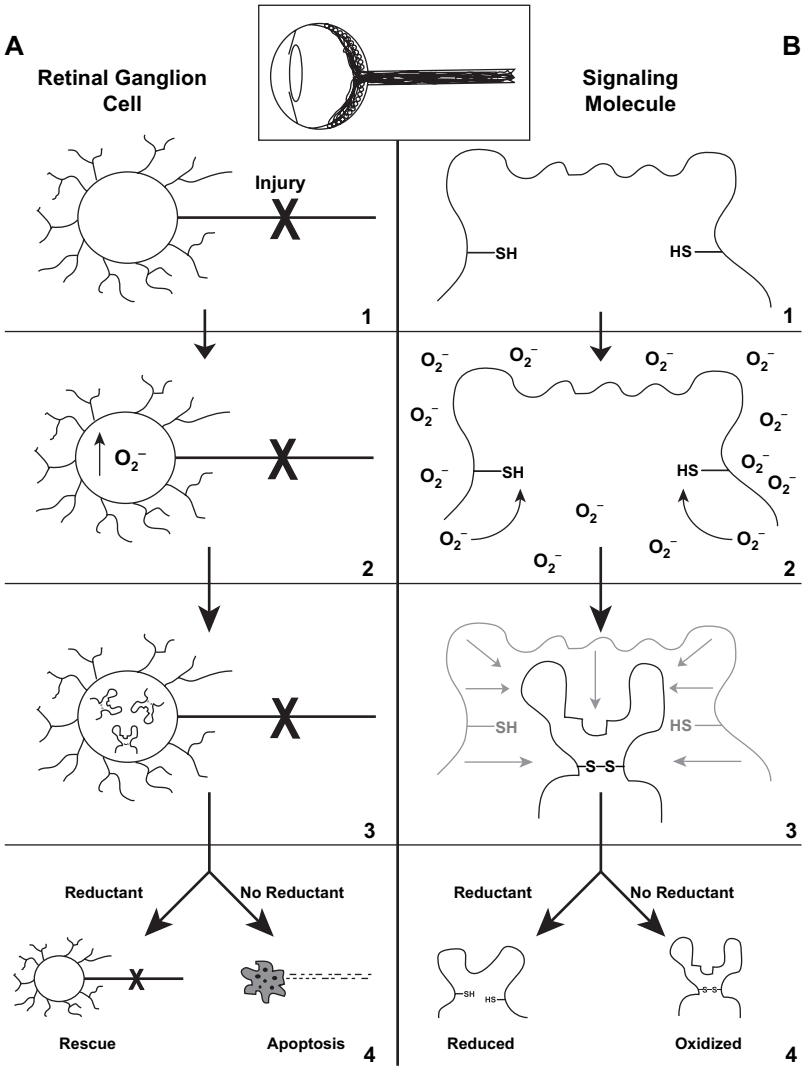

Fig. 8. Schematic of optic nerve injury and resultant protein modification via superoxide. RGC soma and axon after injury (A_1–A_4) and corresponding conformational states of a hypothetical signaling protein (B_1–B_4). The inset displays a schematic drawing of the eye and optic nerve. A healthy RGC (A_1) contains a reduced signaling protein with free sulfhydryl groups (B_1). Optic nerve injury or transection (A_2) initiates a rise in superoxide anion via an as yet unknown mechanism. Intracellular superoxide oxidizes the cysteine sulfhydryls of proteins (B_2). The concentration of superoxide increases over time within the RGC soma (A_3) and disulfide bonds are created as a result, inducing conformational changes and induction of cell death signals (B_3). The RGC will either undergo apoptosis (if untreated) or be rescued (if treated with reducing agents) (A_4). When a reducing agent, such as TCEP, PB1 or PB2, is administered, disulfide bonds are reduced and the protein resumes normal conformation and function (B_4). Without the reducing agent, the signaling of apoptosis will continue.

an upstream event necessary and sufficient to induce RGC death (Lieven et al., 2006). Although the source of ROS has not been determined, our recent findings suggest that it is dependent on functioning mitochondrial complex III (Lieven et al., 2006). ROS can transduce signals by oxidizing sulfhydryls (Cross and Templeton, 2004). Disulfide bond formation within a protein or with other proteins alters its configuration, which can prevent it from performing specific functions or cause it to initiate new reactions. Ultimately, the rise in ROS causes the cell to undergo an irreversible death cascade. RGCs can survive axotomy, however, if treated with reducing agents (Geiger et al., 2002).

The first trialkylphosphines (Ruegg and Rudinger, 1977) were poorly soluble in water and thus not applicable to biological systems. The discovery that TCEP was a potent reducing agent and water soluble (Burns et al., 1991) led to its widespread use. Its properties include high stability at elevated temperatures (Rhee and Burke, 2004) and neutral pH (Getz et al., 1999; Han and Han, 1994), and weak metal chelating activity (Krezel et al., 2003). For these reasons TCEP has favorable characteristics for use in pharmacology. Yet, its polarity prevents it from easily crossing cell membranes, and the unprotected phosphine is highly reactive with oxygen.

PB1 and PB2 have modifications that neutralize the polarity of the compounds and are easily removed by intracellular amines and cytosolic esterases (Fig. 5). The borane protects the phosphine from oxidation, and thus stabilizes the molecule. The phenyl group(s) has three likely effects. First, it is very nonpolar and increases the molecule's ability to cross cell membranes (Table 1). Second, it delocalizes the electrons on the phosphine by resonance, so that after removal of the borane protecting group the phosphine is less susceptible to oxidation by molecular oxygen (but also less reactive towards disulfide bonds). Finally, a phenyl group presents less steric hindrance than does a carboxymethyl group. This factor is likely to be important because reduction of protein disulfide bonds by TCEP is inefficient (Cline et al., 2004). The methyl esters of PB1 and PB2 are likely to be cleaved by cytosolic esterases, yielding a polar intracellular intermediate that would be unlikely to cross cell membranes, and thus would maintain a high intracellular concentration.

Our data indicate that PB1 and PB2 are neuroprotective at very low concentrations *in vitro*. We previously showed that a single intravitreal injection of TCEP increases RGC survival from optic nerve crush in the rat for up to 7 days (Swanson et al., 2005), when instilled to a final concentration of 60 μM. This concentration of TCEP is in the same range as that which maintains RGC viability *in vitro* (Geiger et al., 2002). If the effective *in vitro* concentration of PB1 and PB2 also approximates the effective *in vivo* concentration, then it is conceivable that systemic or topical delivery may achieve sufficient concentrations at the RGC. However, PB1, PB2, and similar molecules may not adequately cross the blood-brain barrier and may require higher dosing to see effects similar to that seen *in vitro*. Despite significant neuroprotection, there was still residual RGC death, suggesting that these molecules either incompletely protected against axotomy-mediated signaling, or that they did not protect against other mechanisms of cell death (e.g. excitotoxicity or ischemia) present in the culture model we used. Finally, toxicity when given to animals has not yet been assessed, and could limit the use of these compounds in clinical disease. Fortunately, the LD_{50} value of triphenyl-phosphine in rats dosed orally is 1.150 mg/kg, which greatly exceeds the concentration needed for efficacy *in vitro*. Only the 100 μM concentration of PB2 was found to be toxic to RGCs in our experiments, which is 10^7 times the minimal effective concentration.

In conclusion, the novel TCEP analogues PB1 and PB2 increase RGC survival after axotomy *in vitro* at nanomolar and picomolar concentrations, respectively. Reducing agents probably interfere with the signaling of cell death in acutely axotomized RGCs, and potentially other models of RGC death. This is a form of neuroprotection. The borane-protected phenylphosphine compounds appear to have important advantages over other neuroprotectants, and if found to be safe, could be useful for the treatment of optic neuropathies and other axonal diseases.

Acknowledgments

NIH R01 EY12492 (LAL), R01 GM44783 (RTR), Retina Research Foundation (LAL), and an unrestricted departmental grant from Research to Prevent Blindness, Inc. RTR and LAL are co-inventors on a patent application for the molecules described in this article, assigned to the Wisconsin Alumni Research Foundation.

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