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Intracellular disulfide reduction by phosphine-borane complexes: Mechanism of action for neuroprotection

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ABSTRACT

Phosphine-borane complexes are novel cell-permeable drugs that protect neurons from axonal injury *in vitro* and *in vivo*. These drugs activate the extracellular signal-regulated kinases 1/2 (ERK1/2) cell survival pathway and are therefore neuroprotective, but do not scavenge superoxide. In order to understand the interaction between superoxide signaling of neuronal death and the action of phosphine-borane complexes, their biochemical activity in cell-free and *in vitro* assays was studied by electron paramagnetic resonance (EPR) spectrometry and using an intracellular dithiol reporter that becomes fluorescent when its disulfide bond is cleaved. These studies demonstrated that bis(3-propionic acid methyl ester) phenylphosphine-borane complex (PB1) and (3-propionic acid methyl ester) diphenylphosphine-borane complex (PB2) are potent intracellular disulfide reducing agents which are cell permeable. EPR and pharmacological studies demonstrated reducing activity but not scavenging of superoxide. Given that phosphine-borane complexes reduce cell injury from mitochondrial superoxide generation but do not scavenge superoxide, this implies a mechanism where an intracellular superoxide burst induces downstream formation of protein disulfides. The redox-dependent cleavage of the disulfides is therefore a novel mechanism of neuroprotection.

1. Introduction

Reactive oxygen species (ROS) cause cell death via two disparate mechanisms, direct damage to macromolecules and activation of intracellular pathways that transduce a cell death signal (Simon et al., 2000). In previous studies we demonstrated that superoxide anion is a signaling molecule for death of a specific central nervous system (CNS) neuron, the retinal ganglion cell (RGC), after injury to its axon contained within the optic nerve (Kanamori et al., 2010a, 2010b; Lieven et al., 2006; Scott et al., 2010). Despite near-complete reduction of intracellular levels of superoxide by scavengers such as pegylated superoxide dismutase (PEG-SOD) (Kanamori et al., 2010a) and metallocorroles (Catrinescu et al., 2012; Kanamori et al., 2010b), there is much less neuroprotection of neurons in animal models of optic neuropathy. This disparity between the degree of observed signaling by superoxide and the incomplete neuroprotection when superoxide is scavenged has three possible explanations.

First, the superoxide burst could be a result of apoptosis and not its cause, e.g. if cytochrome c release during apoptosis caused reduction of proximal intermediates in the mitochondrial electron transport chain. The reduced intermediates would react with molecular oxygen to produce superoxide (Cai and Jones, 1998). Against this possibility are data from *in vitro* (Lieven et al., 2012) and longitudinal *in vivo* studies (Kanamori et al., 2010a) demonstrating that the superoxide burst in fact precedes cytochrome c release and

Abbreviations: CNS, central nervous system; DABCO, 1,4-diazobicyclo[2.2.2]octane; DEPMPO, 5-(diethoxyphosphoryl)-5-methyl-1-pyrroline-N-oxide; DTNB, 5,5'-dithiobis-(2-nitrobenzoic acid); DTT, dithiothreitol; EPR, electron paramagnetic resonance; ERK1/2, extracellular signal-regulated kinases 1/2; P1, bis(3-propionic acid methyl ester) phenylphosphine; P2, (3-propionic acid methyl ester)diphenylphosphine; PAMPA-BBB, parallel artificial membrane permeation assay for the prediction of blood-brain barrier penetration; PAMPA, parallel artificial membrane permeation assay for the prediction of blood-brain barrier penetration; PB1, bis(3-propionic acid methyl ester) phenylphosphine-borane complex; PB2, (3-propionic acid methyl ester) diphenylphosphine-borane complex; PEG-SOD, poly(ethylene glycol)-conjugated superoxide dismutase; RGC, retinal ganglion cell; ROS, reactive oxygen species; TCEP, tris(2-carboxyethyl)phosphine.

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phosphatidylserine externalization, respectively. Second, there are oxidation-independent stress pathways such as the endoplasmic reticulum stress signaling pathways, that operate independently of superoxide signaling. Third, the intracellular superoxide burst could be rapidly followed by activation of the downstream effects of superoxide, e.g. oxidation of one or more critical signaling macromolecules. If scavenging superoxide occurs after the downstream pre-apoptotic pathways are activated, then levels of neuroprotection are likely to be incomplete.

One such downstream target for superoxide induced by axotomy is oxidation of cysteine thiols, with consequent formation of disulfide bonds that modify protein structure and function (Carugo et al., 2003; Park and Raines, 2001). Previous studies demonstrated that the disulfide reducing agent dithiothreitol (DTT) can increase *in vitro* survival of CNS neurons in mixed retinal culture (Geiger et al., 2002). Likewise, studies with tris(2-carboxyethyl)phosphine (TCEP), a disulfide-reducing phosphine, demonstrated that reversing sulfhydryl oxidation prevents neuronal death after axotomy *in vivo* (Geiger et al., 2002) and after optic nerve crush in rats (Swanson et al., 2005). Such results are consistent with disulfide formation being a downstream pathway for cell death induced by axonal injury.

Based on the observed neuroprotection with DTT and TCEP, we synthesized membrane permeable derivatives of TCEP, phosphine-borane complexes bis(3-propionic acid methyl ester) phenylphosphine-borane complex (PB1) and (3-propionic acid methyl ester) diphenylphosphine-borane complex (PB2) (Fig. 1). These compounds have a positive, dose-dependent effect on neuronal viability after axonal injury at concentrations much lower than that of non-derivatized TCEP (Schlieve et al., 2006). PB1 and PB2 are neuroprotective *in vivo* in two rat models of CNS axonal injury, optic nerve transection and experimental glaucoma, with a biological mechanism of action that involves activation of the extracellular signal-regulated kinases 1/2 (ERK1/2) pathway (Almasieh et al., 2011).

Although phosphine itself is toxic, alkyl phosphines are far less toxic. The LD_{50} value of TCEP in rats dosed orally is 3500 mg/kg, and an LD_{50} greater than 1024 mg/kg when administered by i.p injection (Hampton Research, 2016). Previous toxicity testing of PB1 and PB2 *in vitro* and PB1 *in vivo* demonstrated no toxicity to RGCs and retinal endothelial cells up to 1 mM with PB1 and 100 μM with PB2, which are 10^5 and 10^8 times the optimal reducing concentration for neuroprotection (Schlieve et al., 2006).

The phosphine-borane complexes PB1 and PB2 are neuroprotective *in vitro* and *in vivo*, and their structural similarity to TCEP is consistent with an ability to reduce disulfide bonds. Yet their biochemical mechanism of action could result from either scavenging of the upstream superoxide burst that signals neuronal death or reduction of intracellular disulfides. Inhibition of either pathway would be associated with increased neuronal survival. In order to distinguish these two possibilities, electron paramagnetic resonance, intracellular disulfide reducing probes, and superoxide assays were used to assess the biochemical effects of these neuroprotective molecules. Their pharmacological characteristics were tested in order to determine whether they would be able to penetrate the blood-brain barrier and cell membranes, consistent with *in vivo* neuroprotective activity. Their reducing activity was compared to DTT and TCEP at various pH to determine their activity at a biologically relevant pH. Phosphine-borane complexes were potent intracellular reducing agents, with pharmacological and pharmaceutical properties that would predict activity as CNS neuroprotectants.

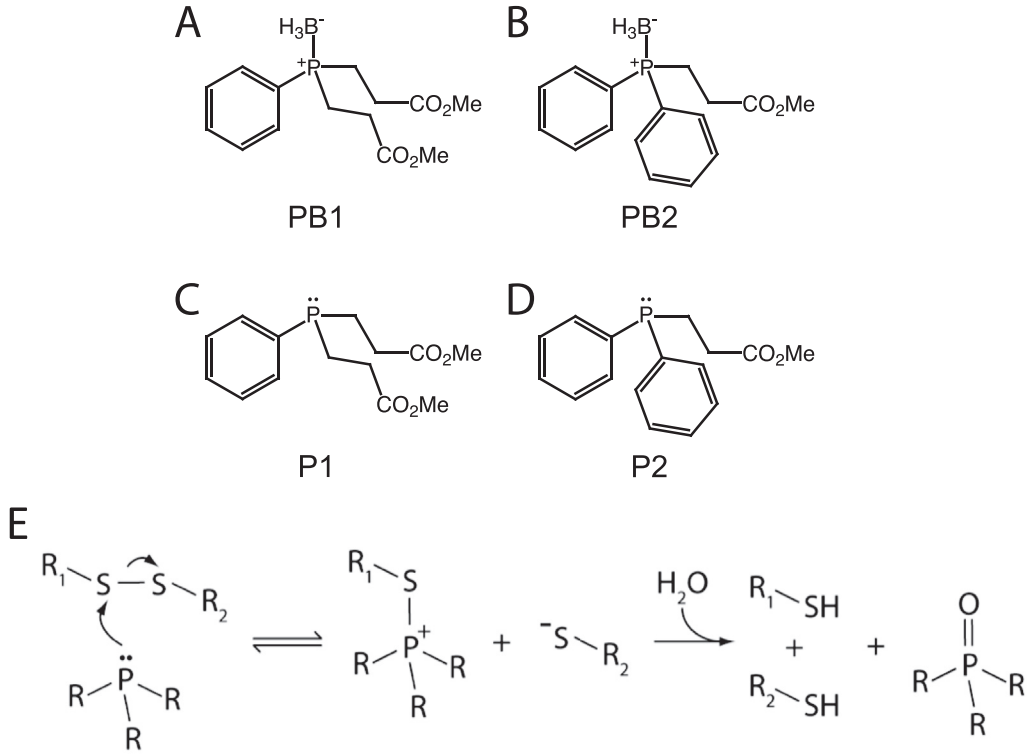

Fig. 1. Biochemistry of phosphine-borane complexes used in the studies. (A) bis(3-propionic acid methyl ester) phenylphosphine-borane complex (PB1); **(B)** (3-propionic acid methyl ester) diphenylphosphine-borane complex (PB2); **(C)** bis(3-propionic acid methyl ester) phenylphosphine (P1); **(D)** (3-propionic acid methyl ester) diphenylphosphine (P2). **(E)** Mechanism of reduction of disulfide by phosphine in water, modified from Burns et al. (1991). Panels A and B are redrawn from Schlieve et al. (2006).