Effects of Phenelzine Administration on Mitochondrial Function, Calcium Handling, and Cytoskeletal Degradation after Experimental Traumatic Brain Injury

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Abstract

Traumatic brain injury (TBI) results in the production of peroxynitrite (PN), leading to oxidative damage of lipids and protein. PN-mediated lipid peroxidation (LP) results in production of reactive aldehydes 4-hydroxynonenal (4-HNE) and acrolein. The goal of these studies was to explore the hypothesis that interrupting secondary oxidative damage following a TBI via phenelzine (PZ), aldehyde scavenger, would protect against LP-mediated mitochondrial and neuronal damage. Male Sprague-Dawley rats received a severe (2.2 mm) controlled cortical impact (CCI)-TBI. PZ was administered subcutaneously (s.c.) at 15 min (10 mg/kg) and 12 h (5 mg/kg) post-injury and for the therapeutic window/delay study, PZ was administered at 1 h (10 mg/kg) and 24 h (5 mg/kg). Mitochondrial and cellular protein samples were obtained at 24 and 72 h post-injury (hpi). Administration of PZ significantly improved mitochondrial respiration at 24 and 72 h compared with vehicle-treated animals. These results demonstrate that PZ administration preserves mitochondrial bioenergetics at 24 h and that this protection is maintained out to 72 hpi. Additionally, delaying the administration still elicited significant protective effects. PZ administration also improved mitochondrial Ca^{2+} buffering (CB) capacity and mitochondrial membrane potential parameters compared with vehicle-treated animals at 24 h. Although PZ treatment attenuated aldehyde accumulation post-injury, the effects were insignificant. The amount of α -spectrin breakdown in cortical tissue was reduced by PZ administration at 24 h, but not at 72 hpi compared with vehicle-treated animals. In conclusion, these results indicate that acute PZ treatment successfully attenuates LP-mediated oxidative damage eliciting multiple neuroprotective effects following TBI.

Keywords: 4-hydroxynonenal (4-HNE); acrolein; mitochondria; phenelzine; spectrin; traumatic brain injury (TBI)

Introduction

BRAIN TISSUE is normally subject to increased levels of oxidative damage because of high demand for energy and oxygen consumption. Mitochondria, intracellular organelles responsible for generation of energy (ATP) for the cell, are key sources of reactive oxygen species (ROS), and therefore, are also critically susceptible to ROS-mediated damage following injury. Following central nervous system (CNS) injury, it is well known that secondary injury processes involving oxidative stress and free-radical induced damage to cellular and mitochondrial proteins and lipids result in cellular dysfunction and death.^{1–13}

Many studies have strongly suggested a critical role for peroxynitrite (PN), a potent reactive nitrogen species (RNS), in several key pathophysiological and neurodegenerative processes that occur acutely following traumatic brain injury (TBI).^{8,14–18} The PN anion (ONOO^-) is formed from the combination of the superoxide and

nitric oxide radicals ($\text{O}_2^{\bullet-} + \text{NO}^{\bullet} \rightarrow \text{ONOO}^-$). A series of further reactions result in the production of PN-derived radicals ($\bullet\text{OH}$, $\bullet\text{NO}_2$, $\bullet\text{CO}_3$), which can initiate peroxidation of lipids (LP). The LP process^{19,20} is initiated (phase 1) by the reaction of highly reactive free radicals with polyunsaturated fatty acids (e.g., arachidonic acid, linoleic acid, or docosahexaenoic acid), which make up membrane phospholipids. These fatty acids are highly enriched within the brain and are particularly vulnerable to reaction(s) with ROS. The reaction of ROS with these fatty acids form an unstable lipid radical ($\text{L}^{\bullet}$) that will react with O_2 to form a lipid peroxyl radical ($\text{LOO}^{\bullet}$). This peroxyl radical in turn takes a hydrogen atom from an adjacent polyunsaturated fatty acid yielding a lipid hydroperoxide (LOOH) and another lipid radical ($\text{L}^{\bullet}$) thus initiating a series of propagation “chain” reactions (phase 2). The termination (phase 3) of the propagation reaction(s) results in the formation of non-toxic (e.g., malondialdehyde; MDA) and, more importantly, the highly reactive and cytotoxic end products 4-hydroxynonenal

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(4-HNE) and 2-propenal (acrolein).^{21–24} 4-HNE and acrolein can covalently bind to cellular and mitochondrial proteins via Schiff base and Michael adduct reactions, which interfere with their structural and functional integrity²⁵ and impair a variety of mitochondrial and cellular functions,^{26,27} thereby contributing to post-TBI secondary injury.

Under normal conditions, these highly reactive aldehydes (4-HNE, acrolein) are rapidly metabolized^{28–33} to detoxify cells and control aldehyde accumulation. However, under neurodegenerative conditions, it has been shown that the activity of these metabolic pathways was reduced and/or dysfunctional, contributing to aldehyde accumulation.³⁴ It is likely that similar effects may occur following TBI, resulting in reduced aldehyde clearance from damaged neurons, culminating in intracellular and intramitochondrial aldehyde accumulation^{7,35} overwhelming the cell and leading to activation of cell death mechanisms. We recently demonstrated that the temporal decline in mitochondrial function following TBI correlated with accumulation of 4-HNE and acrolein protein adducts.⁷ A number of pharmacological approaches have been utilized to bind and sequester aldehydes, some of which have been shown to elicit neuroprotective effects.^{36–45} Acute administration of a single dose of phenelzine (PZ), an FDA-approved drug which acts as an MAO inhibitor as well as an aldehyde scavenger, was able to partially protect cortical mitochondria from LP-mediated oxidative damage after injury, presumably by reducing accumulation of these protein adducts.^{46,47} In addition, a recent study by Chen and colleagues⁴⁸ demonstrated that administration of PZ after spinal cord injury (SCI) reduced acrolein levels. Based upon these promising results, we decided to further investigate dosages of PZ that would improve mitochondrial function and attenuate the progressive aldehyde accumulation at later time-points following TBI.

Prior work in our lab has demonstrated connections between Ca^{2+} dysregulation, LP-mediated damage, and subsequent calpain-mediated cytoskeletal breakdown after SCI² and TBI.¹³ Calpains, Ca^{2+} -dependent cysteine proteases, are acutely activated following CNS injury. One protein that is particularly susceptible to calpain-mediated degradation after injury is α -spectrin,⁴⁹ a neuronal cytoskeletal protein. Given these known connections between post-traumatic mitochondrial Ca^{2+} dysregulation and proteolytic cytoskeletal degradation, we also decided to look at the effects of PZ administration on Ca^{2+} buffering (CB) as well as breakdown products of calpain-mediated spectrin degradation.

The overall goal of this study was to test the hypothesis that PZ treatment following TBI would attenuate LP-mediated damage and aldehyde accumulation thereby improving mitochondrial integrity and reducing cytoskeletal breakdown. Accordingly, the experiments were conducted with the specific aim of determining if a different dosing paradigm(s) for PZ could improve upon previously observed protective effects demonstrated by our laboratory.

Methods

Animals

Young adult male Sprague-Dawley rats (Harlan, Indianapolis, IN; 10 weeks old, 300–325 g) were used for these studies (see Table 1 for numbers). Dually housed animals had access to food and water *ad libitum*. All animal protocols met the guidelines of the National Institutes of Health (NIH) and complied with the policies and regulations approved by the University of Kentucky Institutional Animal Care and Use Committee, in accordance with the NIH Guidelines for the Care and Use of Laboratory Animals.

TABLE 1. EXPERIMENTAL PARAMETERS AND ANIMAL NUMBERS

	Sham	Veh	PZ	PZ _{delay}
Experiment 1				
24 hr Mito Fxn	12	12	12	4
72 hr Mito Fxn	14	16	16	
Experiment 2				
24 hr Mito Ca^{++}	6	6	6	
Mito 4-HNE WB	18	22	22	4
Mito Acrolein WB	12	22	22	4
Experiment 3				
24 hr Cellular WB	3	6	6	
72 hr Cellular WB	3	6	6	

4-HNE, 4-hydroxynonenal; PZ, phenelzine.

Controlled cortical impact traumatic brain injury (CCI-TBI)

The animals were subjected to a unilateral cortical contusion using an electronically controlled pneumatic impact device (Precision Systems & Instrumentation, Fairfax Station, VA) as previously described by our laboratory.^{46,47} All surgical procedures were performed by the same experimenter. Specifically, the animals were anesthetized with isoflurane (2–4%) and placed in a stereotaxic frame (Kopf Instruments, Tujunga, CA); the skin was retracted and a 6-mm unilateral craniotomy was performed that was centered between bregma and lambda. The skull cap was removed without disruption of the underlying dura. The exposed brain was injured using a 5-mm beveled tip that impacted the cortex at ≥ 3.5 m/sec to a depth of 2.2 mm. Following the injury, surgical foam (Surgicel, Johnson & Johnson) was laid upon the dura and the craniotomy site was sealed with a sterile 8-mm plastic disc glued to the skull with Mascot tissue adhesive (Gesswein, Bridgeport, CT). The wound was closed with wound clips, the animal was removed from the stereotaxic device, and the animal regained consciousness in a recovery cage on a circulating water heating pad.

Chemicals

Sodium pyruvate, malate, rotenone, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP), magnesium chloride ($MgCl_2$), sucrose, mannitol, EGTA, EDTA, bovine serum albumin (BSA), glycerol, HEPES potassium salt, Triton-X, Tris Buffered Saline with Tween 20 (TBS-T), TrisHCl, NaCl, KCl, and potassium phosphate monobasic anhydrous (KH_2PO_4) were obtained from Sigma Aldrich (St. Louis, MO); oligomycin was obtained from Enzo Life Sciences (Farmingdale, NY); and protease inhibitors (Complete Mini Protease Inhibitor Cocktail tablet) were obtained from Roche Diagnostics (Indianapolis, IN).

Drug preparation and dosing

Phenelzine sulfate salt (PZ; MP Biomedicals, Solon, OH) was freshly diluted each day in 0.9% saline. For subcutaneous (s.c.) injections, dilutions were made to deliver the assigned dose in an injection volume of 0.2 mL/100 g rat. A 10 mg/kg dose of PZ was administered at 15-min post-TBI followed up by a maintenance dose of 5 mg/kg at 12 h post-TBI. For the pilot delay study, a 10 mg/kg dose of PZ was administered at 1 h post-TBI followed by a maintenance dose of 5 mg/kg at 24 h post-TBI. These dosages were used based upon previously published work.^{46,47} A separate, blinded experimenter administered treatments (either PZ or vehicle) to the animals.

Isolation of Ficoll-purified total cortical mitochondria

Rat brain mitochondria were isolated from the ipsilateral (injured) cortex as previously described^{4,7,46,50} at 24 and 72 h post-injury (hpi) or from sham (craniotomized, but not injured) rats at the corresponding post-surgery time-points. In brief, rats were deeply anesthetized with CO₂ and decapitated, and cortical tissue was rapidly dissected using an 8-mm tissue punch to collect the injury epicenter and surrounding peri-contusional cortical tissue. Brain tissue was then homogenized using a Potter-Elvehjem homogenizer containing ice-cold isolation buffer (215 mM mannitol, 75 mM sucrose, 0.1% BSA, 20 mM HEPES, 1 mM EGTA). The homogenate was centrifuged twice at 1300rcf for 3 min, then pelleted at 13,000rcf for 10 min at 4°C. The crude mitochondrial pellet was re-suspended in isolation buffer and subjected to nitrogen bombing at 1200 psi and layered on top of a discontinuous 7.5% and 10% Ficoll gradient and centrifuged at 32,000 rpm for 30 min at 4°C. The final mitochondrial pellet was rinsed with isolation buffer without EGTA and then re-suspended in an appropriate volume of isolation buffer without EGTA to yield a protein concentration of 10 µg/µL. Mitochondrial protein concentration was determined using a Pierce BCA protein assay kit (Thermo Fisher Scientific, Omaha, NE) measuring absorbance at 562 nm with a BioTek Synergy HT plate reader (Winooski, VT).

Measurement of ex vivo mitochondrial respiratory function

Mitochondrial respiratory rates were measured using a Clarke-type electrode in a continuously stirred, sealed, and thermostatically controlled chamber (Oxytherm System, Hansatech Instruments Ltd., Norfolk, England, UK) maintained at 37°C as previously described.^{4,12,18,51,52} Mitochondria loaded into the chamber were normalized based on protein content: 40–100 µg of isolated mitochondrial protein were placed into the chamber with 250 µL KCl-based respiration buffer (RB; 125 mM KCl, 2 mM MgCl₂, 2.5 mM KH₂PO₄, 20 mM HEPES, and 0.1% BSA, pH 7.2) warmed to 37°C and allowed to equilibrate for approximately 1 min. State II respiration was fueled by addition of the Complex I substrates, 5 mM pyruvate and 2.5 mM malate. One minute later, two boluses of 150 µM ADP were sequentially added to the mitochondria to initiate State III respiration, followed by the addition of 2 µM oligomycin, an inhibitor of ATPase, to monitor State IV respiratory rate for an additional 2 min. To assess the uncoupled respiratory rate (State V (I)), 2 µM FCCP was added to the mitochondria, followed by the addition of 1 µM rotenone to shut down Complex I-driven respiration completely. Complex II-driven respiration (State V (II)) was then initiated with the addition of 10 mM succinate, a Complex II substrate.⁴ The slope of the oxygen consumption trace corresponded with the respiratory rate and the respiratory control ratio (RCR) was calculated by dividing the State III oxygen consumption rate (defined as the maximal rate of respiration in the presence of ADP, second bolus addition) by State IV oxygen consumption rate (defined as the rate of respiration in the presence of oligomycin, using the last 30 sec of the 2 min after the addition of the substrate). Mitochondria were isolated and prepared fresh for every experiment and were used immediately for respiration assays and then stored at –80°C for further analysis.

Real-time evaluation of mitochondrial Ca²⁺ buffering and membrane potential

Mitochondrial CB and membrane potential (MP) experiments were performed simultaneously in a stirred cuvette loaded into a spectrofluorimeter (Shimadzu RF-5301) maintained at 37°C. Mitochondria (~0.1 mg protein) were added to 2 mL RBat pH 7.2 with a combination of the fluorescent Ca²⁺-sensitive dye (CaG5N 100 nM; Molecular Probes, Eugene, OR) to monitor extra-mitochondrial

concentrations of Ca²⁺ and tetra-methyl rhodamineethyl ester (TMRE; 150 nM) for estimating transmembrane potential ($\Delta\psi_m$) as previously described.⁴ There was sequential addition of the following each minute: pyruvate (5 mM)/malate (2.5 mM), ADP (150 µM), then oligomycin (1 µM) (Figs. 1 and 2). At the fifth minute, continuous Ca²⁺ infusion (32 mM CaCl₂) was initiated using a 50-µL Hamilton syringe on an infusion pump at a rate of 0.5 µL/min.

Calculation of mitochondrial Ca²⁺ buffering capacity

The responses of isolated cortical mitochondria to Ca²⁺ infusion were calculated from recorded CaG5N traces (Fig. 1A, inset) as previously described.^{4,12,53} As the mitochondria begin to buffer the Ca²⁺ being infused into the chamber, the CaG5N trace levels out. To determine the steady state of CB, linear regression was used to plot and generate equations. As the mitochondria in the chamber are no longer able to buffer anymore Ca²⁺, and the membrane permeability transition pore (mPTP or MPT) opens, the CaG5N trace goes up again. Using 1-min slope intervals, the peak slope was calculated during the phase of the trace where the mitochondrial are failing to buffer Ca²⁺; again linear regression was used to plot and generate equations. The linear regression intersection was calculated algebraically to obtain the time to MPT, then to convert the x-value (time) to nmol Ca²⁺/mg protein/min.

Calculation of mitochondrial membrane potential

The changes in MP in response to sequentially added substrates (see above) and infused Ca²⁺ were calculated from recordings of fluorescence quenching of the cationic dye TMRE, a mitochondrial MP indicator (Fig. 2A, inset). The mitochondrial MP increases with the addition of Pyr/Mal (TMRE intensity decreases), decreases with ADP (TMRE intensity increases), and increases maximally with the addition of Oligo. The mitochondrial MP remains relatively stable while the mitochondria are still able to buffer Ca²⁺, but as they reach MPT, the mitochondria lose their MP. Once the TMRE trace begins to level off and peak close to baseline levels (highest TMRE intensity), the uncoupler FCCP was added to complete the trace and confirm loss of MP (not shown on trace). After additions of substrates/inhibitors, average TMRE intensities were quantified. Additionally, the gradual loss of MP was evaluated by calculating the change in TMRE intensity over time (area under the curve [AUC] using Graph Pad Prism).

Preparation of cortical mitochondrial samples for measurement of oxidative damage

A portion of the isolated cortical mitochondria (see Table 1 for numbers) was used for the quantitative measurements of 4-HNE and acrolein as an index of LP-mediated oxidative damage at 24 and 72 h post-TBI. Mitochondrial samples were centrifuged at maximum speed for 10 min to remove residual isolation buffer. The pellets were re-suspended in 20–30 µL ice-cold Triton lysis buffer (1% Triton-X, 20 mM tris-HCl, 150 mM NaCl, 5 mM EGTA, 10 mM EDTA, 10% glycerol) with protease inhibitors. Samples were then briefly sonicated, repeatedly vortexed, and incubated on ice for 45 min; then they were centrifuged at maximum speed for 30 min at 4°C. Supernatants were collected for protein assay and stored at –20°C.

Preparation of cortical and hippocampal homogenates for measurement of oxidative and cytoskeletal damage

Rats were deeply anesthetized with CO₂, and decapitated; the injured ipsilateral cortical tissue (left hemisphere; 8-mm tissue punch; as described above) and the underlying ipsilateral hippocampus were rapidly dissected and immediately transferred to

FIG. 1. Oxygen consumption rates for each state of mitochondrial respiration were quantified and representative graphs (A–F) demonstrate the changes in cortical mitochondrial dysfunction in response to a 24- versus 72-h PZ treatment paradigm following severe TBI in male rats. There was a significant reduction in oxygen consumption rates at 24 h for most states of respiration that persisted and deteriorated further by 72 h post-injury (hpi) in isolated cortical mitochondria from vehicle-treated animals compared with sham (stats in results). More importantly, administration of PZ after TBI was able to significantly recover these deficits at both the 24- and 72-h time-points for most states of respiration compared with vehicle. Delayed administration of PZ also significantly improved some states of mitochondrial respiration at 72 h following TBI compared with 72-h vehicle-treated animals (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). PZ, phenelzine; TBI, traumatic brain injury.

tubes on ice pre-filled with Triton lysis buffer containing protease inhibitors. Samples were briefly sonicated, repeatedly vortexed, and incubated on ice for 45 min. The samples were centrifuged at maximum speed for 30 min at 4°C and supernatants were collected for protein assay and stored at –20°C.

Measurement of mitochondrial reactive aldehydes by Western blot

Forty microgram aliquots of cortical mitochondrial protein samples were resolved on 12% Bis-Tris Criterion™ XT pre-cast sodium dodecyl sulfate polyacrylamide (SDS-PAGE) gels (BioRad, Hercules, CA; 20 μ L total volume) and transferred onto a nitrocellulose membrane using a semi-dry electro-transferring unit set at 15 V for 60 min at 4°C. Following gel transfer, membranes were incubated in a TBS blocking solution with 5% milk for 1 h at room temperature (RT). The gels were stained with Coomassie Blue and imaged to validate sample loading. For detection of

proteins, the membranes were incubated with one of the following primary antibodies: rabbit anti-4-HNE (1:2000; Alpha Diagnostics Intl. [ADI], San Antonio, TX), rabbit anti-acrolein (1:2000; Abcam, Cambridge, MA), or rabbit anti-VDAC (1:30,000; Millipore) in TBS-T with 5% milk on an orbital shaker overnight at 4°C. After washing with TBS and TBS-T, the membranes were incubated for 1 h at RT with an infrared labeled secondary antibody goat anti-rabbit IRDye 800 (Li-Cor, Lincoln, NE) to bind to the primary antibody. The bound complex was detected using the Odyssey Infrared Imaging System (Li-Cor). The images were analyzed using the Odyssey Application Software (Image Studio) to obtain the integrated intensities.

Measurement of cellular oxidative markers by Western blot

Fifty microgram aliquots of each sample, combined with an XT Reducing Agent (BioRad), were resolved on 4–12% Bis-Tris gels

FIG. 2. Representative CaG5N spectrophotometer traces (A) illustrate differences to increasing Ca²⁺ load between isolated cortical mitochondria from the experimental groups at 24 h post-TBI. The inset (A) illustrates how the traces were analyzed. Evaluation of the CaG5N traces for the time to (B) reveal a slight decrease in the time to MPT, however, PZ treatment had no effect. Additional analysis of CaG5N traces (C) reveal a significant decrease in Ca²⁺ buffering capacity (increased CB slope) in isolated cortical mitochondrial from vehicle-treated animals compared with sham (**p* < 0.05) and PZ administration appeared to partially restore the CB capacity back down to within sham levels. PZ, phenelzine; TBI, traumatic brain injury.

and transferred as described above. The membranes were incubated with the following antibodies: rabbit anti-4-HNE (ADI; 1:2000) or rabbit anti-acrolein (Abcam; 1:2000) in combination with a second primary antibody, mouse anti- α -tubulin (Abcam, Cambridge, MA; 1:10,000) in TBS-T with 5% milk. After washing, the membranes were incubated with infrared labeled secondary antibodies goat anti-rabbit IRDye 800 and goat anti-mouse IRDye 680 (Li-Cor) to bind to the primary antibodies. The images were obtained using the Li-Cor and analyzed using Image Studio (as described above).

Measurement of α II-spectrin degradation by Western blot

For evaluation of the 150 and 145 kD spectrin breakdown products, 10- μ g aliquots of each sample were used. Prepared cortical and hippocampal tissue samples were combined with an XT Reducing Agent (BioRad), resolved on 3–8% Tris-Acetate gels and transferred as described above. The membranes were incubated with a mouse anti- α -spectrin antibody (ENZO; 1:5000) in combination with a rabbit β -tubulin (Abcam; 1:10,000) in TBS-T with 5% milk. After washing, the membranes were incubated with infrared labeled secondary antibodies goat anti-rabbit IRDye 800 and goat anti-mouse IRDye 680 (Li-Cor) to bind to the primary antibodies. The images were again obtained using the Li-Cor and analyzed using Image Studio (as described above).

Statistical analysis

Statistical analysis was performed with Prism version 7.0 (Graph Pad, San Diego, CA). The data were analyzed using an initial one-

way analysis of variance (ANOVA) followed by Student-Newman-Keuls (SNK) or Tukey's *post hoc t* tests. For all data sets, a *p*-value of <0.05 was defined as statistically significant. For all ANOVAs, if the Brown-Forsythe test showed significant differences in variance, then the Mann-Whitney (M-W) test was used to specifically look at the pharmacological effect of PZ compared with the vehicle for each time-point. Quantitative analysis revealed significant differences among groups for multiple outcome measures in this article; however, the emphasis of graphical representation was designed to highlight the statistically significant differences of the pharmacological treatment paradigm(s); therefore, significant differences as compared with the sham group are mostly noted in the Results section, and not annotated on the graphs. All data for graphical representation are expressed as mean $\pm$ standard deviation (SD) (ANOVA) or median $\pm$ 95% confidence interval (CI) (M-W).

Results

Acute administration of PZ preserves mitochondrial function out to 72 h following TBI

Following TBI, secondary injury mechanisms such as the process of LP, results in the formation of aldehydes such as 4-HNE and acrolein. We have previously demonstrated that the accumulation of these aldehydes in both purified mitochondrial and total cellular populations peaks at 72 hpi.⁷ Pre-treatment of isolated cortical mitochondria with PZ prior to 4-HNE exposure *in vitro* could protect mitochondria against aldehyde-mediated damage. In

FIG. 3. Representative TMRE spectrophotometer traces (A) illustrate differences between the experimental groups in the quenching of this fluorescence dye resulting from changes in mitochondrial membrane potential (MP) for cortical mitochondrial samples isolated at 24 h post-TBI. The inset (A) illustrates trace analysis parameters. Analyses of the TMRE traces reveal Ca²⁺-independent (B) and Ca²⁺-dependent changes (C) in mitochondrial MP. The total MP (B; difference from the lowest MP at baseline to maximum MP with the addition of oligomycin) showed no significant difference between the groups. Quantification of the TMRE fluorescence during Ca²⁺ infusion revealed a significant decline for the vehicle-treated group, and the PZ-treated group was significantly improved by comparison (C; * $p < 0.05$). PZ, phenelzine; TBI, traumatic brain injury; TMRE, tetra-methyl rhodamineethyl ester.

addition, in an *in vivo* TBI model, a single dose of PZ given at 15 min post-injury, was shown to have protective effects at 3 hpi.⁴⁶

The goal of these experiments was to determine if additional dosing of PZ would increase the protective effect on mitochondrial bioenergetics beyond 3 hpi and to assess the therapeutic window of administration. Isolated cortical mitochondrial respiratory function was assessed in animals at 24 and 72 h following a severe TBI. Specifically, the addition of pyruvate and malate to initiate State II (Fig. 3A) was significantly reduced in vehicle-treated samples at both 24 and 72 hpi compared with sham animals ($p < 0.001$), which was improved in PZ-treated animals at both time-points and delayed administration of PZ still showed a protective effect at 72 h (ANOVA; $F = 35.8$; $df = 5, 78$; $p < 0.001$). Quantification of maximal State III rates, initiated with the addition of ADP (Fig. 3B), revealed a significant deficit in vehicle-treated animals at 24 and 72 h ($p < 0.001$), and PZ-treated animals, including the delayed dosing group, showed significantly higher rates (ANOVA; $F = 34$; $df = 5, 78$; $p < 0.001$). State IV, initiated by oligomycin (ATP synthase inhibitor; Fig. 3C), showed significant improvement in PZ-treated compared with vehicle-treated animals at 24 hpi (M-W, $U = 28$; $HL\Delta = 11$; $n_1 = n_2 = 12$; $p = 0.01$ [two-tailed]) and at 72 hpi (M-W, $U = 38$; $HL\Delta = 8.3$; $n_1 = n_2 = 16$; $p < 0.001$ [two-tailed]). Quantification of maximal State V (Complex I; FCCP-driven; Fig. 3D) oxygen consumption showed no significant improvement at 24 hpi; however, there was significant improvement in PZ-

treated compared with vehicle-treated animals at 72 hpi (M-W, $U = 59$; $HL\Delta = 69.3$; $n_1 = n_2 = 16$; $p = 0.008$ [two-tailed]) and delayed administration of PZ maintained the significant improvement (M-W, $U = 8$; $HL\Delta = 94.2$; $n_1 = 16$, $n_2 = 4$; $p = 0.021$ [two-tailed]). The addition of rotenone, a Complex I inhibitor, followed by the addition of succinate, allows for Complex II-driven State V. Maximal State V (Complex II) rates (Fig. 3E) were not significantly improved at 24 hpi; however, there was significant improvement in PZ-treated compared with vehicle-treated animals at 72 hpi (M-W, $U = 69$; $HL\Delta = 24$; $n_1 = n_2 = 16$; $p = 0.026$ [two-tailed]) and delayed administration of PZ maintained the significant improvement (M-W, $U = 7$; $HL\Delta = 25.8$; $n_1 = 16$, $n_2 = 4$; $p = 0.016$ [two-tailed]). The RCR is an index of how well the electron transport chain (ETC) is coupled to oxidative phosphorylation. Analysis of calculated RCRs (Fig. 3F) shows a significant reduction in the 24 ($p < 0.05$) and 72 h ($p < 0.001$) vehicle-treated groups, which was recovered in the PZ-treated animals, including the delayed dosing group, at 72 hpi (ANOVA; $F = 9.71$; $df = 5, 78$; $p < 0.001$).

PZ treatment partially restores mitochondrial Ca²⁺ buffering capacity and the ability of damaged mitochondria to maintain membrane potential

Following TBI, damaged mitochondria lose their ability to buffer cytosolic or extra-mitochondrial Ca²⁺. We had previously

demonstrated that scavenging lipid peroxyl radicals ($\text{LOO}^\bullet$) with the LP-inhibitor U-83836E had a protective effect on CB capacity at 3 h post-TBI in mice.¹² Therefore, the second set of experiments were undertaken to evaluate the potential protective effect of PZ administration on mitochondrial CB capacity and MPT as well as its ability to preserve MP at 24 h after TBI in rats. In a small set of pilot experiments, we determined that mitochondrial function at 72 h was too impaired to effectively evaluate CB (data not shown); therefore, 24 hpi was the only time-point evaluated. The CaG5N traces (Fig. 1A) were evaluated (as illustrated in inset) for differences in the slope of CB and the time to MPT as extra-mitochondrial Ca^{2+} increased. The time to MPT was reduced in injured animals; however, the deficit was not significant, and PZ did not appear to have a protective effect (Fig. 2B). Comparisons of how much Ca^{2+} the mitochondria could buffer as a function of time (CB slope: $\Delta\text{CaG5N}/\Delta\text{time}$) revealed a significant increase in the vehicle-treated animals ($p < 0.05$) that was partially restored in PZ-treated animals (Fig. 2C; ANOVA; $F = 4.88$; $df = 2, 15$; $p = 0.0233$).

The TMRE traces were evaluated (Fig. 2) for Ca^{2+} -independent changes in MP prior to initiation of infusion. There was no difference in the ADP-dependent change in MP between the groups (data not shown), nor was there a significant change in the total amount of MP between sham or injured mitochondria, as calculated from the difference between the lowest MP at baseline and the highest MP following the addition of oligomycin (Fig. 2B). Importantly, however, injured mitochondria showed a reduction in the difference between the MP generated by the addition of P/M and the loss of MP resulting from the addition of ADP, and PZ was able to partially restore this deficit (data not shown; $p < 0.05$). Following the initiation of Ca^{2+} infusion, the Ca^{2+} -dependent changes in MP were evaluated for changes in TMRE fluorescence over time (AUC). These Ca^{2+} -dependent changes in MP for vehicle-treated mitochondria were significantly lower compared with sham ($p < 0.01$; not annotated on graph), and PZ treatment was able to significantly improve the maintenance of MP compared with the vehicle-treated group ($p < 0.05$; Fig. 2C) (ANOVA; $F = 7.62$; $df = 2, 15$; $p = 0.0052$).

PZ administration protects cortical mitochondria from LP-mediated oxidative damage after TBI

Previous work had shown that increased levels of the reactive aldehydes 4-HNE and acrolein *in vitro* have a dose-dependent inhibitory effect on mitochondrial respiratory function.^{46,47} In addition, acute administration of the LP-inhibitor U-83836E was able to attenuate 4-HNE and 3-NT levels in mitochondrial protein samples at 3 h post-TBI in mice.¹² Therefore, we sought to determine if improvements seen in mitochondrial function following PZ administration *in vivo* (Fig. 3) resulted from reductions in the accumulation of 4-HNE and acrolein in mitochondrial protein samples. The effect of PZ was analyzed by Western blot analysis on the accumulation of 4-HNE and acrolein in the same mitochondrial samples at 24 and 72 hpi as an index of LP-mediated oxidative damage in the ipsilateral cortical mitochondria. The accumulation of 4-HNE and acrolein in isolated cortical mitochondria at 24 and 72 h after CCI-TBI is demonstrated in the representative blots at the top of the figure (Fig. 4A,C). *Post hoc* statistical analysis (Tukey's) revealed a significant increase in 4-HNE accumulation in vehicle-treated animals (Fig. 4B; [M-W, $U = 0$; $HL\Delta = 1.47$; $n_1 = 18$, $n_2 = 12$; $p < 0.001$ (two-tailed)]; data not annotated on graph) that was significantly reduced by PZ administration at 72 hpi ($p < 0.01$), including the PZ_{delay} group ($p < 0.05$) (ANOVA; $F = 6.95$; $df = 2$,

25; $p = 0.004$). *Post hoc* analysis also revealed a significant increase in acrolein accumulation in vehicle-treated animals at 72 hpi compared with sham (Fig. 4D; [M-W, $U = 0$; $HL\Delta = 1.09$; $n_1 = n_2 = 12$; $p < 0.001$ (two-tailed)]; data not annotated on graph). Acute and delayed administration of PZ was able to significantly lower the accumulation of acrolein at 72 hpi compared with the vehicle-treated group ($p < 0.001$) (ANOVA; $F = 12.9$; $df = 2, 25$; $p < 0.001$).

Published findings have suggested the importance of the additive contribution to tissue damage by multiple aldehydes following injury, termed "aldehyde load."^{54,55} Therefore, for the sake of analysis, the cumulative OD values for both 4-HNE and acrolein levels were presented as a representation of this (Fig. 4E, graph on left) as well as the percent contribution by either aldehyde to that total load (Fig. 4E, table on right). At 24 and 72 hpi, *post hoc* analysis (SNK) found the total aldehyde load was significantly increased in all injury groups ($p < 0.05$, $p < 0.01$, $p < 0.001$; Fig. 4E, graph on left) (ANOVA; $F = 51.1$; $df = 4, 37$; $p < 0.001$). There was a significant temporal increase in total aldehyde load between 24 and 72 h vehicle-treated aldehyde load levels ($p < 0.001$), which is in agreement with previous findings.⁷ PZ administration significantly reduced the cumulative aldehyde load at 72 hpi ($p < 0.001$). The percent (%) of the load carried by acrolein at 24 hpi was significantly greater compared with the percent (%) carried by 4-HNE ($p < 0.01$, $p < 0.001$), as revealed by *post hoc* analysis (Tukey's), but by 72 hpi the aldehyde load was relatively balanced (two-way ANOVA; significant interaction effect: $F = 18$; $df = 4, 74$; $p < 0.001$; significant aldehyde [column] effect: $F = 24.2$, $df = 1, 74$, $p < 0.001$). Overall, the results show injury-dependent increases in LP-derived oxidative damage to mitochondrial proteins, as previously shown,⁷ with PZ treatment significantly reducing the amount of 4-HNE and acrolein at 72 hpi, which are in agreement with the protective effects of PZ on mitochondrial respiratory dysfunction (Fig. 3), and delaying the administration of PZ did not significantly alter this reduction in aldehyde accumulation. Moreover, the total aldehyde load was found to be significantly decreased at 72 hpi by PZ.

LP-mediated oxidative damage to cortical and hippocampal tissue and PZ-mediated protective effects

Previous experiments performed in our lab have demonstrated cellular protection against LP-mediated oxidative damage 3 h after TBI using U-83836E in mice¹² and 24 h after SCI using the nitroxide antioxidant tempol in rats.³ Additionally, we knew that acute administration of PZ could protect mitochondrial proteins from LP-mediated oxidative damage. So, another set of experiments was carried out to determine if our acute PZ dosing paradigm could protect cellular proteins from the detrimental formation of 4-HNE and acrolein adducts. Cortical and underlying ipsilateral hippocampal tissue homogenates from these sets of sham and injured animals were sacrificed at 24 and 72 h after CCI-TBI and were used for quantitative measurements of LP: 4-HNE and acrolein. The accumulations of 4-HNE (Fig. 5A) and acrolein (Fig. 5D) are demonstrated in the representative blots (only cortical sample blots shown). Western blot quantification revealed that as early as 24 hpi 4-HNE levels in the injured cortex and hippocampus of vehicle-treated animals (Fig. 5B) were elevated and increased significantly by 72 hpi compared with sham levels ($p < 0.05$). PZ treatment was unable to significantly lower 4-HNE levels at either time-point in either cortical (ANOVA; $F = 5.27$; $df = 4, 25$; $p = 0.003$) or hippocampal samples (hippocampal data not shown; ANOVA; $F = 2.85$;

FIG. 4. PZ treatment reduces the amount of injury-dependent increases in LP-related accumulation of 4-HNE- and acrolein-modified mitochondrial proteins at 72 h after TBI. This finding is in agreement with the protective effects of PZ on mitochondrial respiratory dysfunction at the same time-point (Fig. 3). Representative Western blots demonstrate differences in the accumulation of 4-HNE (A) and acrolein (C) in mitochondrial proteins. Quantification of 4-HNE bands between 150 and 75 kD reveals significant elevation in 4-HNE accumulation by 72 h post-TBI that is significantly reduced by PZ (B; $**p < 0.01$). Delayed administration of PZ also significantly reduced the amount of 4-HNE accumulation at 72 h following TBI compared with 72-h vehicle-treated animals ($*p < 0.05$). Quantification of acrolein bands between 150 and 75 kD reveal significant elevation in acrolein accumulation at both 24 and 72 h post-TBI that was significantly reduced by PZ compared with vehicle-treated animals at 72 h (D; $**p < 0.01$). Delayed administration of PZ also significantly reduced the amount of acrolein accumulation at 72 h following TBI compared with 72-h vehicle-treated animals ($***p < 0.01$). Cumulative levels of mitochondrial 4-HNE + acrolein accumulation (E), referred to as aldehyde load, were significantly elevated in all injury groups ($*p < 0.05$, $**p < 0.01$, $***p < 0.001$) compared with sham. Aldehyde levels were significantly elevated in vehicle-treated animals at 72 h compared with 24 hrs ($^{***}p < 0.001$). Whereas PZ administration reduced accumulation of aldehydes at 24 h, the reduction was not significant until 72 h post-TBI ($^{####}p < 0.001$). The table of cumulative mitochondrial aldehyde load percentages shows acrolein carrying a significantly greater percentage at 24 h that is offset by increasing levels of 4-HNE by 72 h post-TBI ($^ap < 0.01$; $^bp < 0.001$). 4-HNE, 4-hydroxynonenal; LP, lipid peroxidation; PZ, phenelzine; TBI, traumatic brain injury.

df=4, 25; $p=0.045$). Western blot quantification revealed that acrolein levels in the injured vehicle-treated cortex were significantly elevated at 24 ($p < 0.05$) and 72 h ($p < 0.001$) compared with sham (Fig. 5E; ANOVA; $F=6.32$; df=4, 25; $p=0.001$). In agreement with our previous findings,⁷ there was a significant temporal increase in the levels of acrolein accumulation in the vehicle-treated cortical samples at 72 h compared with the levels seen in the vehicle-treated samples at 24 hpi ($p < 0.05$) and in the hippocampus, acrolein levels were significantly elevated by 72 hpi compared with sham animals ($p < 0.01$) (ANOVA; $F=3.64$; df=3, 68; $p=0.017$). Unfortunately, similar to the insufficient effect of PZ-treatment on lowering levels of 4-HNE (shown in Fig. 5B), PZ-treatment was unable to significantly reduce acrolein accumulation either (shown in Fig. 5E).

As shown in Figure 4E, the “aldehyde load” for both 4-HNE and acrolein levels (i.e., cumulative OD values) were presented for cortical (Fig. 5C, graph) and hippocampal (Fig. 5F, graph) tissues samples. The percent (%) contribution by either aldehyde to the total load for cortical (Fig. 5C, table) and hippocampal (Fig. 5F,

table) samples are shown as well. At 24 and 72 hpi, *post hoc* analysis (SNK) found the total aldehyde load in cortical tissue samples was significantly increased in vehicle-treated groups compared with sham ($p < 0.05$, $p < 0.01$, $p < 0.001$); however, although PZ administration was able to reduce these levels, they were not significantly lower than either time-points’ respective vehicle-treated group (Fig. 5C, graph on top; ANOVA; $F=6.7$; df=4, 25; $p < 0.001$). For the cortical samples, the aldehyde load appears to be relatively evenly balanced between 4-HNE and acrolein contributions (Fig. 5C, table below), as no significant differences were found by *post hoc* analysis (Tukey’s) even after TBI, (two-way ANOVA; significant interaction effect: $F=3$; df=4, 50; $p=0.027$).

In the underlying ipsilateral hippocampus (Western blot images not shown), *post hoc* analysis (SNK) found the total aldehyde load at 72 hpi was significantly increased in vehicle-treated groups as compared with sham ($p < 0.01$), and although PZ administration was able to reduce it, it was not significantly lower than the vehicle-treated group (Fig. 5F, graph on top; ANOVA; $F=3.8$; df=4, 25;

FIG. 5. The administration of PZ does not significantly lower the accumulation of 4-HNE- and acrolein-modified cellular proteins at 24 or 72 h after TBI. Representative Western blots (cortical samples shown) demonstrate differences in the accumulation of 4-HNE (A) and acrolein (D) in total cellular protein samples. Quantification of 4-HNE bands between ~120 and 60 kD was used to find injury- and PZ-dependent changes in cortical samples. At 72 h post-injury (hpi), there was a significant elevation in 4-HNE accumulation in vehicle-treated animals compared with sham animals (B; $*p < 0.05$); however, there was no significant reduction in PZ-treated cortical samples. Quantification of acrolein bands between 100 and 60 kD was used to find injury- and PZ-dependent changes in cortical samples. At 24 hpi, there was a significant elevation in cortical acrolein levels in vehicle-treated animals and PZ-treated animals (E; $*p < 0.05$). At 72 hpi, there was a significant elevation in cortical acrolein accumulation in vehicle-treated and PZ-treated animals compared with sham animals ($**p < 0.01$, $***p < 0.001$). Whereas PZ was able to reduce the levels of acrolein, the reduction compared with the vehicle group was insignificant. Cumulative levels of 4-HNE + acrolein accumulation, or aldehyde load, were significantly elevated in cortical protein samples (E) for both vehicle-treated groups ($*p < 0.05$, $***p < 0.001$) compared with sham and PZ administration was successful at lowering these levels; however, the reduction was insignificant at either time-point. The corresponding table of percentages for cortical cumulative aldehyde load shows a relatively even contribution from both aldehydes for all experimental groups. The aldehyde load in the hippocampal protein samples (F) (Western blots not shown) was not significantly elevated until 72 hpi ($**p < 0.01$), and although PZ administration was able to reduce the accumulation, it was insignificant. The corresponding table of percentages for cumulative hippocampal aldehyde load shows higher amounts of acrolein in sham animals ($^b p < 0.001$) that settles to a relatively even contribution from both aldehydes for all experimental TBI groups. 4-HNE, 4-hydroxynonenal; PZ, phenelzine; TBI, traumatic brain injury.

$p = 0.015$). The percent (%) of the load carried by acrolein in the hippocampus of sham animals was significantly greater compared with the percent (%) carried by 4-HNE ($p < 0.001$) as revealed by *post hoc* analysis (Tukey's), but after injury, the aldehyde load appears relatively balanced between 4-HNE and acrolein contributions (Fig. 5F, table below), as no significant differences were found by *post hoc* analysis (Tukey's) (two-way ANOVA; significant interaction effect: $F = 6.01$; $df = 4, 50$; $p < 0.001$; significant aldehyde [column] effect: $F = 48$; $df = 1, 50$; $p < 0.001$).

PZ treatment lowers calpain and caspase-3 mediated-spectrin degradation

Intracellular Ca^{2+} overload resulting from PN-mediated oxidative damage and mitochondrial dysfunction activates the protease calpain. Calpain activation results in proteolytic cleavage of the neuronal cytoskeletal protein α -spectrin forming two fragments (145 kD and 150 kD) from the full length (280 kD). The 145 kD α -spectrin breakdown product (SBDP) is generated specifically by

calpain and the SBDP 150 is generated by both calpain and caspase 3-mediated proteolysis. It has been previously determined that calpain-mediated cytoskeletal damage temporally coincides with increased oxidative damage at both the cellular and mitochondrial level.⁷ In an effort to link pharmacological reduction in oxidative damage with pathological calpain activation and spectrin degradation, we looked to see if our PZ-treatment altered calpain-mediated cytoskeletal damage. Cortical and hippocampal tissue homogenates were assessed for these SBDPs at 24 and 72 h after CCI-TBI as shown in the representative blot (Fig. 6A). There were no significant differences at 24 or 72 hpi for either the 150 or the 145 kD SBDPs between vehicle- or PZ-treated animals (Fig. 6B,C; 24-h ANOVA; $F=2.36$; $df=3, 20$; $p=0.102$; 72-h ANOVA;

$F=2.19$; $df=3, 20$; $p=0.121$). Although the effect of PZ was insignificant, there was a dramatic reduction in the levels of spectrin degradation at 24 hpi for both the 150 and 145 kD fragments (Fig. 6B); this effect did not carry out to 72 hpi (Fig. 6C). Additionally, there was no reduction in the levels of SBDPs in either vehicle- or PZ-treated animals in hippocampal samples (data not shown; 24-h ANOVA; $F=0.725$; $df=3, 20$; $p=0.549$; 72-h ANOVA; $F=1.21$; $df=3, 20$; $p=0.333$). In summary, coincident with the above-described protective effects of PZ administration on mitochondrial respiratory failure and LP mitochondrial and cellular oxidative damage, there was an injury-induced increase in calpain-mediated degradation of the neuronal cytoskeletal protein α -spectrin that was diminished by PZ administration at 24 hpi, indicative of neuronal preservation; however, the effect was not sustained out to 72 hpi.

Discussion

Mitochondria and oxidative stress/damage

Following TBI the primary mechanical insult is closely followed by an acute increase in free-radical production (ROS and reactive nitrogen species [RNS]) and activation of an array of secondary injury processes. The contribution of secondary oxidative damage and subsequent mitochondrial dysfunction to pathophysiological and neurodegenerative mechanisms has been well established in several models of traumatic CNS injury.^{1–13,56} Mitochondria are particularly susceptible to free radical or oxidative damage as they intrinsically produce free radicals as a by-product of oxidative metabolism (leakage from the ETC), in addition to the intracellular accumulation of free radicals (ROS/RNS) from extramitochondrial sources. One of the most validated secondary injury mechanisms occurs through the LP process resulting from increased free-radical production, specifically PN, following CNS injury.^{8,14–18} LP-mediated damage has been closely linked to mitochondrial dysfunction post-TBI.⁵⁷ In addition, PN-derived radicals can react with susceptible amino acids (e.g., cysteine, lysine, arginine) causing protein oxidation and resulting in the formation of protein carbonyls, also elevated following CNS injury. Finally, the PN radical nitrogen dioxide ($\bullet\text{NO}_2$) can react with aromatic amino acid residues, primarily tyrosine moieties, resulting in the formation of 3-nitrotyrosine (3-NT)⁵⁸ thereby interrupting crucial protein and enzymatic functionality, also elevated following CNS injury.^{7,16}

Pharmacological interventions targeting sources of PN-mediated damage have clearly demonstrated its role in both mitochondrial dysfunction and cellular pathology following injury. Pharmacological strategies include various approaches such as: (1) the inhibition of the initiation of LP by reducing the formation of free radicals (i.e., NOS inhibitors)¹⁹; (2) scavenging free radicals using anti-oxidants such as vitamin E^{19,59,60} or tempol^{3,17,61}; (3) the inhibition of the LP process by binding lipid radicals using tirilazad^{62,63} or U-83836E^{12,13,64}; and finally, (4) the scavenging of reactive aldehydes to reduce protein carbonyl formation using PZ,^{46–48} hydralazine,^{41,65} or penicillamine.¹⁸ Over the past few years, our approach has been aimed at these last two points of intervention. Removal/sequestration of reactive aldehydes seems to play a more pivotal role in mitochondrial protection as compared with pharmacological anti-oxidant therapeutic approaches as they have been found to be more clinically relevant because the therapeutic window for anti-oxidant interventions is very narrow as the post-traumatic oxidative “burst” occurs very rapidly after TBI. Although there are a number of interventional strategies that are

FIG. 6. The administration of PZ does not significantly lower the spectrin breakdown products (SBDP) at 24 or 72 h after TBI. Representative Western blot (A) demonstrates differences in the 150 kD and 145 kD SBDP at 24 (B) and 72 h post-injury (hpi) (C) in cortical cellular protein samples. Quantification of SBDP was used to identify injury- and PZ-dependent changes in cortical samples. At both 24 and 72 hpi, there were significant elevations in cortical spectrin breakdown compared with sham levels (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). PZ administration partially protected cortical tissue from injury-dependent spectrin breakdown at 24 hpi by reducing the levels back down to within sham levels (150 kD); however, this effect did not persist out to 72 hpi. PZ, phenelzine.

being investigated for the therapeutic uses of aldehyde scavenging in the context of neurological disorders and trauma, there are still many unanswered questions. The results from this study further our understanding as to the effective use of PZ as a pharmacological neuroprotective strategy against the detrimental effects of LP-mediated oxidative damage following TBI in young adult male rats.

PZ and mitochondrial protection

In recent years, the use of PZ as a neuroprotectant has proven effective in a number of animal models including TBI,^{46,47,66} SCI,⁴⁸ ischemic-reperfusion,⁵⁵ and experimental autoimmune encephalomyelitis (EAE).^{67,68} PZ, a well-known MAO inhibitor, has also been shown to exert its neuroprotective effect through the covalent binding of its hydrazine moiety (-NH-NH₂) to the carbonyl functional group (R-CHO) of reactive LP-derived aldehydes 4-HNE and acrolein in a dose-dependent manner⁴⁶ as well as 3-AP (refer to section on polyamine metabolism).⁵⁵ In our previous work, we had shown that a single dose of PZ at 15 min post-TBI slightly, but insignificantly, increased the rate of O₂ consumption in the presence of ADP (State III respiration) and did not alter State IV respiration (in the presence of oligomycin) in cortical mitochondria isolated at 3 hpi; however, the ratio of these two rates, the RCR, was significantly improved in the PZ groups compared with the vehicle-treated group.⁴⁶ Additionally, we tested a multi-dosage paradigm of PZ with repeated dosing every 12 h, and mitochondrial respiratory function was assessed at 72 h.⁴⁷ As with the acute study, we only found an increase in the RCR, whereas neither State III or State IV respiration were individually improved by PZ using this paradigm (personal communication; unpublished data); however, there was a reduction in mitochondrial 4-HNE. Although these results were somewhat disappointing, given the promising results from our previous *in vitro* work,^{46,47} clearly demonstrating PZ's effectiveness at protecting mitochondria from aldehyde-mediated damage, we continued to pursue the use of PZ as a therapeutic neuroprotectant. Therefore, this current set of experiments was carried out in an attempt to determine a more optimal dosing paradigm for improving mitochondrial function at 24 h. For these studies, we chose to look at both the 24- and 72-h time-points to determine if the effect of PZ on mitochondrial protection observed at 24 hpi was maintained out to 72 hpi when mitochondrial function is even more impaired.⁷

The animals were administered an initial s.c. dose of PZ at 15 min post CCI-TBI at 10 mg/kg followed by a maintenance dose at 12 h at 5 mg/kg. We chose to use this dosing paradigm based upon our previous work with this drug. We found that at 24 hpi, two doses of PZ were able to restore mitochondrial respiratory deficits to within sham levels. As previously observed, mitochondrial respiratory dysfunction significantly declined at 72 hpi compared with 24 hpi for all states of respiration, including RCR (see Fig. 3; stats not illustrated, ANOVA, $p < 0.05$ – $p < 0.001$). Although the two doses of PZ at 15 min and 12 h were able to significantly improve respiratory function over vehicle-treated levels, PZ treatment was not able to maintain the near complete preservation of mitochondrial bioenergetics observed at 24 h. Specifically, when we look at State II for example: the acute administration of PZ elicited a 32% improvement in O₂ consumption rates at 24 hpi; similarly, there was a 30% improvement in the PZ-treated group at 72 hpi compared with vehicle. This trend was consistent for most of the mitochondrial respiratory data (see Fig. 3). These findings suggest that the therapeutic protective effect of PZ observed at 24 h, in terms of the amount of preservation of most

TABLE 2. PZ PRESERVATION OF MITOCHONDRIAL FUNCTION

	PZ Preservation of Mito Function (%)		
	24 hr Veh vs PZ	72 hr Veh vs PZ	72 hr Veh vs PZ _{delay}
State II	32%	30%	21%
State III	22%	27%	23%
State IV	27%	21%	12%
State V (I)	21%	18%	20%
State V (II)	13%	19%	21%
RCR	7%	15%	23%

PZ, phenelzine; RCR, respiratory control ratio.

states of mitochondrial respiratory function, was maintained out to 72 h (see Table 2).

Another possibility is that the PZ dosing is able to maintain and/or preserve a certain percentage and/or population of the mitochondria within the injury site and penumbra, and that these preserved mitochondria still remain at 72 hpi. We have found that when the synaptic mitochondria were separated from the non-synaptic population, the synaptic mitochondrial respiratory function was significantly reduced compared with the non-synaptic mitochondria at 24 hpi.³⁵ In this same study, we also looked at 72 h after TBI and found that in addition to the respiratory function of *both* populations being significantly reduced the non-synaptic mitochondrial population was no longer functioning significantly better compared with the synaptic mitochondria. In addition, the synaptic mitochondrial proteins show higher levels of aldehyde accumulation by 72 hpi and, therefore, are more susceptible to LP-mediated damage at this later time-point. In light of these results, it is therefore possible that in the context of the current study, acute dosing of PZ may be preferentially protective of one of these populations over the other. Our working hypothesis is that because synaptic mitochondria carry a larger percentage of the aldehyde load following TBI,³⁵ they are going to benefit the most from the aldehyde scavenging capability of PZ administration. More studies are currently underway to test this hypothesis and to determine whether PZ is indeed preferentially more protective for the synaptic mitochondrial population.

Mitochondrial Ca²⁺ homeostasis and aldehyde-mediated alterations

Mitochondria play an important role in the maintenance and regulation of intracellular Ca²⁺ homeostasis by storing and releasing Ca²⁺ using various mechanisms.^{69–77} Following TBI, intracellular ion concentrations are altered, with Ca²⁺ levels rising rapidly.^{74,78–80} Mitochondria absorb the increased Ca²⁺ influx thereby serving as Ca²⁺ “sinks” in an attempt to maintain homeostatic cytosolic Ca²⁺ levels after injury.^{74,81} The mPTP in the inner mitochondrial membrane is triggered to open as the concentration of Ca²⁺ within the mitochondrial matrix rises.⁸² Once this pore opens, the mitochondria can no longer contain or “buffer” the sequestered Ca²⁺, resulting in release of the accumulated Ca²⁺, as well as ROS and other pro-apoptotic substances, back into the cytosol.^{83–86} In addition, as mitochondria take up excessive Ca²⁺ mitochondrial nitric oxide synthase (mtNOS) is activated, resulting in the overproduction of the nitric oxide radical (•NO) and with O₂[•] leakage from complex I, eventually leading to the formation of PN (ONOO[•]) (see Fig. 7). PN-induced oxidative damage and accompanying mitochondrial dysfunction^{2–4} further contribute to

FIG. 7. Diagram illustrating (A) the hallmarks of LP-mediated oxidative damage following CNS injury and (B) the presumptive mitochondrial and neuroprotective effects of acute phenelzine (PZ) administration. In an untreated animal, injury to the CNS (TBI/SCI) causes an increase in intracellular Ca^{2+} and mitochondrial Ca^{2+} uptake, which stresses the mitochondria and causes activation of mitochondrial nitric oxide synthase (mtNOS) resulting in the production of peroxynitrite (PON; ONOO^-) and the generation of the highly reactive free radicals: hydroxyl radical ($\bullet\text{OH}$), nitrogen dioxide ($\bullet\text{NO}_2$), and carbonate radical ($\bullet\text{CO}_3$). These free radicals interact with mitochondrial and cellular membranes thereby initiating the process of lipid peroxidation (LP). LP-mediated oxidative damage interferes with normal mitochondrial and cellular functionality leading to reduced mitochondrial Ca^{2+} buffering capacity and release of Ca^{2+} back into the cytoplasm; damage to the ATPase within the cell membrane compromises the ability of the cell to extrude cytoplasmic Ca^{2+} further exacerbating post-traumatic intracellular Ca^{2+} overload. Increased Ca^{2+} activates cytoplasmic calpain initiating proteolysis of cytoskeletal proteins (e.g., spectrin in neurons). The administration of PZ (B) has been shown in CNS injury models (TBI/SCI) to interfere with LP-mediated oxidative damage, thereby eliciting various protective effects on mitochondrial and cellular integrity and functionality. CNS, central nervous system; PZ, phenelzine; SCI, spinal cord injury; TBI, traumatic brain injury.

Ca^{2+} dysregulation that eventually leads to activation of cell death mechanisms.⁸⁷

Post-traumatic mitochondrial Ca^{2+} overload results in increased levels of mitochondrial ROS.^{88–91} In addition, aldehyde exposure *in vitro* has also been shown to increase both ROS production⁹² and intracellular Ca^{2+} levels.^{92–94} Overproduction of ROS initiates LP-mediated excessive aldehyde accumulation after TBI resulting in intracellular Ca^{2+} build-up that subsequently increases the Ca^{2+} load on mitochondria. In addition, mitochondrial and cellular membrane integrity is compromised by aldehyde-adduct-mediated permeabilization resulting in loss of mitochondrial membrane potential.^{95–98} In the current study, the partial restoration of CB capacity (Fig. 1) and MP (Fig. 2) demonstrates that scavenging aldehydes acutely after TBI with PZ can improve mitochondrial membrane integrity and Ca^{2+} homeostasis acutely. After several failed attempts to perform the CB/MP assay(s) on cortical mitochondria isolated from animals at 72 hpi, we decided to terminate the experiments as the mitochondrial buffering capacity was too low to reliably quantify (data not shown). This was somewhat expected as we had previously observed significant mitochondrial respiratory dysfunction at 72 hpi.⁷ Further investigation of the ef-

fect(s) of PZ on CB and MP when the mitochondrial functionality is higher, maybe 36–48 h, would provide a clearer idea as to the persistence of the preservation elicited by PZ administration beyond the current 24-h window.

Mitochondrial membrane susceptibility to LP and aldehyde generation

Cells within the brain are subjected to increased levels of oxidative damage not only because of their high demand for energy and oxygen consumption,⁹⁹ but also because of the high levels of polyunsaturated fatty acids (PUFAs) that make up membrane phospholipids. Post-traumatic increases in cytosolic Ca^{2+} can induce activation of phospholipase A_2 (PLA_2), which mediates PUFA release from the membrane. PUFAs such as arachidonic acid (AA), linoleic acid (LA), and docosahexaenoic acid (DHA) are highly susceptible to attack by free radicals (ROS/RNS) because hydrogen atoms at their bis-allylic carbons are readily extractable making them vulnerable to LP. Additionally, the oxidizability (k_p ; rate constant) of PUFAs varies with AA and DHA being among the more oxidizable, therefore, more vulnerable.¹⁰⁰ Upon interaction

with ROS/RNS, these PUFAs undergo LP to generate the reactive aldehydes 4-HNE and acrolein, which interfere with mitochondrial and cellular membrane integrity and functionality.

Mitochondria are unique in that their inner membranes exclusively contain the phospholipid cardiolipin (CL).^{101–103} The unique structure of CL with four fatty acyl chains makes it a primary target for ROS-mediated LP. Tyurina and colleagues¹⁰⁴ recently demonstrated that brain CL contains a relatively equal abundance of LA and AA, with DHA in distant third (at about 20% the abundance of the other two PUFAs). Interestingly, acrolein is an LP end-product formed from AA, and at 40-fold greater as compared with formation of 4-HNE; however, 4-HNE can also be formed from LA and linolenic acid.¹⁰⁵ Although the phospholipid source of 4-HNE and acrolein detected by WB after injury cannot be determined from our results, the increased susceptibility of CL makes it a prime suspect in overall mitochondrial susceptibility. Therefore, differences in phospholipid-PUFA composition of mitochondrial membranes as compared with other membranes could explain the shifts in the percentages of the aldehyde load being carried by acrolein compared with 4-HNE between the different samples (Fig. 4E) as compared with a more even balance in cellular protein samples from the cortex (Fig. 5C,F). Because the propagation phase of the LP process is dependent upon the oxidizability (k_p) of the fatty acid (FA), and AA in particular has a high k_p , it stands to reason that CL, containing predominantly AA and LA, will more readily propagate the reaction¹⁰⁰ within mitochondrial membranes as opposed to other membrane phospholipids with other FA side chains. Whereas this provides a plausible explanation for why mitochondria are biochemically susceptible to LP, this also could explain why PZ had a better protective effect on reducing mitochondrial aldehyde load, but was unable to provide a significant protective effect for cellular protein samples.

Acrolein. Because acrolein is the most reactive of the aldehydic by-products of LP and polyamine metabolism (PAM; see below),^{21,23,54,55,106,107} it stands to reason that acrolein-mediated toxicity would be central to post-traumatic induced mitochondrial dysfunction. Acrolein and 4-HNE have been shown to increase oxidative stress in isolated mitochondria by increasing mitochondrial ROS production (in non-synaptic mitochondria) and to decrease levels of glutathione (GSH), an endogenous antioxidant.^{107–110} In addition, it has been previously shown that acrolein (and 4-HNE) can directly interfere with mitochondrial bioenergetics.^{24,47,111} In addition, we previously showed increased levels of acrolein-protein adducts in cortical tissue as early as 8 h following TBI, peaking at 2–3 days, and remaining above sham levels out to at least 7 days post-injury.⁷ In the current study we found that although acrolein levels in mitochondrial protein samples were elevated by 24 hpi, they did not reach significance until 72 hpi as compared with sham levels (see Fig. 4D). The same trend was seen in cortical tissue protein samples (see Fig. 5D), in accordance with our previous work.⁷ These increases in acrolein-bound proteins within the first several days following injury further support acrolein's critical role in secondary injury mechanisms, namely oxidative damage, occurring during at least the first 72 h after TBI. In agreement with this presumption, acrolein's half-life is much longer than the rapid ROS/RNS-mediated oxidative burst acutely following injury, on the order of hours to days,¹¹² and its smaller size and greater diffusibility increase its potential for damaging injured and penumbral tissue in a prolonged and persistent manner. In agreement with our current findings, recent work by Chen and associates⁴⁸ also showed a

reduction in acrolein after SCI following two doses of PZ (similar dosing paradigm to our delay). However, they use an antibody that only detects acrolein-lysine adducts (FDP-lysine; see Fig. 8A).^{48,113} The antibody that we chose (Abcam: ab 37110) is not specific for this lysine-only adduct; therefore, we believe that our protein-adduct profile provides a more comprehensive view of acrolein-adduction following TBI. We observe numerous acrolein adducts formed after TBI in both mitochondrial and cellular protein samples.^{7,35} Nevertheless, a more detailed proteomic analysis would need to be performed to specifically identify these susceptible proteins, as has been nicely characterized for numerous 4-HNE adducts.^{95,114–121}

From these lines of evidence we conclude that acrolein plays a critical and direct role in mitochondrial dysfunction, presumably by increasing ROS production following TBI. Whereas we did not directly measure ROS generation in this study, given the well-known link between ROS and LP, increased levels of LP are indicative of increased ROS and oxidative-mediated damage, as evidenced by increased aldehyde levels post-TBI. We recently found acrolein levels in synaptic mitochondria isolated from the injured cortex to be significantly higher as compared with non-synaptic mitochondria, which corresponded with a concomitant decline in synaptic mitochondrial respiratory function.³⁵ In addition, it has been demonstrated that acrolein exposure (at levels considered to be cytotoxic at $>1 \mu\text{M}$ concentrations¹²²) inhibited both mitochondrial electron transport and adenine nucleotide translocase (ANT) activity.¹⁰⁸ It is, as yet, unknown if acrolein inhibits *in vivo* ANT activity following TBI, although it is highly likely based on these findings. However, more work needs to be done to determine the effect of aldehyde scavenging by PZ on ANT activity after injury, presumably by attenuating post-traumatic, ROS-aldehyde-mediated mitochondrial bioenergetic crisis.

4-HNE. Whereas 4-HNE is more abundant, it is less reactive than acrolein.^{21,47,123} Despite this, it is still a highly reactive end-product of ROS-mediated phospholipid breakdown (LP) and, like acrolein, plays a critical role in post-traumatic mechanisms resulting in cellular and mitochondrial toxicity and dysfunction. Under normal physiological conditions, 4-HNE has been shown to act as a biological signaling substance that is generated and metabolized without cytotoxic consequence to the cell. This “normal” range for 4-HNE lies below a cellular concentration of $1\text{--}2 \mu\text{M}$.^{122,124} Following cellular damage, increased levels of free radicals (e.g., $\text{OH}\cdot$, $\text{O}_2\cdot^-$) result in increased tissue/cellular 4-HNE concentrations upwards of $10 \mu\text{M}$ to 1 mM ,¹²² suggesting that oxidative stress is a key factor in upsetting the aldehyde-cytotoxicity balance. A multitude of studies using concentrations within the lower range have demonstrated non-cytotoxic effects involved with cell survival and proliferation,^{125–128} whereas studies using the higher range have shown more cytotoxic effects including, but not limited to, disruption of Ca^{2+} and protein homeostasis, altered membrane fluidity, as well as activation of caspase pathways and cell death.^{116,128–131} More detailed studies need to be performed to identify and translate these non-toxic and toxic concentrations for 4-HNE and acrolein (and possibly 3-AP) into the post-TBI brain tissue environment. However, we can conclude from previous work in our lab that 4-HNE (and presumably acrolein) levels are likely within cytotoxic range, at least within the mitochondria, within 3 h post injury, as reflected by significant reductions in mitochondrial respiratory function, which, as aldehyde levels progressively increase over time, continues to deteriorate.⁷ By

FIG. 8. An overview of the chemical reactions involved in the formation of reactive aldehyde-protein adduct formation (A) (modified from Sousa and colleagues [2017]¹⁶⁷ and reprinted with permission) and the proposal mechanisms of action for phenelzine (PZ)-mediated protection following TBI through aldehyde sequestration (B) (4-HNE and acrolein; modified from Singh and associates [2013]⁴⁶ and reprinted with permission) and/or the inhibition of monoamine oxidase (MAO) enzymatic activity (C) (modified from Binda and co-workers [2008]¹⁶⁸ and reprinted with permission). 4-HNE, 4-hydroxynonenal; TBI, traumatic brain injury.

5 days post-injury, as these aldehydes are presumably metabolized (see below) and protein-adduction declines, mitochondrial function recovers, and caspase-3/calpain-mediated spectrin breakdown begins to dwindle, suggesting intrinsic attenuation of aldehyde-mediated neurodegenerative processes.

Mitochondrial susceptibility to LP-mediated damage by aldehyde adduction and PZ

An array of proteins have been identified as susceptible to HNE reactivity with particular amino acid residues; with binding preference as follows: Cys>>His>Lys (refer to the reactions shown in

Fig. 8A).¹³² These proteins range from plasma membrane transporters and growth factor receptors to neurotransmitters and mitochondrial proteins, as well as chaperones, proteasomal proteins, and cytoskeletal proteins.^{116,133} Following CNS injury, protein synthesis is known to be dramatically and temporally altered with some being upregulated, whereas others are downregulated or degraded as injury mechanisms progress. Hence, the proteins (and lipids) available for 4-HNE, and acrolein, to react with, change over time. In addition, because of the preferential binding of cysteine residues, proteins with susceptible cysteines are adducted first, then proteins with susceptible lysine residues, etc. Therefore, after TBI, the aldehyde-protein adduct profile not only shifts as it moves on to

binding with other amino acids, but it also increases over time. This is clearly visible in the shifting intensities in different bands on the WBs for both 4-HNE and acrolein (Figs. 4A,C and 5A,D). Although we quantify the OD value based upon the total intensity across a range (~ 150 – 50 kD), it is important to note the appearance of different 4-HNE and acrolein-protein adducts at 72 hpi as compared with sham animals. As discussed above, there is evidence to suggest likely targets based upon previous proteomic work, such as tubulin, DRP, mitochondrial ETC proteins, etc.; however, additional detailed proteomic assays need to be performed to identify and validate these susceptible mitochondrial and cellular proteins that are accumulating after TBI.

Moreover, scavenging aldehydes with PZ treatment after TBI is likely also going to alter the aldehyde-protein adduct profile as well. Because PZ covalently binds and traps reactive aldehydes (4-HNE, acrolein, and 3-AP) with its hydrazine moiety (refer to Fig. 8B), this reduces the aldehydes available to react with susceptible proteins. In this study, we found that PZ administration was able to reduce 4-HNE and acrolein protein adducts. However, PZ administration appeared to provide a stronger protective effect for mitochondrial proteins against LP-mediated aldehyde toxicity as compared with cellular proteins. We show that the cumulative aldehyde load levels for cortical mitochondrial protein samples (Fig. 4E) as compared with cortical cellular protein samples (Fig. 5C) show relatively the same accumulation of LP-derived aldehydes at 24 hpi regardless of whether the animals were treated with PZ or not. Although PZ was able to reduce the accumulation by just over 19% in both cases, the effect was insignificant. More importantly, however, the cumulative aldehyde level in the mitochondrial protein samples at 72 hpi is almost 30% higher than in cellular protein (Fig. 5C), and PZ was able to significantly lower this by 38% (Fig. 5C) as compared with the aldehyde load in mitochondrial samples from vehicle-treated animals.

Aldehyde metabolism and pathological accumulation: scavenge versus clearance

Due to their electrophilicity, 4-HNE and acrolein are primarily metabolized^{28,29} by the glutathione s-transferases (GSTs)^{28,30,134–137} and the multi-drug resistant protein 1 (MRP1)^{31,32,138} to detoxify cells and control aldehyde accumulation. In addition, these reactive aldehydes, can also be reduced by aldo-keto oxidoreductase enzymes (AKR) to alcohols (glycols),^{33,111,139} or in the case of 3-AP, can be oxidized to their corresponding acid metabolites (carboxylic acids) by aldehyde dehydrogenases (ALDH).^{29,54,140–144} Glutathione (GSH), an endogenous antioxidant, can bind these aldehydes to form a conjugate, and GST metabolizes the aldehydes to water soluble aldehyde-GSH conjugates that can be removed from the cell. In a closed-head model of TBI, GST activity was shown to decrease in cortical tissue at 24 h after impact.¹⁴⁵ In Alzheimer's disease (AD), both GST and MRP1, which are necessary to metabolize and efflux these aldehyde-GSH conjugates from the cell(s), were found to be reduced and/or dysfunctional, thereby contributing to subsequent aldehyde accumulation.³⁴ 4-HNE, acrolein, and 3-AP have all been shown to be produced in excess following TBI.^{7,35,47,146} Based upon these findings, it is likely that similar effects are occurring following controlled cortical impact-TBI (CCI-TBI), resulting in reduced aldehyde clearance from damaged neurons as a result of reduced GSH and GST activity, culminating in these observed intracellular and intramitochondrial aldehyde accumulations.^{7,35} Further studies would need to be performed to determine whether aldehyde reactivity, primarily

4-HNE and acrolein, is interfering with GST function directly, or if the post-traumatic effects are indirect (e.g., altered GST mRNA or protein expression levels). Further, it would be of particular interest to see if scavenging aldehydes with PZ is also protecting and enabling functionality of this endogenous protective process. Although we know that aldehyde metabolism occurs very rapidly, it remains to be determined how pharmacological aldehyde scavenging contributes to this endogenous clearance process. At this point, the effect of PZ administration post-TBI on GSH/GST activity post-TBI is, as yet, undetermined. More work needs to be done to understand how the timing of PZ administration and dosage might align with the timing of endogenous aldehyde clearance.

PZ and spectrin

An additional consequence of LP-mediated cellular protein damage is disruption of intracellular Ca^{2+} homeostasis resulting in decreased Ca^{2+} uptake by failing mitochondria thereby exacerbating cytosolic Ca^{2+} overload and leading to calpain-mediated proteolysis (see Fig. 7). Calpains are calcium-dependent cysteine proteases that are acutely activated following CNS injury. One protein in particular that is susceptible to calpain-mediated degradation is α -spectrin,⁴⁹ a neuronal cytoskeletal protein. Prior work in our lab has demonstrated connections between Ca^{2+} dysregulation, LP-mediated damage, and subsequent calpain-mediated spectrin breakdown after SCI² and TBI.¹³ *In vitro* exposure of PC-12 cells to 4-HNE causes activation of caspase-3 at even lower doses and within a shorter time frame than necessary to induce cell death.⁹² Caspase-3 is one of the enzymes, along with Ca^{2+} -activated calpain, which contributes to spectrin breakdown. This suggests a high level of sensitivity in terms of aldehyde-mediated caspase activation. Oxidation of CL initiates release of cytochrome c into the cytosol, thereby activating caspase-3 and inducing apoptosis. Acrolein, however, has not been shown to initiate cytochrome c release, *in vitro*.¹¹¹ In the current study, we show no significant protection against cytoskeletal breakdown by PZ as compared with the vehicle-treated group at either time-point, although PZ treatment at 24 hpi was able to restore the amount of the 150 kD breakdown product to within sham levels (see Fig. 6B). It therefore seems likely that PZ-mediated effects against post-traumatic cytoskeletal breakdown results more indirectly from mitochondrial-mediated protection.

Additional mechanisms of PZ-mediated protection

Polyamine metabolism (PAM) and aldehyde scavenging. There are multiple metabolic sources of reactive aldehydes, of which lipid peroxidation and polyamine oxidation have been implicated in the neuropathological processes associated with various neurodegenerative diseases and models of neurotrauma. Although the end-products of LP have been previously discussed, the key molecules resulting from PAM include spermine and spermidine, as well as putrescine; hydrogen peroxide (H_2O_2) and cytotoxic aminoaldehydes have been shown to be involved in modulation of NOS activity, regulation of mitochondrial Ca^{2+} transport, and membrane stabilization. The oxidation of spermine and spermidine to putrescine generates 3-aminopropanal (3-AP) and acrolein,^{54,147} both of which have been shown to have neurotoxic effects. In the context of neurotrauma, both aldehydes, and putrescine, have been shown to be upregulated following TBI^{7,35,146} and SCI.⁴⁸ In addition, the aldehydes 4-HNE and acrolein are known to activate the intrinsic apoptotic pathway(s)

independent of lysosomes,^{25,148–150} whereas 3-AP is taken up by lysosomes causing them to rupture and release enzymes resulting in lysosome-dependent cell death.^{151–153} However, because the aminoaldehyde 3-AP, though still very reactive, acts through lysosomal-dependent mechanisms to elicit apoptosis at the pre-mitochondrial phase^{151–154} we chose not to include it in our evaluation for the current study.

Acrolein has been demonstrated to have a much greater capacity for neurotoxicity as compared with 3-AP.⁵⁴ Whereas the levels of 3-AP in the brain following TBI appear to increase,¹⁴⁶ there exists plenty of evidence for their participation in the cumulative “aldehyde load” and subsequent aldehyde-related/mediated cellular and mitochondrial damage following injury. Therefore, it is highly likely that although we only investigated the reduction in 4-HNE and acrolein accumulation with PZ treatment, there is more than likely proportional 3-AP scavenging occurring as well. For example, with a pKa of 5.2,¹⁵⁵ PZ can get into lysosomes and interact with 3-AP accumulating there after TBI and potentially prevent lysosomal rupture¹⁵¹ to protect against mitochondrial dysfunction and neurotoxicity. However, 3-AP reactivity compared with acrolein is significantly lower,^{54,55} so it may or may not matter, although, it may be reducing the amount of PZ available to react with the other two more reactive and damaging aldehydes.

The role of MAO activity and inhibition. Monoamine oxidase (MAO) is an enzyme located in the outer mitochondrial membrane that catalyzes the oxidative deamination of neurotransmitters and dietary amines. There are two isoenzymes, MAO-A and MAO-B. Although the precise localization of these isoforms has yet to be definitively determined, they can be differentiated by their selective substrates and inhibitors. MAO-A catalyzes the oxidative deamination of hydroxylated amines (e.g., noradrenaline, 5-hydroxytryptamine [serotonin]), whereas MAO-B shows greater affinity for non-hydroxylated amines (e.g., benzylamine, beta-phenylethylamine). Therefore, selective MAO-A inhibitors lead to increased synaptic levels of noradrenaline and serotonin, whereas MAO-B inhibitors lead to increased levels of dopamine. The enzymatic action of MAOs produces transient aldehyde intermediates, such as 3-AP, and H₂O₂, resulting in free radical generation, namely production of the superoxide radical (O₂^{•−}) (refer to Fig. 8C). This highly reactive oxygen radical plays a critical role in various biochemical pathways known to contribute to post-traumatic mechanisms of secondary injury.^{19,156}

PZ is a long-standing FDA-approved drug for the treatment of anxiety and depression through its potentiation of monoaminergic neurotransmission by non-selective and irreversible inhibition of MAOs (refer to Fig. 8C). MAO inhibitors have been shown to elicit neuroprotective effects.^{147,157–159} Despite PZ having other physiological effects, it is becoming evident that its actions as an aldehyde scavenger as well as an inhibitor of MAO are both likely sources of its functionality as a neuroprotectant. PZ primarily inhibits MAO-A, at both low and high doses, but as the dosage increases, so does its inhibition of MAO-B in a dose dependent manner.¹⁶⁰ In addition, a single dose of PZ (15 mg/kg) sustained MAO-A and MAO-B inhibition out to 24 h post-IP injection.¹⁶⁰ Selegiline (or deprenyl), an MAO inhibitor that lacks the hydrazine and cannot scavenge aldehydes, has still been shown to increase SOD, reduce free-radicals,¹⁶¹ and protect mitochondrial function by decreasing mitochondrial Ca²⁺ and stabilizing membrane potential,^{161,162} as similarly shown here with PZ (see Fig. 2). Importantly, these other effects of deprenyl occur when used at concentrations insufficient to inhibit MAO activity.^{161,162} The

current thinking is that selegiline is only MAO-B selective at low dose, whereas higher doses inhibit both MAO-A and MAO-B, as similarly seen with PZ. Similar deleterious, high dose-dependent effects presumably related to excessive inhibition of MAO activity were also observed in recent studies in our lab with PZ.^{47,66}

In agreement with these data, an *in vitro* study performed in our lab looked at the protective effects of pargyline, which has MAO inhibitory activity but lacks the hydrazine functional group, compared with PZ, and demonstrated that MAO-B inhibition alone was not able to protect isolated cortical mitochondria from aldehyde-induced loss of bioenergetics.⁴⁷ It's possible that PZ's neuroprotective strength comes from its ability to both scavenge reactive aldehydes and inhibit the activity of both isoforms MAO-A and MAO-B. It is also possible that we are missing an additional component contributing to the neuroprotective effects of PZ administration by not looking at these effects following TBI; further, because 3-AP is also generated by MAO activity⁵⁴ additionally contributing to the aldehyde pool and the potential for aldehyde-mediated neurotoxicity. In further support of this, the hydrazine function of PZ has been shown to react with the polyamine metabolite 3-AP eliciting neuroprotection both *in vitro* and *in vivo*.⁵⁵

Despite the aforementioned protective effects of deprenyl, another study looking across a wide dosage range (100–600 μ M) was unable to show a protective effect against aldehyde-induced (3-AP) neuronal (retinal ganglion cells) damage.⁵⁵ This further supports the idea that the MAO inhibition alone will not protect against aldehyde-mediated damage, and that the protection elicited by MAO inhibition likely relies on its ability to inhibit MAO-A instead of MAO-B. These findings suggest that although MAO inhibition in and of itself can have neuroprotective effects, these effects may be additive to the sequestration of aldehydes by PZ following TBI.

PZ dosing considerations and bioavailability

Recently, the plasma half-life of PZ administered intraperitoneally (IP) to rodents was reported at 29 min,⁴⁸ which is significantly shorter than previously reported in humans at 11.6 h.¹⁶³ The current study's dosing paradigm was designed based upon these data, suggesting a half-life of 12 h; hence the delivery of a maintenance dose at 12 hpi. Based on the apparent shorter half-life for PZ in rodents compared with humans, it would seem logical that increasing the dosing of PZ to either multiple doses/day or continuous infusion to maintain blood and tissue levels at a higher level would be more protective. However, a recent study by our lab using an osmotic pump to deliver 10 mg/kg/day PZ for 72 h showed just the opposite. The results showed only minimal improvement in mitochondrial respiratory rates and modest reductions in mitochondrial aldehyde accumulation.⁶⁶ In the current study, by comparison, a more limited dosing paradigm of PZ produced significantly higher mitochondrial respiration rates compared with the rates from animals that received the continuous infusion out to 72 hpi: 39.7 $\pm$ 7.6% higher rates, on average, across all five individual states of respiration (as shown in Fig. 3; *t* test/state: *p* $\leq$ 0.01). In addition, there was a 12.6% increase (*t* test: *p* $<$ 0.05) for the RCR. These improvements in individual states of mitochondrial respiration, as well as improved RCR, support our hypothesis that limited, acute attenuation/suppression of aldehyde accumulation by PZ is responsible for the neuroprotective effects, whereas persistent administration may be deleterious to these effects. In support of this, we were able to demonstrate similar neuroprotective effects in the delayed treatment group. The protective effects of delayed PZ administration were also shown following delayed administration

of PZ (one dose/day) following SCI.⁴⁸ Based on these findings, we can conclude that the scavenging of reactive aldehydes by PZ following TBI is protective and that the apparent shorter half-life of the drug does not appear to impact its effectiveness.

We had previously demonstrated a 10-fold difference between acrolein (3 μ M) and 4-HNE (30 μ M) in dose-dependent toxicity induced in cortical mitochondrial respiration.⁴⁷ However, the same treatment dosage of PZ (30 μ M) was able to successfully protect mitochondrial bioenergetics against either aldehyde exposure. As previously suggested,⁴⁶ PZ acts to scavenge 4-HNE at a 1:1 equimolar ratio, whereas acrolein requires a 10-fold higher level of PZ to be effective.⁴⁷ This suggests that a higher dosage of PZ could potentially do a better job of scavenging acrolein because of its greater toxicity; however, this does not appear to be the case *in vivo*, as we were able to more sufficiently protect mitochondrial function with this lower dosing paradigm compared with less mitochondrial protection with more PZ (e.g., continuous infusion with osmotic pumps)⁶⁶ and higher doses.⁴⁷ Because PZ has multiple molecules with which it can react, and because these reactions are irreversible, it would be pharmacologically and clinically beneficial to more clearly understand its binding preferences with regards to its hydrazine aldehyde binding and its capacity for MAO inhibition. Future studies may be necessary to determine the differential capacity of PZ to scavenge each of these aldehydes. Although this has been done comparing the ability of PZ to protect against 3-AP and acrolein exposure *in vitro*,⁵⁴ 4-HNE has not been looked at head-to-head. We do not know whether PZ is preferential for one aldehyde over another, or if its preference is to inhibit MAO, then the excess is left to react with aldehydes.

Conclusion

Mitochondrial dysfunction in the injured brain has been linked to a toxic combination of (1) loss of Ca^{2+} homeostasis, (2) increased free radical production, and (3) elevated production of LP-derived aldehydes. These, in combination, are vital contributors to progressive mitochondrial and cellular functional decline, which can ultimately lead to neuronal cell death. Accordingly, the pursuit of pharmacological interventions to reduce the cytotoxic and pathological consequences incurred by aldehyde accumulation (i.e., through aldehyde scavenging) following TBI remains an important neuroprotective strategy after injury. These are just a few of the reasons why inhibiting the action of aldehyde-mediated toxicity is an important pharmacological strategy to continue to pursue. There is a growing body of evidence to suggest that oxidative- and aldehyde-mediated damage play not only a key role in neuropathology associated with trauma, as clearly demonstrated in this study, but also in neurodegenerative diseases such as AD^{33,132,164,165} and Parkinson's disease.^{150,166} In our current study we demonstrated that only two doses of PZ, delivered at 15 min and at 12-h post-TBI (hpi), was more effective than previous dosing paradigms at preserving mitochondrial bioenergetics at 24 h and that this neuroprotective effect was sustained out to 72 hpi. These improvements in PZ-treated animals following TBI were associated with (1) improvements in mitochondrial Ca^{2+} buffering and membrane potential and (2) reduced levels of LP-mediated aldehyde (4-HNE and acrolein) accumulation in mitochondrial protein samples. Further, we found that this dosing paradigm of PZ following TBI was also protective against LP-mediated oxidative damage to cortical cellular proteins by reducing levels of aldehydes (4-HNE and acrolein) accumulation as well as reducing the amount of spectrin breakdown products compared with vehicle-treated animals.

Although it has been previously demonstrated that administration of PZ can reduce the levels of 4-HNE and acrolein following TBI,^{47,66} there was not correlative preservation of mitochondrial bioenergetics in any comprehensive manner (i.e., across all states of respiration) as seen in the current study, thus suggesting sub-optimal PZ dosing. The current series of studies demonstrates a positive correlation between functional mitochondrial protection with a corresponding decrease in 4-HNE and acrolein accumulation following TBI. Whereas this more limited administration of PZ was more successful at reducing aldehyde accumulation in mitochondrial protein samples compared with previous studies, it was not as effective at scavenging aldehydes within the cell as a whole. It did appear that PZ may have been more effective at scavenging acrolein as compared with 4-HNE, but more studies would need to be performed to determine whether PZ is more selective for acrolein. Although PZ is not selective for only 4-HNE and acrolein, its action is likely related to it scavenging of multiple reactive aldehydes all contributing in different manners to exacerbate secondary mechanism of post-traumatic damage within the injury site and penumbra after TBI.

Acknowledgments

This work was supported by NIH grants R01 NS083405 and R01 NS084857 from NINDS and by NIH grant P30 NS051220.

Author Disclosure Statement

No competing financial interests exist.

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