Phenelzine Protects Brain Mitochondrial Function *In Vitro* and *In Vivo* following Traumatic Brain Injury by Scavenging the Reactive Carbonyls 4-Hydroxynonenal and Acrolein Leading to Cortical Histological Neuroprotection

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Abstract

Lipid peroxidation (LP) is a key contributor to the pathophysiology of traumatic brain injury (TBI). Traditional antioxidant therapies are intended to scavenge the free radicals responsible for either initiation or propagation of LP. A more recently explored approach involves scavenging the terminal LP breakdown products that are highly reactive and neurotoxic carbonyl compounds, 4-hydroxynonenal (4-HNE) and acrolein (ACR), to prevent their covalent modification and rendering of cellular proteins nonfunctional leading to loss of ionic homeostasis, mitochondrial failure, and subsequent neuronal death. Phenelzine (PZ) is a U.S. Food and Drug Administration–approved monoamine oxidase (MAO) inhibitor (MAO-I) used for treatment of refractory depression that possesses a hydrazine functional group recently discovered by other investigators to scavenge reactive carbonyls. We hypothesized that PZ will protect mitochondrial function and reduce markers of oxidative damage by scavenging LP-derived aldehydes. In a first set of *in vitro* studies, we found that exogenous application of 4-HNE or ACR significantly reduced respiratory function and increased markers of oxidative damage ($p < 0.05$) in isolated noninjured rat brain cortical mitochondria, whereas PZ pretreatment significantly prevented mitochondrial dysfunction and oxidative modification of mitochondrial proteins in a concentration-related manner ($p < 0.05$). This effect was not shared by a structurally similar MAO-I, pargyline, which lacks the hydrazine group, confirming that the mitochondrial protective effects of PZ were related to its carbonyl scavenging and not to MAO inhibition. In subsequent *in vivo* studies, we documented that PZ treatment begun at 15 min after controlled cortical impact TBI significantly attenuated 72-h post-injury mitochondrial respiratory dysfunction. The cortical mitochondrial respiratory protection occurred together with a significant increase in cortical tissue sparing.

Keywords: 4-hydroxynonenal; acrolein; brain mitochondria; lipid peroxidation; neuroprotection; phenelzine; traumatic brain injury

Introduction

TRAUMATIC BRAIN INJURY (TBI) is a devastating condition that presents serious challenges to the health and welfare of civilians, soldiers, and veterans. In the United States alone, 1.7 million persons are diagnosed with TBI each year¹ and 5.3 million persons in the United States are living with permanent or temporary post-traumatic disability.² Additionally, the military subset population is realizing an unprecedented shift in TBI occurrence since the onset of the wars in Iraq and Afghanistan. Military TBI diagnoses have almost tripled in 8 years, with 30,000 diagnosed soldiers

in 2012, from approximately 10,000 soldiers in 2004.³ The Centers for Disease Control and Prevention has previously advocated TBI as a “major public health epidemic,” calculating an overwhelming economic burden of over \$16 billion a year and \$56 billion overall.⁴

Appropriately, significant efforts are underway to further elucidate TBI pathophysiology and identify more-effective neuroprotective strategies. One of the most understood injury mechanisms post-TBI involves free-radical–induced lipid peroxidation (LP). Free radicals post-TBI are created after a loss of calcium homeostasis leading to subsequent peroxidation of lipids. Typically, following the primary injury, a mechanical depolarization

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of neurons leads to the loss of Ca^{2+} homeostasis and subsequent elevation of intracellular Ca^{2+} .⁵ As a result, superoxide ($\text{O}_2^{\bullet-}$) production elevates attributable to a single electron (e^-) reduction of molecular oxygen (O_2). $\text{O}_2^{\bullet-}$ is capable of stealing an electron (an oxidizing agent) or donating an electron (a reducing agent); this dual nature may be the source of $\text{O}_2^{\bullet-}$ modest reactivity. However, $\text{O}_2^{\bullet-}$ can undergo several fates leading to generation of more-prolific and excessively volatile radicals. Should $\text{O}_2^{\bullet-}$ undergo either enzyme-mediated (superoxide dismutase) or spontaneous dismutation to create hydrogen peroxide (H_2O_2), iron from brain tissue hemorrhage can cause iron-catalyzed Fenton reactions to generate excessive hydroxyl radicals ($\bullet\text{OH}$).⁶ The other fate of $\text{O}_2^{\bullet-}$ is to react with nitric oxide radical ($\text{NO}\bullet$), which is readily produced in mitochondria upon activation of mitochondrial nitric oxide synthase (mtNOS).^{7,8} Should $\text{O}_2^{\bullet-}$ react with $\text{NO}\bullet$, a temporary peroxynitrite anion (ONOO^-) is formed, which, in the context of TBI pathology, leans toward protonation or reaction with CO_2 . In the later circumstance, CO_2 exposure will generate unstable nitrosoperoxocarbonate (ONOOCO_2), whose degradation products generate carbonate radical ($\text{CO}_3^{\bullet-}$) and nitrogen dioxide radical ($\bullet\text{NO}_2$). In the former circumstance of protonation, peroxynitrous acid is formed and degraded into $\bullet\text{OH}$ and $\bullet\text{NO}_2$.

Free radicals (e.g., $\bullet\text{OH}$, $\bullet\text{NO}_2$, and $\text{CO}_3^{\bullet-}$) induce the LP of polyunsaturated fatty acids, including arachidonic, linoleic, docosahexaenoic, and eicosapentaenoic acids, within cell and organellar membranes. The LP begins with the abstraction of an electron from an allylic carbon by a free radical (i.e., "initiation"). Subsequent reactions during "propagation" contribute to the overall decomposition of the fatty acid into a degraded aldehydic product, such as 4-hydroxynonenal (4-HNE) or 2-propenal (acrolein; ACR). Whereas LP does compromise membrane integrity and interrupts phospholipid dependent proteins (e.g., ion channels and electrogenic ion pumps), the aldehydic breakdown products have been well characterized as toxic mediators of cellular damage in neurotrauma models.^{9–11}

Specifically, 4-HNE and ACR possess carbonyl functional groups capable of covalently binding lysine, histidine, and cysteine amino acids of cellular and mitochondrial proteins through Schiff base and/or Michael adducts.^{12,13} These alterations induce conformational changes in protein structure compromising their function, which contributes to overall cellular demise. Free-radical-induced LP is one of the most deleterious contributors to acute post-TBI pathophysiology.^{14–19}

Given the richness in the environment of polyunsaturated fatty acids in brain tissue and the intrinsic ability to generate reactive oxygen species in both normal and pathological conditions, mitochondria are targets for not only LP, but also subsequent aldehydic attacks. Increasingly, 4-HNE and ACR have been identified as reactive carbonyls capable of impairing mitochondrial respiration and contributing to accumulation of oxidative damage markers.^{5,10,11,20} The impaired mitochondria become incapable of regulating ionic homeostasis, have severely reduced or completely inhibited ability to generate adenosine triphosphate through oxidative phosphorylation, and will initiate apoptotic cell death, thereby killing the neuron in which they reside.^{10,16,21–24}

A crucial component to keeping the neuron alive is to stabilize mitochondrial function. And, the most definitive way to protect mitochondrial from dysfunction is to prevent either free radical production or the subsequent LP cascade. For many years, researchers have demonstrated that pharmacological antioxidants, such as tirilazad, can scavenge lipid peroxyl ($\text{LOO}\bullet$) free radicals

and interrupt the LP propagation phase.²⁵ However, a timely pharmacological intervention by $\text{LOO}\bullet$ scavengers is limited by the narrow therapeutic window intrinsic to scavenging radicals that very rapidly react with biomolecules with a diffusion rate-limited rate constant. Additionally, $\text{LOO}\bullet$ scavengers only interrupt the LP cascade; they are not capable of reversing it. Fortunately, an alternative means exists to prevent free-radical-induced LP by targeting the latest stage of the cascade: the generation of the aldehydic breakdown-products. Scavengers that target the deleterious breakdown products, that is, reactive carbonyls (4-HNE and ACR), are known as "carbonyl scavengers." The use of carbonyl scavengers may have the potential to expand the clinical therapeutic window and, possibly, reverse some aspects of LP-mediated damage.

The most effective carbonyl scavengers identified to date are capable of covalently binding carbonyls through a hydrazine functional group ($-\text{NH}-\text{NH}_2$).^{26–28} Two such compounds are hydralazine (HZ) and phenelzine (PZ), which already possess U.S. Food and Drug Administration (FDA) approval for clinical use. HZ is used generally to treat hypertension as a potent arterial vasodilator whereas PZ has been used in a variety of conditions, primarily as a monoamine oxidase (MAO) inhibitor (MAO-I) for treatment of depression that is refractory to newer serotonin or norepinephrine reuptake inhibitors. Both compounds possess a hydrazine moiety and have demonstrated the ability to inhibit carbonyl toxicity in cell-culture models.^{27,29–31} Additionally, HZ and PZ have both been shown to exhibit neuroprotective effects in multiple models of neurotrauma. In particular, HZ was demonstrated to function as a neuroprotectant in the context of spinal cord injury while simultaneously reducing acrolein accumulation.³² On the other hand, PZ has been reported to be a neuroprotectant in focal and global ischemia-reperfusion stroke models and similarly reduced a deleterious "aldehyde load."³¹ Although both HZ and PZ are promising candidates for carbonyl scavenging in treatment of TBI, HZ may be contraindicated because of its ability to induce hypotension, which may already exist in patients who have sustained a TBI.

Therefore, the intent of this study was to demonstrate that the most clinically relevant drug, PZ, is able to protect mitochondrial function and reduce oxidative damage markers. Additionally, we investigated the importance of the hydrazine functional group by which PZ is believed to be capable of scavenging reactive carbonyls. Although our lab has previously demonstrated the ability of 4-HNE to inhibit mitochondrial respiration and, subsequently, PZ's ability to prevent oxidative damage,⁵ the current *in vitro* studies have never been conducted in the high-throughput assay format utilizing the Agilent Technologies (Santa Clara, CA) XFe24 analyzer, which better enables the determination of the dose-response relationship for antagonism of 4-HNE and ACR by comparing the mitochondrial protective effects of PZ, which contains the hydrazine group, against the structurally similar compound PG, which lacks the hydrazine group. Figure 1 shows the structures of 4-HNE and ACR as well as the comparative structures of PZ and PG. Moreover, given that both compounds are MAO-Is, the comparison of the two allows for a determination of whether MAO inhibition might contribute to the protective effects of PZ.

Last, we have expanded our previously published pilot studies with PZ in a rat controlled cortical impact (CCI) TBI model,⁵ more extensively confirming the ability of early post-injury administration to reduce brain mitochondrial respiratory failure and increase cortical tissue sparing.

FIG. 1. (A) Structure of 4-hydroxynonenal (4-HNE) and acrolein (ACR), each possessing a nucleophilic aldehyde (i.e., reactive carbonyl). 4-HNE and ACR are the aldehydic breakdown products of free-radical-induced lipid peroxidation. Carbonyls refer to compounds that contain a double-bonded oxygen (CHO) functional group, to include reactive aldehydes: 4-HNE and ACR. (B) Structure of phenelzine (PZ), a monoamine oxidase inhibitor containing a hydrazine functional group capable of scavenging reactive carbonyls. (C) Structure of pargyline (PG), a monoamine oxidase inhibitor, which does not contain a hydrazine functional group.

Methods

Animals

All experiments were performed with adult male Sprague-Dawley (SD) rats (Charles River Labs International, Portage, MI) weighing 300–350 g. Rats were provided food and water *ad libitum*. All protocols associated with this study were approved by the University of Kentucky (Lexington, KY) Institutional Animal Care and Use Committee consistent with the National Institutes of Health Guidelines for the Care and Use of Animals.

Chemicals

ACR was purchased from Sigma-Aldrich (Supelco, Inc., Bellefonte, PA). 4-HNE was purchased from EMD Chemicals Inc. (Merck KGaA; Darmstadt, Germany). PZ sulfate salt was purchased from MP Biochemicals (Solon, OH). All other compounds were stored in accord with the manufacturer's recommendations. Working solutions for all compounds were prepared fresh each day

of experimentation. All chemicals used for experimentation in this work were purchased as previously reported.⁵

Isolation of Ficoll-purified brain mitochondria for in vitro studies

Rat brain mitochondria were isolated as previously published.^{5,10,33} Cortical brain tissue dissected from brains extracted from decapitated rats after CO₂ exposure and homogenized using Potter-Elvehjem manual homogenizer in 3 mL of ice-chilled isolation buffer (215 mM of mannitol, 75 mM of sucrose, 0.1% bovine serum albumin (BSA), 20 mM of HEPES, and 1 mM of ethylene glycol tetraacetic acid [EGTA], pH adjusted to 7.2 with KOH). Differential centrifugation at 4°C was used to separate nuclei and cellular components from crude mitochondrial pellet. The pellet was subjected to nitrogen bomb disruption followed by resuspension, then layered onto a discontinuous 7.5% and 10% Ficoll gradient. The resuspension was centrifuged at 100,000g for 30 min at 4°C. The resultant pellet was again resuspended in 25–50 μ L of isolation buffer without EGTA to yield a concentration of

~10 mg/mL. Final protein concentration was determined using a bicinchoninic acid (BCA) protein assay kit measuring absorbance at 562 nm with a BioTek Synergy HT plate reader (BioTek Instruments Inc., Winooski, VT). For each experiment, fresh mitochondria and buffer solutions were prepared and were used immediately for *ex vivo* respiration assays.

Preparation of sensor cartridge and substrates/inhibitors for mitochondrial bioenergetic analysis in in vitro studies

The Seahorse Biosciences Extracellular Flux Analyzer (XFe24; Agilent Technologies) can be used to quantify cell culture or isolated mitochondrial bioenergetics.^{34–38} In the current study, the XFe24 analyzer was used to quantify respiratory function and bioenergetics of mitochondria in intact and well-coupled (respiratory control ratio [RCR] >5). Mitochondria were isolated using Ficoll-gradient centrifugation, as previously described.³⁴ The XFe24 analyzer offers a 24-well (7 μ L/well) microplate format that is ideal for *in vitro* studies of the effects of compounds that either reduce or preserve normal mitochondrial respiration (i.e., complex I- or complex II-driven oxygen utilization) when added directly to isolated brain mitochondria.^{38,39} The 24 wells enable side-by-side inclusion of multiple replicates that is ideal for *in vitro* dose-response studies of compounds that either cause, or protect against, mitochondrial dysfunction. One day preceding experimentation, 1.3 mL of XF Calibrant solution (Agilent Technologies) was added to each well of the 24-well dual-analyte sensor cartridge (Agilent Technologies). The sensor cartridge was stacked on top of a Hydro Booster plate, which was stacked on a Utility Plate (Agilent Technologies). The stacked plates were incubated overnight in a 37°C incubator without CO₂. The sensor cartridge was removed from incubation and placed into the XFe24 on the day of experimentation, the loaded with mitochondrial substrates and inhibitors at 10 $\times$ concentrations. For the initiation of state III respiration (e.g., activation of complex I and IV), Port A of the sensor cartridge received (50-mM) pyruvate/(25-mM) malate/(10-mM) ADP. Port B contained 10 μ M of Oligomycin-A solution for the generation of state IV respiration. Port C contained 40 μ M solution of carbonylcyanide-p-trifluoromethoxyphenylhydrazine (FCCP) for uncoupled, adenosine diphosphate (ADP)-driven (complex I) state IV respiration. Port D contained 100 nM of solution of rotenone/100 mM of succinate for assessment of uncoupled, succinate-driven (complex II) state IV respiration. During the automated sensor calibration, 7.5 μ g of isolated mitochondria were suspended in 50 μ L in mitochondrial isolation buffer and added to each well of the cell-culture plate (Agilent Technologies) excluding background wells, which contained respiration buffer alone. The plate was then centrifuged at 2000 rpm for 4 min at 4°C to attach mitochondria to the 24-well cell-culture plate. After centrifugation 450 μ L of warmed (37°C) respiration buffer (215 mM of mannitol, 75 mM of sucrose, 0.1% BSA, 20 mM of HEPES, 2 mM of MgCl₂, and 2.5 mM of KH₂PO₄, adjusted to pH of 7.2 with KOH) was added gently to the corner of each well for a total volume of 500 μ L. Experiments investigating the pretreatments of PZ, pargyline (PG), 4-HNE, or ACR were conducted once the mitochondria were fixed to the Seahorse culture plate (Agilent Technologies) are described below.

In vitro 4-hydroxynonenal and acrolein dose response to inhibit brain mitochondrial respiratory function

Experiments were performed to determine the optimal concentration of 4-HNE and ACR to inhibit approximately 50% (suboptimal dose) of mitochondrial function. Isolated mitochondria affixed to a culture plate (as described above) were exposed to 4-HNE or ACR, ranging from 10 to 100 μ M and 1 to 10 μ M, respectively. Following 4-HNE or ACR treatment, mitochondria

were assessed by the XFe24 for oxygen consumption rate (OCR; pmoles O₂/min). The OCR was generated by Agilent Technologies algorithms designed to calculate area under the curve. Excel (Microsoft Corporation, Redmond, WA) software was used to isolate time-specific points wherein the generated rate calculations reflected the OCR of complex I and complex II, for example, the specified time points following the autoinjection of pyruvate-malate and succinate, respectively. Because mitochondria possess the capability to eventually utilize all of the ADP substrates and there exists a delay before oligomycin can inhibit respiration, the highest time points for ADP rates and lowest time points for oligomycin rates are used in place of software-generated averages. This is in accord with previously published methods.³⁴ The current studies indicate that optimal doses of 4-HNE and ACR were determined to be 30 and 3 μ M, respectively.

In vitro phenelzine dose response to protect against 4-hydroxynonenal- or ACR-induced mitochondrial respiratory dysfunction

Increasing concentrations of PZ (3, 10, or 30 μ M) were exposed to isolated mitochondria (7.5 μ g) in 24-well culture plates (Agilent Technologies) for 5 min.

Immediately following PZ pre-treatment, mitochondria were exposed to reactive aldehydes, such as 4-HNE and ACR (30 and 3 μ M, respectively) for 10 min. Culture plates containing PZ-pretreated mitochondria with subsequent reactive aldehyde exposure were assessed for their ability to respire oxygen with XFe24, by measurement of OCR (pmoles O₂/min).

In vitro phenelzine or pargyline treatment against 4-hydroxynonenal or acrolein

Similar experiments were used to determine the efficacy of a PZ to prevent 4-HNE- or ACR-induced respiratory inhibition. In these experiments, isolated mitochondrial were exposed to a 5-min pre-treatment of PZ 30 μ M, followed by a 10-min exposure to reactive aldehyde 4-HNE or ACR. PZ pretreatment concentrations were chosen based on the *PZ dose response to 4-HNE or ACR* and in accord with previously published methods published in isolated cortical mouse brain mitochondria.^{5,9} Concentrations of PG were based on molar equivalency with the most effective dose of PZ.

Measurement of 4-hydroxynonenal- or acrolein-induced mitochondrial oxidative damage for in vitro studies

The ability of PZ to protect mitochondria from oxidative damage markers 4-HNE and ACR was measured using western blot analysis. Based upon the BCA protein assay results and normalization of protein concentration across samples, 50- μ g cortical mitochondrial samples were exposed to a 5-min pre-treatment of increasing concentrations of PZ 3, 10, or 30 μ M followed by 15-min exposure to 4-HNE or ACR (30 and 3 μ M, respectively). Equal mitochondrial aliquots to those used for respiration analysis were carefully loaded onto a pre-cast gel (12% Bis-Tris [w/v] acrylamide; Criterion XT; Bio-Rad Laboratories, Inc., Hercules, CA) with XT-MOPS buffer (Bio-Rad Laboratories) for subsequent analysis of 4-HNE content. Mitochondrial samples assayed for ACR adducts were separated on pre-cast gel (4–12% gradient tris-acetate gels) using MOPS buffer (Bio-Rad Laboratories). Proteins assayed for 4-HNE and ACR were transferred to nitrocellulose membranes with a semidry electrotransferring unit (Bio-Rad Laboratories) at 15 volts for 45 min at room temperature. TBS (5.0% milk/Tris-buffered solution) was used to incubate membranes for 1 h at room temperature. Membranes were then incubated overnight at 4°C in TBST (0.5 mM of Tween-20) with respective dilution of 4-HNE or

ACR primary antibody. 4-HNE mouse polyclonal primary antibody (Alpha Diagnostics International Inc., San Antonio, TX) was diluted 1:2000. ACR rabbit monoclonal antibody (Abcam, Cambridge, MA) was diluted at 1:1000. 4-HNE and ACR primary antibodies were detected by 2-h incubation at room temperature with goat antimouse or antirabbit secondary antibody conjugated to infrared dye (1:5000; IRdye-800CW; Rockland, Gilbertsville, PA) in TBST. Membranes were analyzed with the LiCor Odyssey Infrared Imaging System (Li-Cor Biosciences, Lincoln, NE). 4-HNE and ACR protein smears were assessed 150 to 50 kD and compared to untreated lanes as percent control.

In addition to meticulous care in normalization of protein concentration between samples and in loading each gel lane, after transfer, gels were stained with Coomassie blue to enable a visual estimate of equal protein loading across lanes. Moreover, when individual analyses required multiple gels and blots, we normally include a loading control containing a known concentration of 4-HNE- or ACR-modified mitochondrial protein to enable quantitation across multiple blots.

Statistical analysis for in vitro mitochondrial studies

All results were calculated using GraphPad Prism (GraphPad Software Inc., La Jolla, CA), and expressed as means $\pm$ standard deviation. For mitochondrial respiration studies, ACR and 4-HNE dose response and PZ or PG versus ACR or 4-HNE were analyzed by two-way analysis of variance (ANOVA), which, if it was significant, was followed by Student-Newman-Keuls multiple comparisons testing. Oxidative damage markers 4-HNE and ACR measured by western blot were analyzed by two-way ANOVA, followed by Dunnett's multiple comparisons post-hoc testing.

Controlled cortical impact traumatic brain injury model procedures

Young adult male SD rats (Harlan Labs, Indianapolis, IN), weighing 300–350 g, were used in experiments wherein CCI procedures were used to induce a TBI. All of the following mitochondrial isolation studies involving respiratory and oxidative damage measurements were carried out at 72 h. This time point was chosen based upon our time-course studies, which have shown 72 h to be the post-TBI time point at which mitochondrial dysfunction and oxidative damage are at their peak (Hill and colleagues, submitted). Rats were anesthetized with 5% isoflurane (SurgiVet, 100 series; Smiths Medical ASD, Inc. St. Paul, MN) and oxygen (SurgiVet, O₂ flow meter 0–4 liters per minute; Smiths Medical ASD). Heads were shaved and placed into a stereotaxic frame (Kopf Instruments, Tujunga, CA). Isoflurane was reduced to 2% for the remainder of the procedure. Lubrication was applied to the eyes to prevent drying, and a rectal probe was used to maintain body temperature at 37°C by an electronic heat pad under the animal. A midline surgical incision was made to retract skin and expose the skull for a 6-mm unilateral craniotomy. The craniotomy was performed with a Michelle trephine (Miltex, Bethpage, NY) centered between bregma and lambda sutures, lateral to the sagittal suture. The cortex was exposed by removing the skull cap carefully without damaging the dura. The exposed brain was injured with an electronic CCI device (TBI 0310; Precision Systems & Instrumentation, Fairfax Station, VA). The dura was injured by a 5-mm-diameter beveled tip that compressed the exposed cortex 2.2 mm at a velocity of 3.5 m/sec and a dwell time of 500 ms. A thin veil of Surgicel (Johnson & Johnson, Arlington, TX) was placed over the injury. A pre-made plastic cap with an 8-mm diameter was attached directly to the skull over the injury site with commercial cyanoacrylate. The cyanoacrylate was allowed to dry before the wound was closed with staples. Rats were removed from the stereotaxic head holder and placed into an incubation cage, which

provided heat through conduction by an electronic water pump. Rats were monitored until the righting reflex was regained.

Sham groups of rats were subjected to the craniotomy, but not CCI. Vehicle treatment groups were exposed to craniotomy, CCI, and received the vehicle solution (0.9% saline). PZ treatment groups received the craniotomy, CCI, and the drug solution in its respective vehicle. Rats were randomly assigned to designated groups.

Isolation of rat brain mitochondria for in vivo traumatic brain injury studies

Rats were exposed to CO₂ for approximately 1 min until flaccid. Brains were rapidly extracted after decapitation. Ipsilateral cortical tissue was dissected on ice from the rest of the brain and an 8-mm-diameter circular trephine was used to extract tissue corresponding to the site of impact including the penumbra. The cortical tissue was homogenized using a Potter-Elvehjem manual homogenizer in 3 mL of ice-chilled isolation buffer (215 mM of mannitol, 75 mM of sucrose, 0.1% BSA, 20 mM of HEPES, and 1 mM of EGTA, pH adjusted to 7.2 with KOH). Differential centrifugation at 4°C was used to separate nuclei and cellular components from crude mitochondrial pellet. Tissue was exposed to two rounds of centrifugation (1300g for 3 min) at 4°C. After each centrifugation, the supernatant was decanted and diluted in isolation buffer with EGTA. Supernatants were centrifuged at 13,000g for 10 min. The pellet was resuspended carefully in 400 μ L of isolation buffer with EGTA, which was placed inside a nitrogen bomb (Parr Instrument Co., Moline, IL) at 1200 pounds per square inch for 10 min to disrupt synaptosomes. Samples were then added onto a discontinuous 7.5% and 10% Ficoll layered gradient. Samples layered on the Ficoll column were centrifuged at 100,000g for 30 min at 4°C within a Beckman SW 55ti rotor. The resultant pellet was resuspended in 25–50 μ L of isolation buffer without EGTA to yield a concentration of $\sim$ 10 mg/mL. Final protein concentration was determined using a BCA protein assay kit measuring absorbance at 562 nm with a BioTek Synergy HT plate reader (BioTek Instruments).

Mitochondrial bioenergetic analysis for in vivo traumatic brain injury studies

For the *in vivo* TBI studies in which the mitochondria were isolated at 72 h post-injury from the injured cortex for *ex vivo* analysis of respiratory function, the Clark-type oxygen electrode (Oxytherm; Hansatech Instruments, Norfolk, UK) was used to enable a more-detailed analysis of complex I- and complex II-driven oxygen utilization than is possible for the Agilent Technologies XFe24 analyzer we used for the above-described *in vitro* studies. Approximately 180–200 μ g was able to be isolated from injured and uninjured cortical punches. However, 100 μ g of isolated mitochondrial protein were used as previously described⁵ to assess mitochondrial respiratory function. Mitochondria were suspended in respiration buffer (215 mM of mannitol, 75 mM of sucrose, 0.1% BSA, 20 mM of HEPES, 2 mM of MgCl₂, and 2.5 mM of KH₂PO₄, pH adjusted to 7.2) in a volume of 250 μ L, which was continuously stirred in a sealed chamber at a constant 37°C. In accord with previously published articles, respiratory function was measured as the amount of oxygen consumed over time (nmoles/min). Substrates were added to the chamber in the order presented in Table 1.

Western blot analysis of mitochondrial oxidative damage markers for in vivo traumatic brain injury studies

In order to measure markers of oxidative damage (4-HNE), the following western blot analysis was used, which is in accord with

TABLE 1. INDUCERS OF VARIOUS STATES OF RESPIRATION (AT FINAL CONCENTRATIONS) FOR EX VIVO MITOCHONDRIAL BIOENERGETIC ANALYSIS IN THE OXYTHERM

State	Substrates	Amount	Procedure
I	No substrates	n/a	Equilibrate for 1 min Contains only mitochondria and respiration buffer
II	Pyruvate+malate	2.5 μ L of 5 mM of pyruvate +2.5 mM of malate	Complex I substrates allowed to mix with mitochondria and reach a steady rate
III	ADP	1.25 μ L of 150 μ M	Allowed to achieve maximal respiration with a second bolus of ADP
IV	Oligomycin	0.25 μ L of 1 μ M	Allowed to reach maximum effect in approximately 2 min
Va	FCCP	0.5 μ L of 1 μ M	Allowed to reach maximum effect in approximately 2 min
Vb	Rotenone	0.2 μ L of 1 mM	Allowed to inhibit complex I respiration
	Succinate	5 μ L of 10 mM	Complex II substrates allowed to drive respiration

ADP, adenosine diphosphate; FCCP, carbonylcyanide-p-trifluoromethoxyphenylhydrazone; n/a, not applicable.

previously published methods.^{5,9} Mitochondrial samples assayed for 4-HNE adducts were separated on a precast gel (12% Bis-Tris [w/v] acrylamide; Criterion XT, Bio-Rad Laboratories) with XT-MOPS buffer (Bio-Rad Laboratories). Mitochondrial samples assayed for ACR adducts were separated on pre-cast gel (4–12% gradient Tris-acetate gels) using MOPS buffer (Bio-Rad Laboratories). Proteins separated on gel were transferred to nitrocellulose membranes with a semidry electrotansferring unit (Bio-Rad Laboratories) at 15 volts for 45 min at room temperature. Following transfer, membranes were blocked in 5.0% milk in TBS (20 mM of Tris HCl and 150 mM of NaCl) for 1 h at room temperature. Membranes were then incubated at 4°C overnight in primary 4-HNE antibody solution TBST (0.5 mM of Tween-20). 4-HNE rabbit polyclonal primary antibody (Alpha Diagnostics International) was diluted 1:2000.

The 4-HNE primary antibody was detected by 2-h incubation at room temperature with goat-rabbit immunoglobulin G (IgG) or antimouse IgG secondary antibody conjugated to infrared dye (1:5000, IRdye-800CW; Rockland Immunochemicals, Inc., Gilbertsville, PA) in TBST. Membranes were analyzed with the Li-Cor Odyssey Infrared Imaging System (Li-Cor Biosciences). The 4-HNE protein smears were assessed from 150 to 50 kD.

Cortical tissue sparing analysis for traumatic brain injury studies

After 72 h post-TBI, animals were anesthetized with an intraperitoneal overdose of pentobarbital (150 mg/kg) and transcardially perfused with 0.01 M of phosphate buffer solution (PBS) followed by 4% paraformaldehyde (PFA) solution in 0.01 M of PBS at pH 7.4. Brains were rapidly extracted and allowed to soak in 4% PFA. Brains were removed after 24 h and allowed to soak in 20% sucrose+PFA solution for an additional 24 hours. Tissue was rapidly frozen on the microtome platform at –20°C for 10 min. A freezing microtome was used to cut coronal slices 40- μ m thickness at a blade angle of 7.5 degrees to the surface of the tissue. Sections were taken from the septal area (intra-aural level, 10.7) to the posterior hippocampus (intra-aural level, 0.3). Every tenth section was taken for mounting; a total of approximately 12 sections represented one brain. Mounted sections were soaked in CHCl₃ (100mL)+EtOH (400mL of 95% EtOH) for 30 min and then transferred to increasing concentrations of EtOH for dehydration (95%, 70%, and 50%) for 3 min each, then soaked in dH₂O for 3 min. Slides were then soaked in Nissl/cresyl violet solution (5.64 mL of 1 M of acetic acid, 0.816 g of sodium acetate, 500 mL of dH₂O, and 0.2 g of cresyl violet powder [Sigma C1791]) for 5–10 min. Then, slides were dipped eight times in dH₂O, 50% EtOH, 70% EtOH, and 95% EtOH+acetic acid (500mL of 95% EtOH+12 drops of acetic acid), then 3 min in 95% EtOH, 5 min in 100% EtOH, 5 min in Citrisolv stock solution, then 5 min in a second batch of Citrisolv stock so-

lution. Tissue sections were lightly covered with Permount and cover-slipped.

Slides were individually photographed with a bright-field microscope at 1.25 $\times$. Tissue sparing across groups was processed utilizing ImageJ (National Institutes of Health, Bethesda, MD) software. Analysis of rat brains was blinded and used the Cavalieri stereological protocol^{5,10}; the group for which each rat belonged (vehicle, PZ treatment single dose, or PZ treatment double dose) was unknown. Each section was separated by a known distance (d), where d =mean section thickness multiplied by the number of sections between the sampled sections. Total cortical area was measured by pixels and converted to mm² and is defined as the dorsal aspect of lamina I to the dorsal aspect of the corpus callosum, and was measured for each hemisphere. Each area was multiplied by d to calculate a subvolume that, when summed, provided the respective total volume. The volume of the ipsilateral (injured) cortex was compared to the contralateral (uninjured) cortex and expressed as percent of spared tissue (ipsilateral volume/contralateral volume, $\times$ 100). Ipsi- to contralateral comparison provided effective means of measuring spared tissue without having to compensate for variability in tissue fixation or processing across different brains and eliminated the need to euthanize 8 sham animals. Measurements of cortical spared tissue volumes were conducted in a blinded fashion.

Statistical analysis for traumatic brain injury studies

All experiments investigating the effects of TBI on cortical mitochondrial respiration and oxidative damage, cortical α -spectrin degradation, and cortical spared tissue volumes were analyzed by one-way ANOVA, which, if it showed significance, was followed by Dunnett's multiple comparisons post-hoc testing. In all cases, statistical significance was determined as $p < 0.05$.

Results

In vitro studies with normal brain mitochondria exposed to 4-hydroxynonenal or acrolein

4-hydroxynonenal and acrolein inhibit brain mitochondrial respiration. Ficoll-isolated cortical mitochondrial bioenergetics was assessed using an Agilent Technologies XFe24 analyzer 10 min post-exposure to 4-HNE and ACR. *Ex vivo* isolated mitochondria exposed to a 5-min PZ pre-treatment followed by 10-min exposure to increasing concentrations of 4-HNE 10, 30, and 100 μ M or ACR 1, 3, and 10 μ M inhibited mitochondrial respiration in a significant, dose-dependent manner for complex I- and complex II-driven OCR. Mitochondria exposed to the 30- and 100- μ M 4-HNE concentrations showed significantly inhibited complex

I- and complex II-driven OCR ($p < 0.05$). 4-HNE at 30 μM was deemed an optimal concentration to reduce, but not completely inhibit, mitochondrial respiration for either complex I or II. However, ACR was able to significantly reduce complex I and II respiration at all tested concentrations compared to controls: 1, 3, and 10 μM ($p < 0.05$). ACR at 3 μM was deemed optimal. Thus, ACR was found to be approximately 10-fold more potent than 4-HNE in inhibiting mitochondrial complex I and complex II OCRs.

Phenelzine protects against 4-hydroxynonenal- or acrolein-induced mitochondrial dysfunction in a concentration-dependent fashion. The Agilent Technologies XFe24 analyzer was used to determine the ability of PZ to protect cortical, isolated mitochondrial OCR exposed for 10 min to 4-HNE (30 μM) or ACR (3 μM). Concentrations of 4-HNE and ACR were chosen based on previous experiments (Fig. 2). Mitochondria were incubated with a 5-min pre-treatment of increasing concentrations of PZ (3, 10, and 30 μM) then incubated with a 10-min treatment of 4-HNE or ACR.

Mitochondrial OCR rates are reported for complex I and complex II. For mitochondria exposed to 4-HNE, significant protection was observed in complex I-driven OCR with 10- and 30- μM concentrations of PZ ($p < 0.05$; Fig. 3A). As for 4-HNE-insulted mitochondria, complex II-driven OCR was only significantly protected by the maximum tested PZ concentration: 30 μM ($p < 0.05$; Fig. 3B). In similar experiments, PZ, only at the maximum dose tested (30 μM), was able to significantly protect ACR-insulted mitochondria for both complex I- and II-driven OCRs ($p < 0.05$; Fig. 3B,C).

Phenelzine, but not pargyline, protects against 4-hydroxynonenal- and acrolein-induced mitochondrial dysfunction. The comparative ability of PZ or PG to prevent mitochondrial dysfunction was assessed with the Agilent Technologies XFe24 analyzer. Concentrations of 4-HNE (30 μM) or ACR (3 μM) were chosen based on their demonstrated efficacy to significantly inhibit mitochondrial OCR (Fig. 2). Similarly, the concentration of

FIG. 2. 4-hydroxynonenal (4-HNE) and acrolein (ACR) reduces complex I- and complex II-driven respiration in a dose-dependent fashion in cortical mitochondria isolated from young adult, uninjured SD rats. Isolated mitochondria were exposed to increasing concentrations of 4-HNE for 10 min at room temperature and immediately assessed for oxygen consumption rates. 4-HNE at 30 and 100 μM significantly inhibited both complex I- and complex II-driven respiration. ACR at all tested concentrations (1, 3, and 10 μM) was able to significantly inhibit mitochondrial respiration. One-way analysis of variance followed by Dunnett's multiple comparison post-hoc test. * $p < 0.05$. Error bars represent $\pm$ standard deviation. $N = 4$ rats per group. ADP, adenosine diphosphate; Mito, mitochondria; SD, Sprague-Dawley; UT, untreated.

FIG. 3. Isolated mitochondria were exposed to a 5-min phenelzine (PZ) pre-treatment, followed by a 10-min incubation of 4-hydroxynonenal (4-HNE) and acrolein (ACR), and immediately assayed for oxygen consumption rates. PZ pre-treatment was able to protect mitochondrial respiration in a dose-dependent manner. (A) PZ, at 10 μ M and 30 μ M, was able to significantly protect mitochondrial function against an insult of 4-HNE at 30 μ M for complex I-driven respiration. (B) PZ was also able to significantly protect complex II-driven respiration from a 30- μ M insult of 4-HNE. (C and D) PZ, at 30 μ M, was able to significantly protect complex I- and complex II-driven respiration from 3 μ M of ACR. One-way analysis of variance followed by Dunnett's multiple comparison post-hoc test. * $p < 0.05$. Error bars represent $\pm$ standard deviation. $N = 4$ rats per group. ADP, adenosine diphosphate; Mito, mitochondria.

PZ (30 μ M) was chosen based on previous experiments demonstrating significant prevention of mitochondrial dysfunction (Fig. 3). With these conditions established, isolated cortical mitochondria were treated with either a PZ or PG pretreatment for 5 min, followed by ACR or 4-HNE exposure for 10 min. Mitochondrial OCR was expressed as percent control for ADP or succinate-driven respiration rates (e.g., complex I and complex II, respectively). Mitochondria exposed to 4-HNE (30 μ M) or ACR (3 μ M) significantly ($p < 0.05$) inhibited complex I- (ADP) and complex II-driven (succinate) OCR compared to untreated controls (Fig. 4). However, mitochondria exposed to PZ (30 μ M) pretreatment significantly prevented 4-HNE- or ACR-induced mitochondrial OCR for both tested complexes ($p < 0.05$). No significant difference was found between PZ-protected mitochondria and untreated controls. In the same experiment, mitochondria were instead pretreated with an analog compound, PG (30 μ M), followed by 4-HNE or ACR insult. Pargyline possesses a similar structure to

PZ and also serves as a MAO inhibitor, but lacks a hydrazine moiety functional group. Consistent with the hypothesized importance of the hydrazine functional group in carbonyl scavenging, PG-treated mitochondria demonstrated no significant protection against 4-HNE or ACR.

Phenelzine prevents mitochondrial oxidative damage in a dose-dependent fashion. Western blot analyses were used to determine whether increasing concentrations of PZ could reduce oxidative damage to mitochondrial proteins. A 50- μ g aliquot of isolated cortical mitochondria was utilized in the same manner as described above. A 5-min PZ exposure to isolated mitochondria demonstrated dose-dependent protection against exogenously applied 4-HNE (30 μ M) or ACR (3 μ M; Fig. 5). PZ at either 10- or 30- μ M concentrations significantly reduced 4-HNE adducts (i.e., oxidative damage markers) when compared to 4-HNE-only-treated

FIG. 4. Phenelzine (PZ) protects mitochondrial respiration from 4-hydroxynonenal (4-HNE) and acrolein (ACR) insult, but pargyline (PG) does not. Isolated mitochondria incubated with 4-HNE (30 μ M) or ACR (3 μ M) exhibited significantly impaired mitochondrial function for both complex I- and complex II-driven respiration. However, a 5-min pre-treatment of PZ (30 μ M) significantly protected mitochondrial respiration for both complex I- and complex II-driven respiration. Pargyline, which is also a monoamine oxidase inhibitor, but lacks a hydrazine moiety, was not able to effectively protect mitochondrial respiration from 4-HNE or ACR insult. One-way analysis of variance followed by Student-Newman-Keuls post-hoc test. * $p < 0.05$. Error bars represent $\pm$ standard deviation. $N = 6$ rats per group. ADP, adenosine diphosphate; Mito, mitochondria.

mitochondria ($p < 0.05$). Similarly, PZ at 10 and 30 μ M significantly reduced ACR adducts ($p < 0.05$).

Phenelzine, but not pargyline, reduces mitochondrial oxidative damage. Isolated cortical mitochondria exposed to 5-minute pre-treatments of PZ (30 μ M), but not PG, were able to significantly prevent accumulation of oxidative damage markers of 4-HNE or ACR by western blot analysis ($p < 0.05$; Fig. 6). Mitochondria exposed to 4-HNE (30 μ M) or ACR (3 μ M) revealed a significant increase in banding intensity compared to untreated controls. PG+ACR or PG +4-HNE treatments were not significantly different from 4-HNE- or ACR-treated mitochondria, respectively. Additionally, PZ-only- or PG-only-treated mitochondrial (controls) were not significantly different than untreated mitochondria.

Controlled cortical impact traumatic brain injury studies

Phenelzine maintains cortical mitochondrial respiratory control ratio after traumatic brain injury. Cortical mitochondria harvested at 72 h from TBI rats that received only vehicle treatment exhibited a 30% reduction of RCR post-injury when compared to sham controls ($p < 0.05$), as shown in Figure 7. In contrast, cortical mitochondria isolated from the injured brain of PZ-treated rats (10 mg/kg subcutaneously [s.c.] at 15 min post-TBI, followed by repeated 5-mg/kg s.c. maintenance doses at 12, 24, 36, 48, and 60 h post-TBI) at 72 h post-TBI exhibited a complete, statistically significant preservation of the RCR post-injury compared to vehicle-treated rats ($p < 0.05$ vs. vehicle TBI group). Moreover, PZ TBI rats were not significantly different than vehicle- or PZ-treated Sham controls (Fig. 7).

FIG. 5. Phenelzine (PZ) reduces 4-hydroxynonenal (4-HNE) and acrolein (ACR) in a dose-dependent manner. Mitochondria were isolated from cortex of uninjured SD rats and exposed to increasing concentrations of PZ pre-treatment (3, 10, and 30 μ M) for 5 min, followed by 4-HNE or ACR for 10 min. Mitochondria were then assessed for oxidative damage markers 4-HNE or ACR by western blot analysis between 150 and 50 kD. 4-HNE (30 μ M) or ACR (3 μ M) significantly increased oxidative damage. PZ, at 10 and 30 μ M, was able to significantly ameliorate 4-HNE or ACR in mitochondria. One-way analysis of variance followed by Dunnett's multiple comparison post-hoc test. Student *t*-test compared untreated to 4-HNE or ACR. #, * $p < 0.05$. Error bars represent $\pm$ standard deviation. $N = 6$ rats per group. Mito, mitochondria; SD, Sprague-Dawley.

Phenelzine attenuates cortical mitochondrial oxidative damage after traumatic brain injury. In a separate experiment with an additional cohort of rats, the ability of PZ to reduce 4-HNE-mediated mitochondrial oxidative damage at 72 h post-TBI was assessed by western blot analysis. In vehicle-treated TBI rats, mitochondrial 4-HNE accumulation was significantly ($p < 0.05$) elevated compared to either sham or sham+PZ control groups. PZ treatment post-injury was able to significantly ($p < 0.05$) reduce 4-HNE modification of mitochondrial proteins compared to that observed in mitochondria from vehicle-treated injured rat brains, although not to the level of the sham-only group (Fig. 7). Control groups (sham and sham+PZ) were not significantly different from each other.

Phenelzine increases cortical tissue sparing after traumatic brain injury. Ultimately, post-TBI neuroprotective efficacy depends upon a demonstration that PZ can decrease histological damage. Accordingly, we tested whether PZ treatment could increase cortical tissue sparing measured at 72h post-TBI, which is the peak of post-TBI mitochondrial respiratory functional compromise. We evaluated two PZ dosing paradigms designated PZs and PZm. PZs involved administration of a single dose of PZ 10 (mg/kg) injected s.c. 15 min post-TBI. This single dose had previously been shown, by our laboratory, to significantly increase cortical tissue sparing in the rat CCI model when assessed at 14 days post-injury.⁵ Additionally, we tested a repeated maintenance dosing regimen (PZm) involving the administration of the

FIG. 6. Phenelzine (PZ) reduces 4-hydroxynonenal (4-HNE) and acrolein (ACR), but pargyline (PG) does not. Uninjured rat mitochondria were isolated from cortex and were incubated with a 5-min PZ or PG pre-treatment followed by a 10-min incubation of 4-HNE (30 μ M) or ACR (3 μ M). Samples were then assessed by western blot analysis for measures of oxidative damage. 4-HNE- (30 μ M) or ACR-treated (3 μ M) mitochondria exhibited statistically significant elevations of 4-HNE or ACR compared to untreated groups. However, PZ, at 30 μ M, was able to significantly ameliorate 4-HNE or ACR accumulation. One-way analysis of variance followed by Dunnett's multiple comparison post-hoc test. Student *t*-test compared untreated to 4-HNE or ACR. $^{*}p < 0.05$. Error bars represent $\pm$ standard deviation. $N = 6$ rats per group. Mito, mitochondria.

same 10 mg/kg s.c. dose at 15 min post-injury, followed by a 5-mg/kg s.c. maintenance dose every 12 h out to 60 h post-TBI. In both dosing paradigms, rats were euthanized at 72 h post-TBI.

Figure 8A–C shows typical cresyl-violet–stained examples of the degree of cortical tissue loss in vehicle, PZ(S), and PZ(M) TBI rats in brain sections from the epicenter of the CCI-induced injury whereas Figure 8D shows the quantitation of cortical spared tissue in the ipsilateral injured hemisphere as a percentage of the cortical tissue volume in the contralateral noninjured hemisphere. In the vehicle-treated group, mean cortical tissue sparing was 93%. In the PZ(S) group, mean cortical tissue sparing was 96%. In the repeated-

dosing PZ(M) group, mean cortical tissue sparing was 103%. One-way ANOVA showed a significant difference ($F = 8.57$; $df = 2, 20$; $p < 0.002$) across the three groups, which enabled post-hoc testing. Accordingly, Dunnett's testing showed that whereas the single-dose PZ(S) group was not significantly different from vehicle, the multiple-dosing PZ(M) group did achieve significance compared to vehicle.

Discussion

Free radical induction of LP is one of the most validated secondary injury mechanisms in TBI pathophysiology and neurodegeneration.

FIG. 7. Top: Effects of phenelzine (PZ) on cortical mitochondrial bioenergetics 72 h post-injury following severe controlled cortical impact (CCI). Mitochondrial respiration was measured with a Clark-type electrode expressed as respiratory control ratio (RCR). The RCR is the rate of oxygen consumption during state III divided by state IV respiration. Substrates during states III and IV are defined within the text. Animals received PZ (10 mg/kg subcutaneously) 15 min post-injury followed by maintenance dosing (5 mg/kg subcutaneously) every 12 h; rats were euthanized at 72 h. Sham and sham+PZ groups were significantly different compared to vehicle groups. RCR of PZ treatment was significantly increased compared to vehicle and not significantly different from either sham control variant (sham or sham+PZ). One-way analysis of variance ($F=7.7$; $df=3, 24$; $p<0.009$) followed by Student-Newman-Keuls post-hoc test. $*p<0.05$. Error bars represent $\pm$ standard deviation; $n=8-9$ rats per group, except sham where $n=5$ rats per group. Bottom: PZ reduces 4-hydroxynonenal (4-HNE) accumulation in mitochondria after severe CCI-TBI when treated 15 min post-injury at 10 mg/kg, followed by 5 mg/kg every 12 h. Rats were euthanized at 72 h. (A) Quantification of markers of oxidative damage in cortical mitochondria. (B) Mitochondria were assessed for markers of oxidative damage by western blot analysis between 150 and 50 kD. 4-HNE was significantly elevated in the vehicle group compared to both sham groups, respectively. The PZ treatment group exhibited significantly reduced oxidative damage compared to the vehicle group, but did not return to sham levels. Analysis of variance ($F=9.9$; $df=3, 24$; $p<0.0002$) followed by Student-Newman-Keuls post-hoc test. $*p<0.05$. Error bars represent $\pm$ standard deviation; $n=8-9$ rats per group, except sham where $n=5$ rats per group. TBI, traumatic brain injury.

Numerous studies have demonstrated that antioxidant compounds possessing the ability to interrupt the LP cascade are protective in TBI models.²⁵ Most notably, tirilazad mesylate (U74006F) was able to inhibit the propagation phase of LP and improved post-TBI survival in patients with traumatic subarachnoid hemorrhage in a phase III clinical trial.⁴⁰ Unfortunately, these compounds have an inherently limited therapeutic window as their targets rapidly evolve during the course of the LP cascade. However, much of the damage caused from LP is attributed to the deleterious aldehydic breakdown products.⁴¹ Two notorious reactive aldehydes, 4-HNE and ACR, are broadly characterized by their electrophilic functional groups and

relatively long half-lives compared to highly reactive free radicals. Such characteristics allow 4-HNE and ACR to become ideal targets for pharmacological scavenging.

Scavenging reactive aldehydes or “carbonyl scavenging” is a treatment approach that essentially serves to provide sacrificial targets for 4-HNE and ACR. Both 4-HNE and ACR can be scavenged in this way.⁴² However, one compound in particular, PZ, provides the following distinct advantages as a carbonyl scavenger for treatment of TBI. Primarily, PZ is an FDA-approved and is readily available in a clinical setting. Because PZ is not a hypotension-inducing compound, unlike HZ, it would not be contraindicated for acute treatment of TBI, in which hypotension is not uncommonly observed as an injury-induced complication. Finally, PZ possesses the ability to function as an antioxidant against superoxide radical, as well as scavenging both unbound LP-derived carbonyls, and as well as scavenging some of the reactive aldehydes already bound to proteins.²⁷ The scavenging of 4-HNE and ACR bound to proteins by Michael addition will retain an exposed carbonyl capable that is capable of contributing to further damage; however, PZ, in a process dubbed “adduct-trapping,” is able to covalently bind, sequester the carbonyl, and prevent subsequent damage.³⁰

Although the therapeutic advantages of PZ are apparent, studies only recently have begun to elucidate the true implications PZ treatment in neurodegeneration. Additionally, the collapse of mitochondrial functional integrity is tightly associated with neuronal cell death.⁴³ Therefore, the first purpose of the current study was to investigate the ability of PZ to function as a carbonyl scavenger and protect mitochondria in an *ex vivo* model of TBI.

In vitro studies with normal brain mitochondria exposed to 4-hydroxynonenal or acrolein

In order to test PZ’s ability to scavenge reactive aldehydes, it was necessary to first establish that inhibition of mitochondrial respiration by 4-HNE or ACR could be measured in the Agilent Technologies Flux Analyzer and was consistent with previous studies that were conducted with the Hansatech Oxytherm Clark Type electrode.^{5,9} Accordingly, we were able to demonstrate a concentration-dependent relationship for 4-HNE and ACR to inhibit respiration. When mitochondria were exposed to PZ followed by suboptimal doses of 4-HNE or ACR, mitochondrial respiratory dysfunction could be prevented. Additionally, PZ pre-treatments were able to attenuate accumulation of mitochondrial protein oxidative damage markers measured by western blot.

The bases of PZ neuroprotection can exist in several modalities. For instance, the PZ ring structure can function as a scavenger of antioxidant for superoxide radical.⁴⁴ Additionally, the fact that PZ inhibits MAO activity predicts that it may provide mitochondrial, and therefore neuronal, protection by reduction of hydrogen peroxide and other aldehydes.⁴⁴ However, in the present article, we demonstrated that PZ is able to protect against mitochondrial respiratory functional failure caused by 4-HNE or ACR, whereas the nonhydrazine MAO inhibitor, PG, does not. Given that these compounds are structurally and functionally related, but only PZ prevents mitochondrial dysfunction and accumulation of oxidative damage markers in mitochondria, two reasonable conclusions can be made. The first is that PZ scavenges carbonyls through its hydrazine moiety, which is consistent with previous predictions.^{28,31} Second, a mere mechanical separation of protein target and reactive aldehyde is not sufficient to prevent oxidative dysfunction, otherwise PG+ACR or 4-HNE treatment groups would have reduced mitochondrial dysfunction and decreased oxidative damage

FIG. 8. Coronal sections of ipsilateral rat brains taken at $1.2\times$ magnification. (A) Vehicle-treated (0.9% saline) rat brain injected 15 min post-TBI. (B) Phenelzine (PZs) single-dose-treated animal, injected with a *single* dose of PZ, 15 min post-injury at 10 mg/kg. (C) Rat brain of PZ-treated with a multiple dosing paradigm (PZm): single subcutaneous injection of PZ 15 min post-injury, followed by maintenance dosing of 5 mg/kg every 12h thereafter. All groups (vehicle, PZ(S), and PZ(M)) were euthanized 72 h after first injection. Black bar represents 1 mm. (D) Percent of tissue sparing followed by either vehicle (saline), PZ(S), or PZ(M) treatment. Rats were euthanized in all treatment paradigms at 72 h after first injection. PZs did not exhibit a statistically significant amount of cortical tissue sparing when compared to vehicle. However, PZm significantly increased the total volume of spared cortical tissue. One-way analysis of variance ($F=8.5$; $df=2,20$; $p<0.002$) followed by Dunnett's post-hoc test. * $p<0.05$ compared to vehicle. Error bars represent $\pm$ standard deviation; $n=7-8$ rats per group. TBI, traumatic brain injury.

markers. Additionally, PG+ACR or 4-HNE groups were not statistically different than 4-HNE or ACR alone.

It should be noted that whereas there was a 10-fold concentration difference between 4-HNE and ACR used to decrease mitochondrial respiration and increase oxidative damage, this is consistent with our previous studies^{5,9} showing that ACR is 10 $\times$ more potent than 4-HNE. Despite the increased potency of ACR, PZ was still able to prevent mitochondrial dysfunction for both complex I- and II-driven respiration. Although the reported data show that complex II

is insulted more heavily by 4-HNE and ACR, and therefore PZ treatment has a greater protection for complex II, these protein complexes are not operating under the same respiration states. Complex I-driven respiration is measured during state III respiration, wherein oxidation and phosphorylation are coupled. Complex II-driven respiration is reported from state V uncoupled respiration. When inhibition and protection are compared for complex I and complex II respiration, in similar uncoupled states, complex I appears to be primarily insulted (data not shown).

Controlled cortical impact traumatic brain injury studies

Protection of cortical mitochondrial respiratory function. Our previous studies⁵ demonstrated that a single 10-mg/kg s.c. PZ dose administered shortly after CCI-TBI significantly reduced could maintain mitochondrial respiratory function in mitochondria harvested from the injured cortex as early as 3 h post-TBI. However, our recent detailed time-course studies (data now shown) show that whereas there is an initial depression of mitochondrial complex I-driven respiration during the first hours post-TBI, it recovers by 12 h, followed by a progressive secondary decline that begins at 24 h and peaks at 72 h. Thus, we felt it would be important to re-examine the ability of PZ to attenuate mitochondrial respiratory dysfunction at its peak time. Nevertheless, it seemed logical that to do so would require the administration of a repeated maintenance doses with a dosing interval based upon the roughly 12-h PZ half-life. Therefore, given that by 12 h following the initial administration half of the early PZ dose would be eliminated, we reasoned that we should administer repeated 5-mg/kg s.c. doses every 12 h (i.e., 12, 24, 36, 48 and 60 h post-TBI) in order to optimally maintain mitochondrial protection at the confirmed functional nadir at 72 h. By doing so, we observed a complete support of mitochondrial respiration attributed to our making sure that blood and brain levels of PZ would be maintained near the level associated with the 10-mg/kg s.c. dose level.

Attenuation of 4-hydroxynonenal-induced modification of mitochondrial proteins. Not surprisingly, preservation of mitochondrial respiratory function as a result of PZ treatment is paralleled by a PZ-induced decrease in the levels of 4-HNE bound to mitochondrial proteins. This is consistent with past results demonstrating the ability of free radical scavengers and other LP-inhibiting antioxidants to improve mitochondrial respiratory function along with a decrease in the modification of mitochondrial proteins by 4-HNE.^{16,45–47} Moreover, the observed ability for PZ to do the same even though it is not a classical electron-donating antioxidant can only be associated with its action to covalently bind LP-derived neurotoxic carbonyl compounds.

Improved cortical tissue sparing. Finally, our results confirm that PZ's scavenging of LP-derived neurotoxic carbonyl compounds, such as 4-HNE, is mechanistically connected to an increase in cortical tissue sparing, as first demonstrated in our earlier published preliminary studies.⁵ However, our earlier evidence of post-TBI neuroprotective actions of PZ involved administration of a single 10-mg/kg s.c. PZ dose, which significantly improved cortical tissue sparing at 14 days post-injury. In the present instance, we decided to repeat those experiments, but with a comparison of the relative cortical histological neuroprotection afforded by the previously tested single PZ dose compared to that obtained from the addition of maintenance dosing over a 60-h period. The rationale for the repeated dosing adequate to cover a 72 h post-TBI time frame was based upon our earlier documentation that 4-HNE accumulation in the CCI-TBI-injured brain does not peak until 72 h post-injury,⁴⁸ which is also the time at which mitochondrial respiratory dysfunction is at its maximum (Hill and colleagues, manuscript submitted). Thus, it seemed that a single early dose of PZ would not be optimal for intercepting all of the 4-HNE that would be produced and cause brain damage evolving over a 72-h period. As expected, our results clearly show that the repeated PZ dosing sufficient to maintain drug levels at a level associated with a reduction in carbonyl-mediated damage to cor-

tical proteins over that post-traumatic time frame is superior to a reliance on a single PZ dose that would not maintain blood and brain levels of PZ for the 72-h period. The repeated dosing approach produced a significant and complete preservation of cortical tissue volume, whereas the histological effects of the single PZ dose did not reach significance, although a trend toward increased tissue sparing.

We admit that a limitation of our current 72-h tissue sparing analysis is that optimal assessment of final post-injury tissue sparing in the CCI-TBI model should involve waiting until 7 or 14 days post-injury to make sure that final lesion volume expansion is more complete, which is normally done in our rat CCI-TBI neuroprotection studies.^{5,49} Had we waited until those later times in this study, we may have replicated significant tissue sparing with the single PZ(s) dosing as have shown in our initial studies.⁵ However, we maintain that considering the 72-h post-TBI time course of 4-HNE accumulation, the repeated PZ(m) is a better approach to optimize mitochondrial function and histological neuroprotection by the carbonyl scavenging approach.

Future studies. The current results are inadequate to confirm the translational potential for use of PZ or another carbonyl scavenger for attempting to achieve significant and practical neuroprotection in TBI patients. What we are, first of all, in the process of exploring is whether the neurochemical and histological evidence of post-traumatic neuroprotection, which we have reasonably confirmed to be associated with carbonyl scavenging, is associated with an improvement in motor and cognitive functional recovery. Second, we are currently determining whether the protective effects of carbonyl scavenging when the strategy is initiated early post-TBI is able to be achievable with delayed administration of the drug needed for a clinically practical therapeutic window. We are cautiously optimistic based upon recent findings that the maximum post-TBI increase in LP and carbonyl levels does not occur until 72 h in rodent TBI models.^{48,50} This suggests that holding off PZ treatment initiation for multiple hours ought to still be able to intercept the bulk of LP-associated carbonyls generated post-TBI.

Conclusion

In our currently reported *in vitro* studies, we found that exogenous application of 4-HNE or acrolein to noninjured rat brain mitochondria, mimicking the effects of these neurotoxic carbonyls in the injured brain, reduced respiratory function and increased markers of oxidative damage. PZ pre-treatment significantly prevented mitochondrial dysfunction and oxidative modification of mitochondrial proteins in a concentration-related manner. The effect was not shared by a structurally similar MAO-I, PG, which lacks the hydrazine group, confirming that the mitochondrial protective effects of PZ were related to its carbonyl scavenging and not to MAO inhibition. In subsequent *in vivo* studies, we documented that repeated PZ treatment begun shortly (15 min) after CCI-TBI and repeated every 12 h out to 60 h, attenuated 72-h post-injury mitochondrial respiratory failure together with a significant increase in cortical tissue sparing.

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Author Disclosure Statement

No competing financial interests exist.

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