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Tin Protoporphyrin Provides Protection Following Cerebral Hypoxia-Ischemia: Involvement of Alternative Pathways

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The contribution of heme oxygenase (HO)-linked pathways to neurodegeneration following cerebral hypoxia-ischemia (HI) remains unclear. We investigated whether HO modulators affected HI-induced brain damage and explored potential mechanisms involved. HI was induced in 26-day-old male Wistar rats by left common carotid artery ligation, followed by exposure to a humidified atmosphere of 8% oxygen for 1 hr. Tin protoporphyrin (SnPP; an HO inhibitor), ferriprotoporphyrin (FePP; an HO inducer), or saline was administered intraperitoneally once daily from 1 day prior to HI until sacrifice at 3 days post-HI. SnPP reduced ($P < 0.05$) infarct volume compared with saline-treated animals, but FePP had no effect on brain injury. SnPP did not significantly inhibit HO activity at 3 days post-HI, but SnPP increased ($P < 0.001$) total nitric oxide synthase (NOS) activity compared with HI + saline. Both inducible NOS and cyclooxygenase activities were attenuated ($P < 0.05$) by SnPP, whereas mitochondrial complex I and V activities were augmented ($P < 0.05$) by SnPP. SnPP had no effect on NMDA receptor currents. Overall, like other HO inhibitors, SnPP produced many nonselective effects, such as attenuation of inflammatory enzymes and increased mitochondrial respiratory function, which were associated with a protective response 3 days post-HI.

Key words: heme oxygenase; neuroprotection; nitric oxide synthase; cyclooxygenase; mitochondria

Heme oxygenase (HO; EC 1.14.99.3) is a well-established stress protein that responds to a range of noxious stimuli (Wagener et al., 2003). In addition to its primary role of breaking down heme, HO induces the formation of the antioxidant bilirubin, the gaseous second messenger carbon monoxide, and ferrous iron (Tenhunen et al., 1968). Through these products of heme metabolism, HO has the ability to protect against cellular damage induced by oxidative stress (Wagener et al., 2003), which may lead to opportunities for HO-based therapeutics in molecular medicine (Ryter and Choi, 2009). The inducible isoform of HO, HO-1, is induced following transient middle cerebral artery occlusion (MCAO) and hypoxia-ischemia (HI) in rats (Sutherland et al., 2009) and traumatic brain injury and focal cerebral infarction in humans (Beschorner et al., 2000). The constitutive isoform of HO, HO-2, was also recently shown to be up-regulated in the ipsilateral hemisphere following HI brain injury (Sutherland et al., 2009). Consequently, both HO isoforms have been implicated in the neurodegenerative cascade following cerebral ischemia. However, it remains to be ascertained whether these isoforms promote endogenous cytoprotection or simply form part of the inflammatory cleanup subsequent to the cerebral insult.

Compounds that are structurally similar to the heme molecule can modulate the activity of HO, which can subsequently alter the response to oxidative stress. Tin protoporphyrin (SnPP), an inhibitor of HO, exacerbated acute inflammatory pleurisy induced by carageenin, whereas ferriprotoporphyrin (FePP), an HO inducer, inhibited this acute inflammation (Willis et al., 1996). Furthermore, induction of HO by hemin administration protected against cerebral damage to the cortex and striatum but not the hippocampus in animals that had undergone global ischemia (Takizawa et al., 1998). In contrast, zinc protoporphyrin (ZnPP), another HO inhibitor, reduced infarct volume following transient MCAO (Kadoya et al., 1995; Zhao et al., 1996). In the present study, both SnPP and FePP were administered to identify whether the use of a HO modulator could protect against HI-induced brain damage and elucidate the mechanisms of action of any protection.

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MATERIALS AND METHODS

Experimental Design

Animals underwent HI and were treated with an HO inhibitor (SnPP), an HO inducer (FePP), or saline. Three days following HI, animals were assessed for cerebral injury and HO activity and expression. In addition, other inducible enzymes, including nitric oxide synthase (NOS) and cyclooxygenase (COX), were examined as well as mitochondrial energetics. The NMDA receptor was then explored as a mechanistic target.

Materials

The reagents used in this study were supplied by Sigma-Aldrich (St. Louis, MO) or BDH (Auckland, New Zealand) unless otherwise stated. All antibodies were supplied by Santa Cruz Biotechnology (Santa Cruz, CA).

Animals

All procedures were carried out in accordance with the guidelines on care and use of laboratory animals set out by the University of Otago Animal Ethics Committee. All animals were obtained from the University of Otago Animal Facility (Dunedin, New Zealand).

HI Model

The HI procedure in juvenile (26 days old) rats was carried out using established methods previously described (Sutherland et al., 2005). In brief, 26-day-old male Wistar rats were anesthetized, and the left common carotid artery was permanently ligated. Two hours later, conscious animals were exposed to a humidified hypoxic atmosphere of 8% O_2/92% N_2 (BOC Gases, Dunedin, New Zealand) for 60 min. Physiological parameters such as body weight, rectal temperature, and blood glucose were monitored throughout both peri- and postsurgical period and showed no difference between any treatment groups (data not shown). Three days post-HI, animals were euthanized by decapitation and brains were prepared for either histological or biochemical analysis. The 3-day post-HI time point for euthanasia was chosen because at this time the initial necrotic injury has occurred and the subsequent apoptotic injury via inflammation is developing (Northington et al., 2001). This time point has also been used successfully in previous studies (Clarkson et al., 2005; Sutherland et al., 2005).

Dosing Regime

Three groups of animals were utilized for histological analysis: 1) HI + 0.9% saline (n = 6); 2) HI + 30 μmol/kg SnPP (Frontier Scientific, Logan, UT; n = 6), and 3) HI + 30 μmol/kg FePP (Frontier Scientific; n = 8). Nonintervention controls (n = 8) were used to compare physiological and biochemical parameters. The doses used for SnPP and FePP were comparable to the in vivo doses used in previous studies by Willis et al. (1996) and Takizawa et al. (1998). SnPP and FePP solutions were prepared in the dark by dissolution in 0.1 M NaOH and titration with 0.1 M HCl until the solution reached pH 7.4. All three treatment groups were dosed intraperitoneally (i.p.) at fixed intervals: 1 day prior to HI, during HI (injection made between ischemia and hypoxia steps), and 1 day and 2 days post-HI. For experiments to assess mitochondrial complex activity independent of HI, saline or SnPP was administered i.p. once daily for 4 days before euthanasia.

Histology

Animals that were euthanized for histology had the brain extracted and were immediately assayed for infarct size. Brains were sliced into 1-mm sections using an ice-cold brain matrix (ASI Instruments, Warren, MI), incubated in 3% 2,3,5-triphenyltetrazolium chloride (TTC) for 30 min at room temperature and fixed in 10% neutral buffered formalin. The infarct area (in square millimeters) of each section was quantified in Axiovision software (Carl Zeiss Vision, Aalen, Germany), and the infarct volume (in cubic millimeters) of each brain was calculated by multiplying the infarct area by the thickness of the slice (1 mm). All animals produced a clear injury as indicated by a lack of TTC staining.

Sample Preparation for Biochemical Assays

Brains were isolated from animals specifically euthanized for biochemical analysis (n = 8 per group) and dissected into ipsilateral (left) and contralateral (right) hemispheres, snap frozen in liquid nitrogen, and stored at –80°C. Each hemisphere was homogenized on ice in 10 ml protease inhibitor buffer [1 μM pepstatin A, 10 μM leupeptin, and 1 mM phenylmethylsulfonyl fluoride in 50 mM Tris buffer (pH 7.6)]. The homogenates were centrifuged at 4°C at 425*g* for 5 min, and the supernatant was stored at –80°C until biochemical, mitochondrial, and Western blot (WB) analysis was carried out. One aliquot was immediately assayed for protein levels using the Bradford assay (Bradford, 1976).

HO Activity

Brain microsomes were isolated from homogenates (n = 6–7 per group) by centrifugation and retention of the supernatant at 1,000*g* for 5 min, 10,000*g* for 20 min, 20,000*g* for 20 min, and 105,000*g* for 60 min. After the final centrifugation step, the supernatant was discarded, and the pellet was resuspended in 50 mM Tris buffer (pH 7.4) containing 250 mM sucrose. Protein concentrations were standardized to 3 mg/ml. Liver microsomes were also prepared as a source of biliverdin reductase (BVR; EC 1.3.1.24). A rat liver was homogenized in 10 mM potassium phosphate buffer (pH 7.4) and underwent the same centrifugation steps as for brain microsomes. The final pellet was resuspended in 10 mM potassium phosphate buffer (pH 7.4). Protein concentrations were then standardized to 5–7 mg/ml.

For HO activity measurement, the modified method of Ryter et al. (2000) was used. The reaction mixture (total volume of 500 μl) was composed of 3 mg/ml brain microsomal sample, 40 mM glucose-6-phosphate, 50 U/ml glucose-6-phosphate dehydrogenase, 1 mM hemin, 5–7 mg/ml liver microsomal BVR, and 50 mM Tris buffer, pH 7.4, with 250 mM sucrose. To initiate the reaction, 25 μl of 20 mM NADPH was added, and the mixture was incubated at 37°C for 60 min in the dark. The reaction was stopped by the addition of 500 μl chloroform and centrifuged at 3,220*g* for 5 min

at room temperature. This separated the solution into three layers, of which 100 μl was taken from the organic clear bottom layer and transferred to a quartz 96-well plate. The absorbance was measured using a spectrophotometer at wavelengths of 464 nm and 530 nm. HO activity was calculated by the difference in absorbance between the two wavelengths and expressed as picomoles bilirubin/hour/milligram protein.

Nitric Oxide Synthase Activity

Nitric oxide synthase (NOS; EC 1.14.13.39) activity was determined using a method previously described (Sutherland et al., 2005). Both total NOS activity (calcium-dependent) and inducible NOS (iNOS) activity (calcium-independent) were assessed. NOS activity was expressed as picomoles [^{3}H]L-citrulline/30 min/milligram protein.

Arginase Activity

Arginase (EC 3.5.3.1) activity was also determined using the method described by Sutherland et al. (2005). Arginase activity was expressed as micrograms urea/hour/milligram protein.

Cyclooxygenase Activity

The COX (EC 1.14.99.1) radioimmunoassay was conducted as previously described (Kapoor et al., 2004). COX activity was expressed as picograms PGE_2/30 min/milligram protein.

Western Blotting

The WB procedure was performed as previously described (Sutherland et al., 2005), with several modifications. Sample homogenates were prepared in sample loading buffer, and 10 μg of each sample underwent sodium dodecyl sulfate-polyacrylamide gel electropheresis (Mini Protean III; Bio-Rad, Auckland, New Zealand; 7.5%, 10%, or 12% resolving gels) and was transferred onto a 0.2-μm polyvinylidene difluoride membrane (Bio-Rad). The membrane was blocked and then incubated with a primary polyclonal anti-rat antibody [1:5,000 rabbit HO-1 or HO-2, 1:1,000 rabbit neuronal NOS (nNOS) or endothelial NOS (eNOS), 1:500 rabbit iNOS, 1:2,000 rabbit arginase I or arginase II, 1:1,000 goat COX-1 or COX-2, or 1:1,000 mouse β-actin] overnight at 4°C. A horseradish peroxidase-conjugated secondary polyclonal IgG (1:2000 goat anti-rabbit, donkey anti-goat, or chicken anti-mouse) was then added. Bands were detected using the enhanced chemiluminescence detection system (Amersham Biosciences, Amersham, United Kingdom), and Quantity One (Bio-Rad) was used for densitometric analysis. Optical densities were standardized to β-actin optical density.

Mitochondrial Complex and Citrate Synthase Activities

Tissue homogenate samples for mitochondrial enzyme assays were prepared using the method of Clarkson et al. (2004). All of the mitochondrial and citrate synthase activity assays were conducted using established kinetic spectrophotometric assay methods that have previously been described in detail (Sutherland et al., 2005). Mitochondrial complex I (EC 1.6.5.3) activity and complex V (EC 3.6.3.14) activity were expressed as nanomoles NADH/minute/milligram protein. Mitochondrial complex II–III (complex II EC 1.3.5.1; complex III EC 1.10.2.2) activity was expressed as nanomoles reduced cytochrome c/minute/milligram protein. Citrate synthase (EC 2.3.3.1) activity was calculated and expressed as nanomoles 5,5′-dithio-bis 2-nitrobenzoic acid (DTNB)/minute/milligram protein.

Evoked NMDA Receptor Currents in Cortical Neurons

Currents of the NMDA receptor in cultured neocortical neurons were recorded using electrophysiological techniques previously described (Errington et al., 2006). Maximal responses for each neuron were evoked using 1 mM NMDA, and subsequent experiments were performed using the concentration (32 μM NMDA) required to evoke approximately 50% of the maximal current (Errington et al., 2006). $ZnCl_2$ (100 μM), $SnCl_2$ (100 μM), or SnPP (100 μM) was applied by bath perfusion and concurrently with each pulsed NMDA application. Data were acquired at 10 kHz and filtered at 2 kHz using an Axopatch 200 amplifier (Molecular Devices, Sunnyvale, CA) coupled to a micro1401 A/D converter (CED, Cambridge, United Kingdom). The peak amplitude of the current from baseline during the administration of NMDA was measured in Spike 2 software (CED).

Statistical Analysis

Results were expressed as mean ± SEM. A one-way ANOVA was used, followed by a post hoc Tukey's pairwise comparison test if significant effects were found in the ANOVA. $P < 0.05$ was considered statistically significant.

RESULTS

Infarct Area and Volume

Initially, the effects of SnPP and FePP treatment on the development of infarction following HI were investigated. Three days post-HI induction, animals suffered infarcts to cortical, striatal, thalamic, and hippocampal regions of the brain, which were reduced with SnPP (Fig. 1A). Pairwise comparisons showed that SnPP treatment significantly reduced the area of infarction compared with HI + saline animals at 3 mm ($P = 0.005$), 4 mm ($P = 0.01$), and 11 mm ($P = 0.01$) from the frontal pole of the brain (Fig. 1B). SnPP also reduced infarct area significantly compared with HI + FePP animals at 4 mm ($P = 0.048$), 7 mm ($P = 0.04$), 8 mm ($P = 0.027$), and 11 mm ($P = 0.001$) from the frontal pole (Fig. 1B). Total infarct volume calculations revealed that SnPP administration significantly reduced infarct volume compared with HI + saline animals [one-way ANOVA: $F(2,17) = 5.102$, $P = 0.018$; Tukey's: $P = 0.039$; Fig. 1C]. HI + FePP animals had an infarct volume similar to that of HI + saline animals, which was significantly higher than HI + SnPP ($P = 0.026$; Fig. 1C).

HO

Because both SnPP and FePP have been reported to be modulators of HO, their effects on HO activity

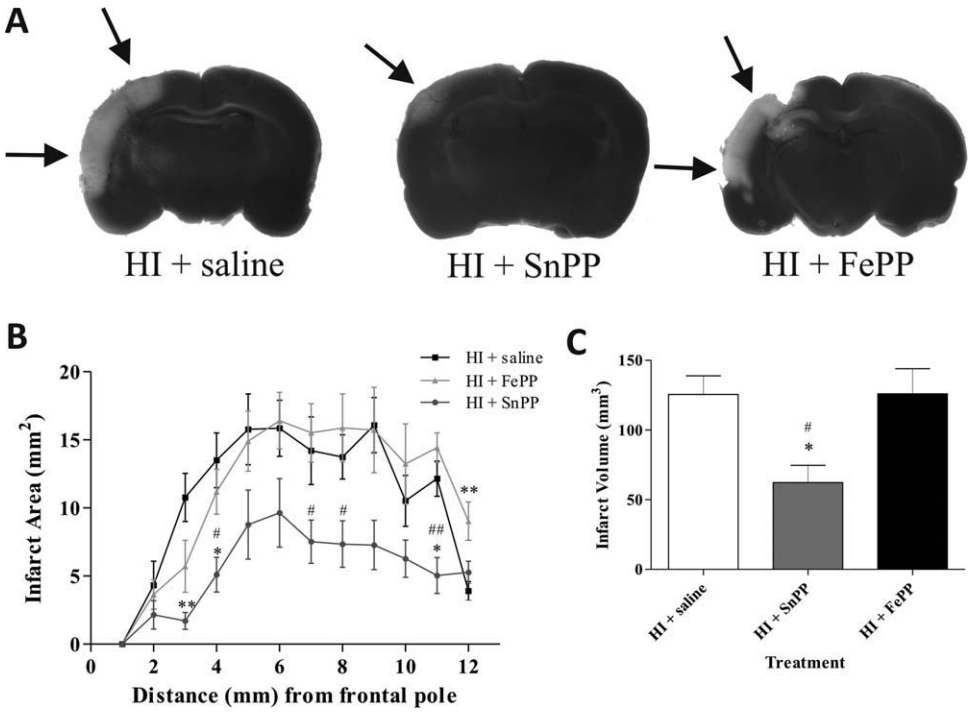

Fig. 1. SnPP reduced infarct area and volume 3 days post-HI. **A**: Three days following HI, brains were dissected out and sectioned. The infarcted area is immediately delineated by a lack of TTC staining (arrows). **B**: Quantification of infarct area following TTC staining. Infarct area was expressed in square millimeters. Black line (squares) denotes HI + saline (n = 6), light gray line (triangles) HI + FePP (n = 8), and dark gray line (circles) HI + SnPP (n = 6). **C**: Infarct volume was quantified by multiplying the area of infarct by the thickness of the slice throughout the brain. Infarct volume is expressed in cubic millimeters. The white bar denotes HI + saline, the gray bar HI + SnPP, the black bar HI + FePP. $^{*}P < 0.05$, $^{**}P < 0.01$ compared with HI + saline. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ compared with HI + FePP.

and isoform expression were examined. In the ipsilateral hemisphere, a one-way ANOVA showed that there was no significant treatment effect on HO activity 3 days following HI [$F(3,21) = 1.749$, $P = 0.188$; Fig. 2A]. Western blots of both HO-1 and HO-2 protein expression (Fig. 2B) in the ipsilateral hemisphere at 3 days post-HI also showed no significant treatment effect [HO-1: $F(3,10) = 1.874$, $P = 0.198$; HO-2: $F(3,10) = 0.848$, $P = 0.499$]. Although it visually appears that HI + saline might have induced both HO activity and HO-1 protein expression at 3 days post-HI compared with nonintervention controls, these differences were not significant (HO activity: $P = 0.207$; HO-1 expression: $P = 0.175$). No significant differences were found between any of the treatment groups in the contralateral hemisphere (data not shown).

NOS

It appears that, at 3 days post-HI, SnPP and FePP had no effect on HO. The metalloporphyrins are known to have many nonspecific effects, including modulation of NOS (Meffert et al., 1994). HO is closely linked with the NOS pathway, sharing many of the same properties (Maines, 1997). Therefore, the effects of SnPP on the NOS pathway were explored. In the ipsilateral hemisphere at 3 days post-HI, there was a significant effect of treatment on total NOS activity [$F(3,26) = 19.89$, $P < 0.001$]. In Figure 3A, pairwise comparisons showed that HI + saline animals had increased total NOS activity in the ipsilateral hemisphere ($P = 0.045$) compared with nonintervention controls. Inclusion of SnPP treatment further increased total NOS activity significantly ($P < 0.001$), whereas HI + FePP had no effect compared with HI + saline levels (Fig. 3A). When protein expression of individual NOS isoforms was examined by WB, no significant differences were found for any isoform between any treatment groups [nNOS: $F(3,9) = 0.761$, $P = 0.544$; eNOS: $F(3,10) = 0.107$, $P = 0.954$; iNOS: $F(3,9) = 0.748$, $P = 0.55$; Fig. 3B]. However, there was a significant treatment effect on iNOS activity [$F(3,20) = 3.377$, $P = 0.039$], and pairwise comparisons revealed that HI + SnPP had reduced ($P = 0.044$) iNOS activity compared with HI + saline (Fig. 3C). There were no significant differences in NOS activity or protein expression between any treatment groups in the contralateral hemisphere (data not shown).

Arginase

The arginase and NOS pathways have a close interaction, sharing the same substrate, so arginase activ-

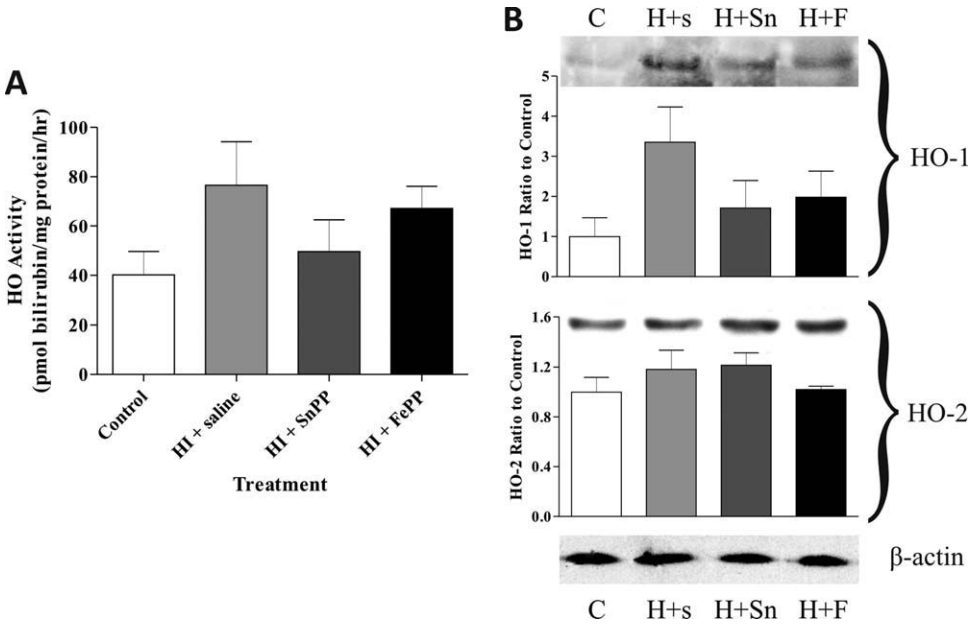

Fig. 2. SnPP and FePP did not significantly modify HO activity or HO-1 and HO-2 expression 3 days post-HI in the ipsilateral hemisphere. **A**: HO activity was analyzed from brain microsomes from the whole ipsilateral hemisphere of each treatment group, including nonintervention controls. HO activity is expressed as picomoles bilirubin/milligram protein/hour (n = 6–7 for each group). **B**: Both HO-1 and HO-2 isoforms were measured for protein expression using WB in the ipsilateral hemisphere. Data are expressed as a ratio of nonintervention control levels. Nonintervention control (C) is denoted by white bars (n = 3), HI + saline (H+s) by light gray bars (n = 4), HI + SnPP (H+Sn) by dark gray bars (n = 4), and HI + FePP (H+F) by black bars (n = 3).

ity and expression were investigated following HI and SnPP treatment. In both hemispheres, arginase I and II protein expression and arginase activity remained unaffected by any of the treatment protocols studied (data not shown).

COX

The COX and HO pathways also interact with each other (Mancuso et al., 2006), so SnPP may be affecting the production of eicosanoids, which was explored. At 3 days post-HI, both COX-1 and COX-2 protein expression were not significantly altered by any treatment group in the ipsilateral hemisphere [COX-1: $F(3,10) = 0.411$, $P = 0.749$; COX-2: $F(3,10) = 0.192$, $P = 0.9$; Fig. 3B]. However, in the ipsilateral hemisphere, there was a significant treatment effect on COX activity [$F(3,12) = 6.642$, $P = 0.007$]. Pairwise comparisons revealed that HI + saline animals had a significantly increased COX activity ($P = 0.008$) compared with nonintervention control animals (Fig. 3D). Meanwhile, SnPP treatment significantly reduced COX activity ($P = 0.02$) compared with HI + saline (Fig. 3D). Interestingly, FePP treatment also significantly attenuated COX activity ($P = 0.033$) compared with HI + saline (Fig. 3D). In the contralateral hemisphere, there were no significant treatment effects on COX activity or protein expression (data not shown).

Mitochondrial Complex Activity

The preservation of mitochondrial function is an important strategy for neuroprotection, so SnPP was examined to see whether it affected mitochondrial complexes. In both ipsilateral and contralateral hemispheres 3 days post-HI, there was a significant treatment effect on complex I activity [ipsilateral: $F(3,21) = 3.571$, $P = 0.031$; contralateral: $F(3,19) = 9.895$, $P < 0.001$]. Pairwise comparisons revealed that SnPP treatment significantly ($P = 0.047$) increased complex I activity compared with HI + saline in the ipsilateral hemisphere (Fig. 4A). In addition, SnPP treatment significantly increased complex I activity in the contralateral hemisphere compared with both HI + saline animals ($P = 0.015$) and nonintervention controls ($P = 0.002$; Fig. 4B). Interestingly, FePP treatment also increased complex I activity in the contralateral hemisphere significantly ($P = 0.026$ compared with HI + saline, $P = 0.003$ compared with nonintervention controls; Fig. 4B). To test whether SnPP alone was sufficient to increase complex I activity, SnPP or saline was administered to naïve animals. SnPP significantly increased complex I activity ($P = 0.045$) compared with saline treatment (Fig. 4C). Three days post-HI, there was no significant treatment effect on complex II–III activity in either the ipsilateral or the contralateral hemisphere (data not shown). Figure 4D shows that, in the ipsilateral hemisphere, there was a treatment effect on complex V activity [$F(3,21) = 4.762$, $P = 0.011$] and that SnPP treatment

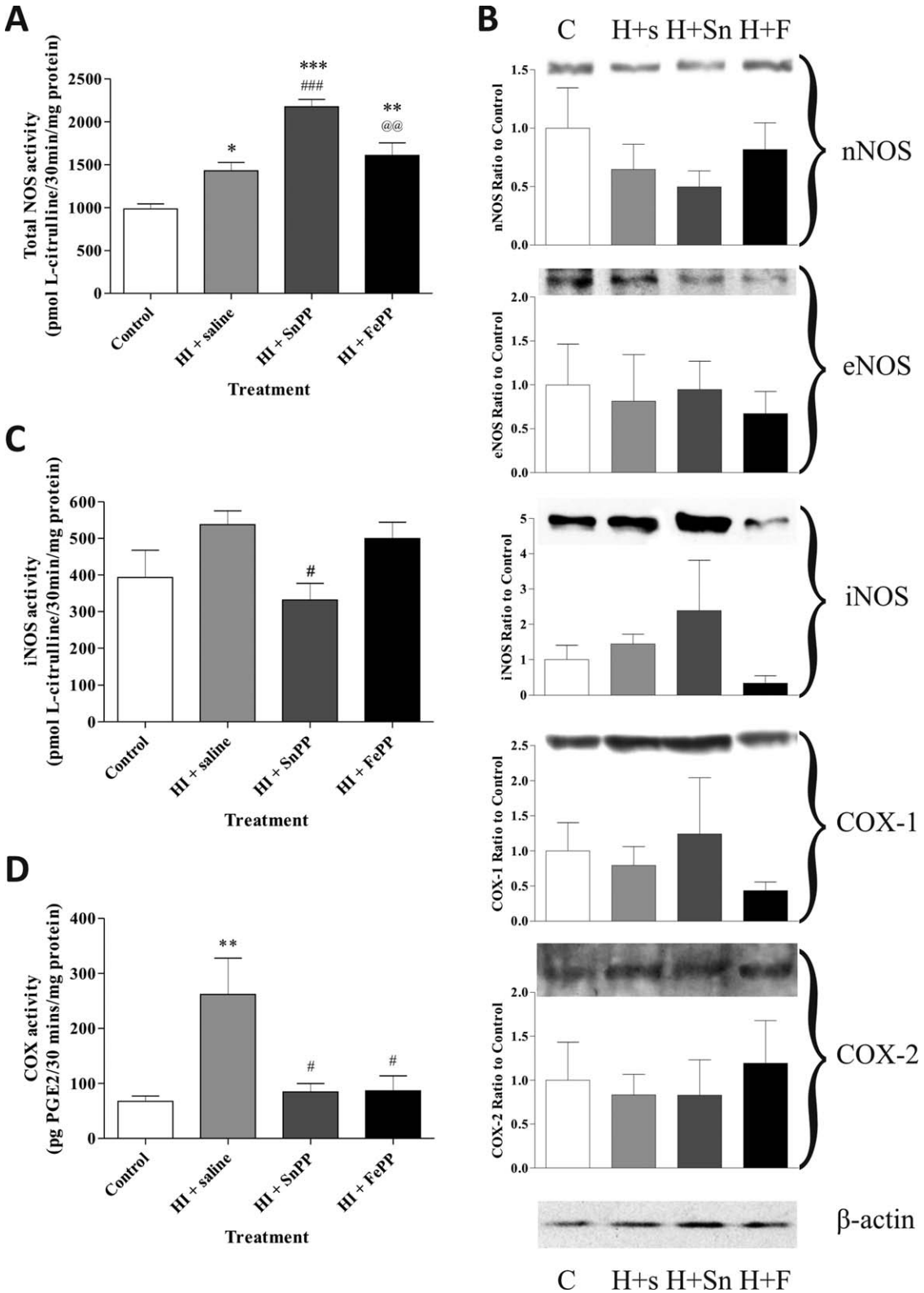

Fig. 3. SnPP increased total NOS activity but inhibited iNOS and COX activities 3 days post-HI in the ipsilateral hemisphere. For all of the graphs in this figure, white bars signify nonintervention controls (n = 6), light gray bars HI + saline (n = 8), dark gray bars HI + SnPP (n = 8), and black bars HI + FePP (n = 8). **A**: Total NOS activity was analyzed from whole-brain homogenates from the ipsilateral hemisphere. Total NOS activity was expressed as picomoles L-citrulline/30 min/milligram protein. **B**: Representative blots and quantitative protein expression for individual isoforms of NOS (nNOS, eNOS and iNOS) and COX (COX-1 and COX-2) that were assessed by WB in the ipsilateral hemisphere. Data are expressed as a ratio of nonintervention control levels. C, nonintervention control (n = 3); H+s, HI + saline (n = 4); H+Sn, HI + SnPP (n = 4); and H+F, HI + FePP (n = 3). **C**: Brain homogenates from the ipsilateral hemisphere were assessed for iNOS activity, which was expressed as picomoles L-citrulline/30 min/milligram protein. **D**: Whole-brain homogenates from the ipsilateral hemisphere were utilized to calculate COX activity, which was expressed as picograms PGE_2/30 min/milligram protein. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared with control. $^{\#}P < 0.05$, $^{\#\#\#}P < 0.001$ compared with HI + saline. $^{@@}P < 0.01$ compared with HI + SnPP.

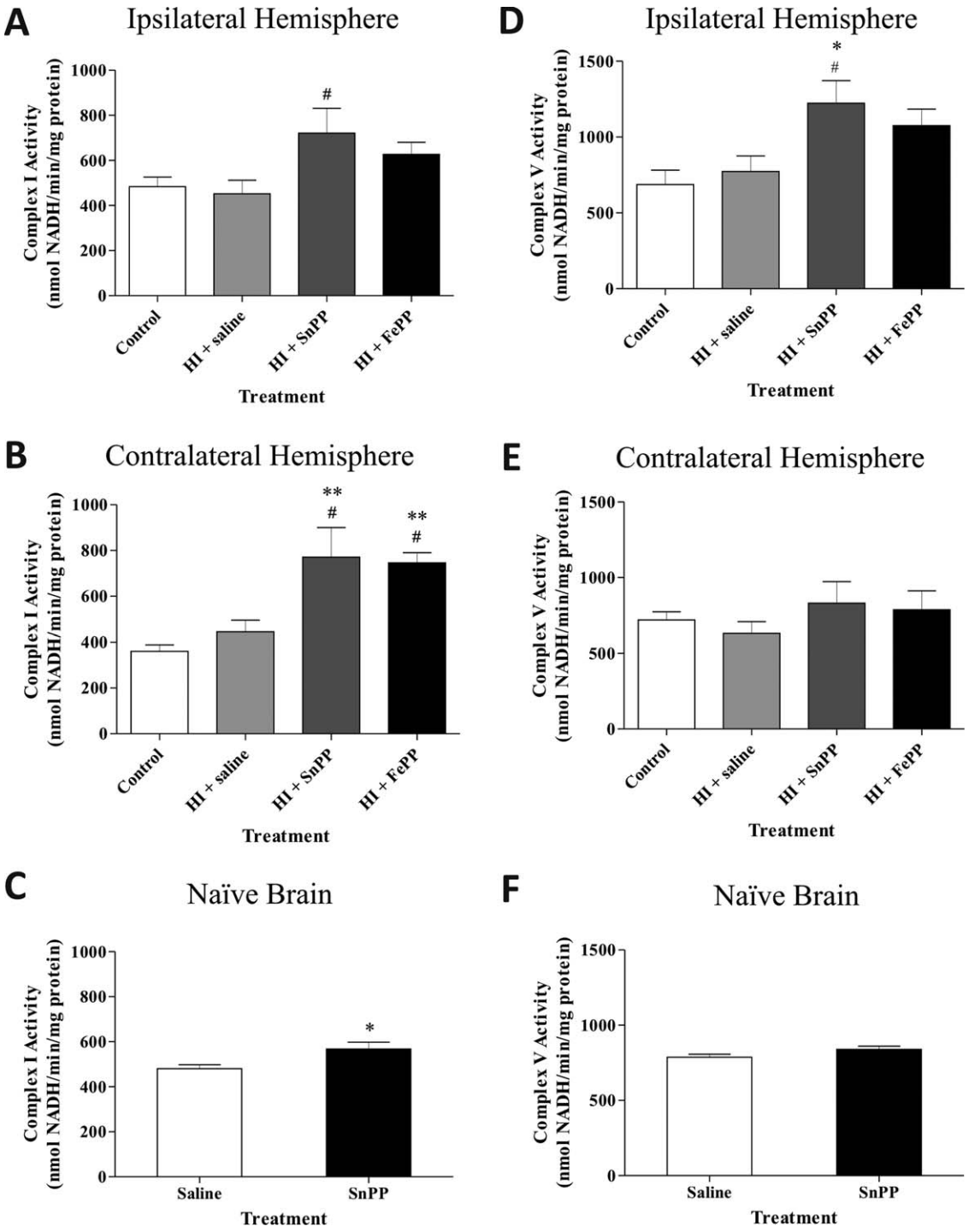

Fig. 4. SnPP significantly augmented complex I activity in both hemispheres and complex V activity in the ipsilateral hemisphere 3 days post-HI. In A,B,D,E, white bars signify nonintervention controls (n = 7), light gray bars HI + saline (n = 8), dark gray bars HI + SnPP (n = 6), and black bars HI + FePP (n = 8). Mitochondria were isolated and analyzed for complex I activity in the ipsilateral hemisphere (**A**) and the contralateral hemisphere (**B**). The same isolated mitochondria were also used to assess complex V activity in the ipsilateral (**D**) and contralateral (**E**) hemispheres. Mitochondria were also isolated from naïve animals treated with either saline (white bars, n = 4) or SnPP (black bars, n = 4) and assessed for complex I (**C**) and complex V (**F**) activities. Complex I and complex V activities are expressed as nanomoles NADH/minute/milligram protein. $^{*}P < 0.05$, $^{**}P < 0.01$ compared with control. $^{\#}P < 0.05$ compared with HI + saline.

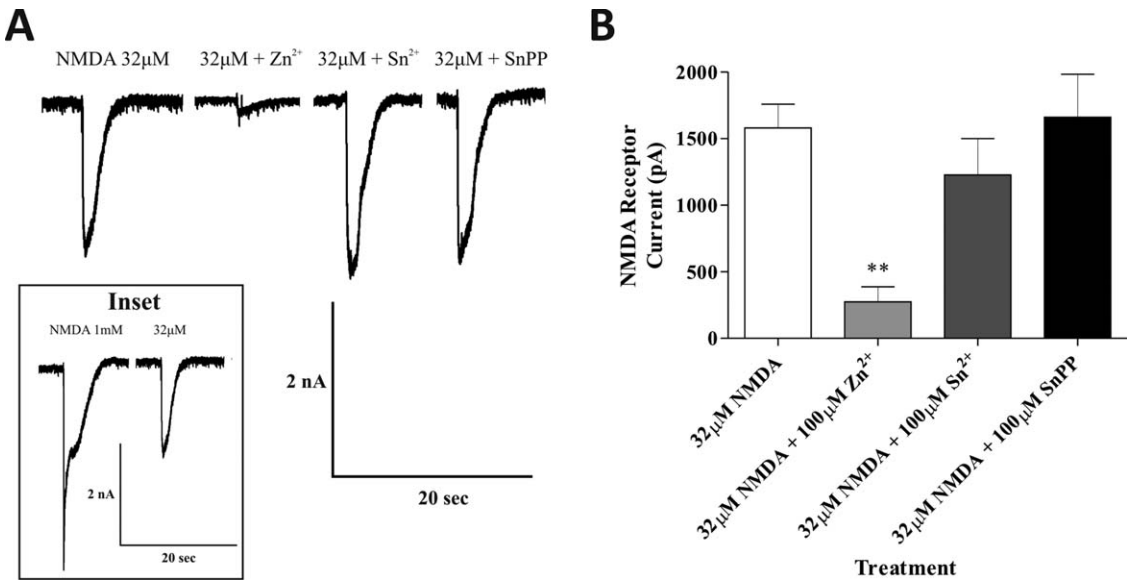

Fig. 5. SnPP and $SnCl_2$ do not significantly modulate evoked NMDA receptor currents in neocortical pyramidal neurons. **A**: Representative traces from experiments showing the significant ($P < 0.05$) reduction of NMDA receptor evoked inward currents by $ZnCl_2$ (100 μM, n = 4) but not SnPP (100 μM, n = 5) or $SnCl_2$ (100 μM, n = 5) compared with control 32 μM NMDA applications. **Inset** shows the concentration-dependent effect of NMDA. Thirty-two micromolar NMDA produces 50% of the maximal response under control conditions. **B**: Bar chart summarizing the effects of $ZnCl_2$, SnPP, and $SnCl_2$ on evoked NMDA receptor currents. $^{**}P < 0.01$ compared with 32 μM NMDA.

significantly increased complex V activity compared with nonintervention controls ($P = 0.018$) and compared with HI + saline ($P = 0.047$). There was no significant difference in complex V activity in the contralateral hemisphere between any treatment groups [$F(3,22) = 0.621$, $P = 0.609$; Fig. 4E]. Again, to test whether SnPP alone was sufficient to increase complex V activity, SnPP or saline was administered to naïve animals. SnPP did not alter complex V activity ($P = 0.127$) compared with saline treatment (Fig. 4F).

Citrate Synthase Activity

Citrate synthase activity is a marker of mitochondrial integrity. There was no significant difference in citrate synthase activity between treatment groups in either the ipsilateral or the contralateral hemisphere (data not shown).

Evoked NMDA Receptor Currents

A critical component of the neurodegerative cascade following HI is activation of NMDA receptors. The effects of $ZnCl_2$, $SnCl_2$, and SnPP on evoked NMDA receptor currents were assessed in cultured neocortical pyramidal cells. Under control conditions, 500-msec pulsed applications of NMDA produced concentration-dependent inward currents in voltage-clamped pyramidal neurons, as has been shown previously (Errington et al., 2006). Maximal currents evoked by 1 mM NMDA were 3,590 ± 238 pA (Fig. 5A, inset), whereas currents evoked by 32 μM NMDA were significantly smaller (1,581 ± 178 pA; $P < 0.001$; Fig. 5A). There was a significant treatment effect on NMDA receptor evoked currents [$F(3,19) = 5.858$, $P = 0.005$]. In the presence of 100 μM $ZnCl_2$, currents evoked by 32 μM NMDA were significantly attenuated in comparison with 32 μM NMDA alone ($P = 0.003$; Fig. 5B). In contrast, NMDA evoked currents were not significantly modulated in the presence of 100 μM $SnCl_2$ or 100 μM SnPP compared with 32 μM NMDA alone (Fig. 5B).

DISCUSSION

The effects of HO modulation on HI brain injury remains unclear. Here we provide clear evidence that SnPP reduced infarct volume 3 days post-HI, whereas FePP produced no effect on infarct volume (Fig. 1). Kadoya et al. (1995) and Zhao et al. (1996) also showed that ZnPP, another HO inhibitor, was neuroprotective following transient MCAO, but this might have been due to the inhibition of interleukin (IL)-1, not HO. There is also some in vitro evidence of HO inhibitor cytoprotection, wherein SnPP protected against hippocampal damage from hypoxia (Panizzon et al., 1996), and tin mesoporphyrin (SnMP) inhibited hydrogen peroxide-mediated astroglial injury (Dwyer et al., 1998). Conversely, Dore et al. (2000) showed that SnPP infusion in mice increased infarct volume following MCAO, which would suggest that HO activity is beneficial following cerebral ischemia. Further in vivo evidence of neuroprotection with an HO inducer was observed following global cerebral ischemia (Takizawa et al., 1998). Therefore, it appears that HO inhibition or induction

could be neuroprotective, depending on the model used and drug administration regime.

Since 1981, SnPP has been established as a potent competitive inhibitor of HO (Drummond and Kappas, 1981), whereas FePP/hemin is an established HO inducer as well as a substrate of this enzyme (Maines, 1997). Interestingly, Bing et al. (1995) showed that HO activity was reduced 6 hr following a single i.p. administration of SnPP, but daily i.p. SnPP administration for 4 days failed to maintain this HO inhibition. This suggests that repeated administrations of SnPP might have reduced efficacy for inhibition of HO. In the present study, HO activity and HO-1 and HO-2 protein expression were not significantly altered by daily treatment with SnPP, 4 days following the initial dose (Fig. 2), although SnPP tends toward a decrease. This does not preclude the possibility that there were transient effects on HO activity and protein expression by SnPP prior to the end of the 4 days of administration as suggested by Bing et al. (1995), but these measurements were not performed. Therefore, SnPP protected against injury from HI but not necessarily through reducing HO activity, whereas FePP had no effect on infarct volume, which may be due to HO activity not being affected by FePP. Another reason why HO activity and expression were not altered with SnPP or FePP could be the whole hemisphere being analyzed rather than individual brain regions such as the cortex or hippocampus. By using the whole hemisphere, both healthy tissue and injured tissue were analyzed together, which would diminish the effects on HO by these compounds.

All metalloporphyrins, including SnPP, produce many nonspecific effects exclusive of their HO modulatory properties (Grundemar and Ny, 1997), including modulation of NOS (Meffert et al., 1994) and soluble guanylate cyclase (Luo and Vincent, 1994). SnPP can also inhibit interleukin-6 and tumor necrosis factor-α up-regulation by hypoxia/reoxygenation in rat macrophages (Tamion et al., 1999). The HO system has a number of interactions with other inducible enzymatic pathways such as NOS and COX (Maines, 1997; Mancuso et al., 2006). Because of these close associations and the lack of selectivity of the metalloporphyrin compounds, we believe that SnPP might have been producing its protective effects against HI-induced brain injury through other inducible proinflammatory enzymes such as NOS and COX. Only when more selective HO modulators are produced will we be able to assess the role of HO in a number of contexts more adequately.

The HO and NO systems have many similarities and can regulate each other's activities (Maines, 1997), and so SnPP may have been producing its effects through the NO pathway. Similar to previous results obtained in our laboratory with other protective agents (Clarkson et al., 2004, 2005), reduced infarct volume with SnPP was associated with a significant upregulation of total NOS activity (Fig. 3A) and inhibition of iNOS activity (Fig. 3C). iNOS-derived NO contributes to late cerebral injury (Iadecola et al., 1995), so inhibition of iNOS activity by SnPP would protect neurons from NO neurotoxicity. The increase in total NOS activity by SnPP must have been due to nNOS or eNOS activity, but no change in expression was observed (Fig. 3B), and the NOS activity assay used does not delineate between nNOS and eNOS activity. eNOS up-regulation has been correlated with a reduction in cerebral infarction following HI (Sutherland et al., 2005) and is now a therapeutic target for cerebral ischemia because of its vasodilatory properties (Endres et al., 2004). The fact that SnPP-induced modulation of NOS activity does not involve altering NOS expression indicates that SnPP may be increasing/decreasing access for the substrate or cofactors of NOS without affecting NOS protein synthesis.

HO activity can also regulate COX activation and subsequent PGE_2 production (Mancuso et al., 2006), so SnPP might have been exerting its effects through the COX pathway. The COX pathway has been implicated in the neuroinflammatory cascade following cerebral ischemia, particularly the COX-2 isoform, insofar as knockout mice had a reduced infarct volume after MCAO (Iadecola et al., 2001). SnPP significantly inhibited COX activity (Fig. 3D) but did not alter COX-1 or COX-2 protein expression (Fig. 3B). Other HO inhibitors, ZnPP and SnMP, also decreased PGE_2 levels in hypothalamic explants (Mancuso et al., 1997) and astrocytic cultures (Vairano et al., 2001). This suggests that SnPP treatment for HI reduces PGE_2 production, which could lead to the protective response observed, but this does not involve the alteration of COX isoform protein synthesis. Interestingly, FePP treatment also reduced COX activity (Fig. 3D). Reduction of PGE_2 levels has also been shown by HO inducers (Haider et al., 2002), but in another study hemin increased PGE_2 levels (Mancuso et al., 1997). However, neuroprotection in this setting might have required the alteration of NOS or both NOS and COX activities, which may explain why SnPP was protective, given that HO activity was not affected.

The preservation of mitochondrial function and ATP levels is an important strategy that can prevent cell death following an ischemic insult. The activity of complex I is crucial for maintaining the ATP synthesis action of complex V, insofar as inhibition of complex I results in a rapid decrease of oxidative phosphorylation (Davey et al., 1998). The up-regulation of both complex I (both hemispheres) and complex V (ipsilateral hemisphere only) activities following SnPP administration (Fig. 4) could preserve mitochondrial energetics and reduce cell death in vulnerable cells after HI. Further experiments showed that SnPP, independent of HI, was sufficient to up-regulate complex I activity, which could potentially prime its protective effect and explain complex I up-regulation in both hemispheres (Fig. 4). In contrast, HI appears to be a prerequisite for an SnPP-induced increase in complex V activity (Fig. 4). FePP treatment also significantly increased complex I activity in the contralateral hemisphere following HI, but, because neither FePP nor SnPP affected HO activity in this paradigm,

this augmentation may be related to some other mechanism. The inhibitory action of SnPP on iNOS activity (Fig. 3C) might also have prevented the breakdown of mitochondrial complexes, which are susceptible to nitric oxide-based free radicals (Bolanos et al., 1995; Davey et al., 1998), following HI. Mitochondrial protection against oxidative damage with an HO inhibitor (SnMP) has also been demonstrated in astroglial cultures (Song et al., 2006). Therefore, the use of an HO inhibitor appears to maintain mitochondrial function during an HI insult, but further research is required to delineate how SnPP could be up-regulating mitochondrial complex activity.

The effects of SnPP on these biochemical parameters may not be direct but may be secondary responses related to the reduced cerebral damage that was produced by SnPP. In particular, NOS and COX are inflammatory enzymes that are up-regulated following HI, but, because SnPP reduced infarct volume (Fig. 1), NOS and COX activities were bound to be altered. Also, changes were observed only in the ipsilateral hemisphere, suggesting that these differing responses were due to the lesser extent of brain damage with SnPP and were not direct effects on these enzymatic pathways. Only when a study is conducted with multiple time-points of assessment for SnPP alongside other known neuroprotective agents will this question be answered.

A critical component of the ischemic injury cascade is the activation of NMDA receptors. Zn^{2+} is a non-competitive antagonist of the NMDA receptor (Peters et al., 1987) as evidenced by the reduction in NMDA receptor currents (Fig. 5). Both Zn^{2+} and ZnPP were neuroprotective against MCAO-induced brain damage (Zhao et al., 1996), and in the present study SnPP protected against HI-induced injury (Fig. 1). We hypothesized that Sn^{2+} or SnPP may alter NMDA receptor currents, which may contribute to the protective properties of SnPP, but no changes in NMDA receptor current were observed (Fig. 5). However, Li and Clark (2002) reported that administration of SnPP inhibited NMDA-induced pain behavior in rats, suggesting that SnPP might have an inhibitory effect on the NMDA receptor that has not been shown electrophysiologically.

In conclusion, SnPP (an established HO inhibitor) protected the brain from HI-induced cerebral damage, whereas FePP (an established HO inducer) had no effect. The results indicate that the mechanism by which SnPP exerted protection might not be related to HO inhibition but might involve the inhibition of iNOS and COX activities, while increasing total NOS activity. SnPP also augmented mitochondrial complex I and V kinetics, possibly as a consequence of its inhibitory action on iNOS. SnPP, like many metalloporphyrins, appears to have nonspecific effects, and the mechanism of SnPP protection against HI-induced injury may be through inhibition of iNOS and COX activities. Using a compound with multimodal actions that can modulate multiple pathways is more likely to reduce neuronal death and improve functional recovery following HI, but more selective HO modulators are required in order to assess the role of HO more adequately following HI.

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