SUPPLEMENTAL MATERIAL

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Primary neuronal cultures and oxygen-glucose deprivation

All experimental procedures were approved by the Institutional Animal Use and Care Committee of the University of Pittsburgh. Primary cultures of cortical neurons were prepared from 17-day-old Sprague-Dawley rat embryos as previously described [1, 2]. Experiments were conducted at 13 days in vitro (DIV), when cultures consisted primarily of neurons (approximately 95-97% MAP2-immunoreactive cells). To mimic an ischemia-like condition in vitro, primary cultures were exposed to combined oxygen and glucose deprivation (OGD) [1, 2]. Control glucose-containing cultures were incubated for the same periods of time at 37°C in humidified 95% air and 5% CO2. Neuronal death was measured at 24 hr after OGD using the lactate dehydrogenase (LDH) release assay (Sigma-Aldrich), Alamar Blue assay (Accumed International, Westlake, OH) and blind cell counting after Hoechst staining [1, 2].

CDDO-Im preparation, HO-1 inhibition and Nrf2 knockdown in cultures

The stock solution of CDDO-Im was prepared by dissolving CDDO-Im in dimethyl sulfoxide (DMSO) at a concentration of 10 mM [3]. To study its effects on HO-1 expression, the cultures were pre-treated with CDDO-Im for 2, 3, 6 or 16 hr at final concentrations of 30, 50, 100 or 300 nM. For Nrf2 nuclear translocation and neuroprotection studies, the cultures were treated with CDDO-Im for 2, 6 or 16 hr at a concentration of 100 nM.

Primary neurons were treated with 10 μM tin protoporphyrin IX (Sn-PPIX, Frontier Scientific, Logan) 2 hr after OGD. Sn-PPIX is a specific inhibitor of HO-1 activity [4, 5]. To assess the role of Nrf2 in the transcription of HO1, we transfected neurons with Nrf2 shRNA lentiviral particles (Santa Cruz, CA), which contain 3 rat-specific constructs, three days before the experiments at a titer of 2x105 IFU. Knockdown of Nrf2 was assessed by Western blot. Lenti-scramble was used as a control.

Rat model of global cerebral ischemia, CDDO-Im infusion and CA1 neuronal counting

Adult male Sprague-Dawley rats (Hilltop Lab Animals, Scottdale, PA) weighing 300-330 g were used to model transient global ischemia. Rats were randomly divided into sham and ischemia groups and anesthetized with 1.5-2% isoflurane in a mixture of 30% O2 and 70% N2O. Transient global ischemia was induced using a previously described model of four-vessel occlusion that lasted 12 min [6]. Rectal and brain temperatures and blood parameters were monitored and remained in the normal range throughout the experiments. Electroencephalography (EEG) was recorded to ensure isoelectricity at the onset of ischemia using a PowerLab system (ADInstruments, Colorado Springs, CO). Rats would be

excluded from study if their EEG failed to reach isoelectricity. Sham operations were performed in additional animals using identical surgical procedures, except that the common carotid arteries were not occluded.

Working solutions of CDDO-Im were prepared by diluting the stock solution with 0.5% bovine serum albumin (BSA) in phosphate-buffered saline (PBS). At indicated time-points before experiments, CDDO-Im was infused into the right intracerebral ventricle (ICV) at doses of 0.5, 1.0 or 1.5 μg in 10 μL. A Hamilton syringe was lowered into the brain at the following coordinates from bregma: anteroposterior, −0.8 mm; lateral, 1.5 mm; ventral, 3.5 mm. In selected experiments, rats were subjected to additional ICV infusion of 30 μg Sn-PPIX in 10 μL PBS at 2 hr after ischemia. To detect hippocampal CA1 neuronal death, rats were sacrificed 3 days after ischemia; the brains were removed and frozen in cold isopentane. Coronal sections of 20 μM were collected for hematoxylin and eosin (H&E) staining or DNA polymerase I-mediated biotin-dATP nick-translation (PANT) labeling [6]. In brief, sections were incubated at 37°C for 60 min with the PANT reaction mixture. The reaction was terminated by PBS washes. The slides were then incubated with dichlorotriazinyl aminofluorescein (DTAF, 1:1000, AnaSpec, Fremont, CA). The numbers of healthy or dead neurons in the entire CA1 were counted microscopically by two investigators blind to the experimental conditions [6].

Mouse model of focal cerebral ischemia, CDDO-Im injection and infarct volume measurement

Temporary focal cerebral ischemia was induced by intraluminal occlusion of the middle cerebral artery (MCAO) as previously described [1]. Male 25-30 g C57/B6 mice (Jackson Laboratories) were randomly divided into vehicle and CDDO-treatment groups and anesthetized with 1.5% isoflurane in a 30% oxygen/70% nitrous oxide mixture through a facemask under spontaneous breathing. Rectal temperature was maintained in a normal range during and after surgery via a temperature-regulated heating pad. Mean arterial blood pressure was monitored during MCAO through a tail cuff connected to a PowerLab system. Regional cerebral blood flow (rCBF) was monitored with a laser Doppler flowmetry (Perimed). Mice will be excluded from study if the rCBF failed to decrease to 20% of baseline during ischemia or failed to recover to 80% during reperfusion. The animals underwent left MCAO for 60 min and then reperfusion for 48 hr. Neurological dysfunction was evaluated by two investigators blind to the experimental conditions before the sacrifice using the 5-point method [7]. At 48 hr after MCA occlusion, brains were removed and sliced into 7 coronal sections each 1 mm thick. Sections were stained with a 2% solution of 2,3,5-triphenyltetrazolium (TTC). Infarct areas were measured blinded using the NIH Image J software (Bethesda, MD), and summed to infarct volumes. To detect the protective effects of CDDO-Im, the working solution was prepared by diluting the stock solution with 0.5% BSA in PBS. In total volumes of 0.5 ml, 10, 25, 50 or 100 μg of CDDO-Im was injected intraperitoneally immediately after withdrawal of the sutures. The vehicle-treated mice were injected intraperitoneally with the same volume of diluents. For the HO-1 inhibition study, Sn-PPIX was ICV injected 2 hr after ischemia, using the following coordinates from bregma: anteroposterior, 0.6 mm; lateral, 1.1 mm; and ventral, 2.2 mm.

Western blot

Primary neurons or brain tissues were collected at indicated time-points after CDDO-Im treatment or ischemia (n=4 per experimental condition), and then stored at -80° C until analysis. Tissues were homogenized in lysis buffer and sonicated. Total protein was extracted and subjected to Western blot analysis using standard methods [2, 6]. Blots were probed with antibodies recognizing HO-1 (1:3000, Enzo Life Science, Plymouth Meeting, PA). Gel analysis was performed with NIH Image J. To analyze the nuclear translocation of Nrf2 in primary neurons, the cultures were harvested and the nuclear fraction was extracted using a nuclear extraction kit (Thermo Scientific, Waltham, MA) and then subjected to Western blots using an Nrf2 antibody (Enzo, 1:1000).

Immunohistochemistry

In the CDDO-Im group, 1.0 μg was injected into the right ICV, and the animals were sacrificed and brains removed after 16 hr. In the ischemic group, rats were sacrificed at 24 or 72 hr after ischemia (n=3 per experimental condition). Sections at the level of the dorsal hippocampus were selected for immunohistochemical stains as described [2, 6]. The same HO-1 antibody (1:1000) as mentioned above was used for immunohistochemistry. HO-1 was visualized with a secondary antibody conjugated to Cy3. Cellular markers, including NeuN (Chemicon, 1:500), GFAP (Sigma, 1:500) and Iba1 (Wako, 1:1000), were co-stained with HO-1 and visualized with Alexa Fluor 488 (Invitrogen). DAPI was used for counterstaining. For the assessment of nonspecific staining, alternating sections from each experimental condition were incubated as above, but without the primary antibody.

Fluoro-Jade B Staining

Degenerated neurons are detected by Fluro-Jade B (FJB) (Millipore, USA) as described previously [8]. Briefly, brains were harvested two days of MCAO in mice. Paraffin sections were cut and immersed in 100% alcohol for 3 min and 70% alcohol for 1min and then washed with distilled water. After incubated in 0.06% potassium permanganate for 15 min and washed distilled water, the sections were stained with 0.001% FJB in 0.09% acetic acid for 20 min. Stained sections were observed under fluorescence microscope, and positive cells in three adjacent sections were counted blindly using 20x magnification.

HO-1 activity assay

HO-1 activity was measured as reported with minor modifications [4, 9]. In brief, brain tissues were harvested 24 hr after CDDO-Im injection or ischemia. Sn-PPIX was ICV infused 2 hr after the treatments. Normal liver was used as positive control. These tissues were homogenized at 4°C in 0.1 M potassium phosphate buffer, and total proteins were extracted. The reaction mixture was 200 μl, containing 300 μg protein, 5 μM hemin, 0.8 mM glucose-6-phosphate, 1 unit/ml glucose-6-phosphate dehydrogenase (Sigma), and 2 units of biliverdin reductase (Sigma). The reaction was initiated by adding 0.5 mM nicotinamide adenine dinucleotide phosphate (NADPH, Sigma). After one hour of incubation at 37 °C in the dark, absorbance was measured with a spectrophotometer at 464 nm and 530 nm. HO-1 activities were calculated as the difference between the two absorbances and expressed as a fraction of HO- activity in the liver.

Data analysis

All data are presented as mean±SE. Comparisons of relative levels of optical density (OD) in Western blots, CA1 neuronal countings and infarct volumes were made with analysis of variance (ANOVA) and Fisher's post hoc tests. A level of p ☐ 0.05 was considered statistically significant.

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SUPPLEMENTAL FIGURE

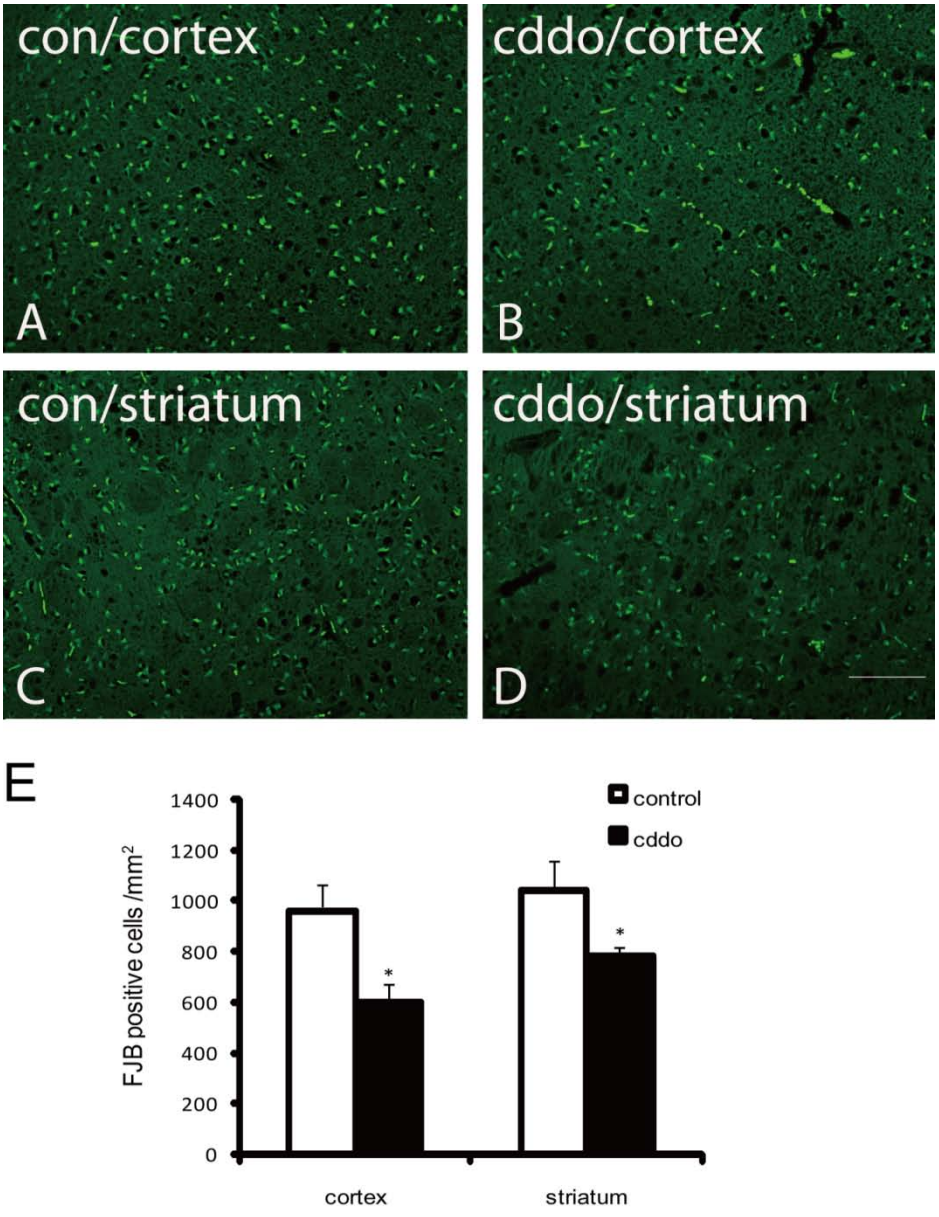

Figure S1: CDDO-Im reduces neuronal death in mouse brain after MCAO.

Brain sections were prepared 48 h after MCAO in mice and FJB staining was performed. Representative microphotos of the cortex (A) and striatum (C) from the vehicle-treated group as well as cortex (B) and striatum (D) of the CDDO-Im-treated (50 μg) group were presented. Scale bar=100μM. (E) Quantitative analysis of degenerative neurons in each group (n=3 per group, *p<0.05).