Pharmacological Induction of Heme Oxygenase-1 by a Triterpenoid Protects Neurons Against Ischemic Injury

Feng Zhang, MD, PhD; Suping Wang, MD; Meijuan Zhang, MD; Zhongfang Weng, MD; Peiying Li, MD, PhD; Yu Gan, PhD; Lili Zhang, MD; Guodong Cao, PhD; Yanqin Gao, PhD; Rehana K. Leak, PhD; Michael B. Sporn, MD; Jun Chen, MD, PhD

Background and Purpose—Heme oxygenase-1 (HO-1) is an inducible Phase 2 enzyme that degrades toxic heme; its role in cerebral ischemia is not fully understood. We hypothesize that chemically induced HO-1 upregulation with the novel triterpenoid CDDO-Im (2-cyano-3,12-dioxooleana-1,9-dien-28-oyl imidazoline), a robust inducer of Phase 2 genes, protects neurons against ischemic injury.

Methods—Using 3 different models of ischemia, including oxygen–glucose deprivation in neuronal cultures, global ischemia in rats, and focal ischemia in mice, we determined (1) whether CDDO-Im induces HO-1 expression and protects against ischemic injury; and (2) whether HO-1 inhibition disrupts the neuroprotective effect of CDDO-Im.

Results—CDDO-Im treatment (50–300 nmol/L) resulted in 8-fold HO-1 upregulation in cultured neurons and protected against oxygen–glucose deprivation. The protection was abolished when the cultures were transfected with nuclear factor (erythroid-derived 2)-like-2–shRNA or coincubated with tin protoporphyrin IX, a specific HO-1 inhibitor. In the rat model of global ischemia, intracerebroventricular infusion of CDDO-Im (0.5–1.5 μg) augmented HO-1 expression in hippocampal neurons and resulted in significant increases in CA1 neuronal survival after global ischemia. To further strengthen the clinical relevance of the CDDO-Im treatment, we tested its effects in the mouse model of temporary focal ischemia (60 minutes). Postischemic intraperitoneal injection of CDDO-Im (10–100 μg) enhanced HO-1 expression and significantly reduced neurological dysfunction and infarct volume. Intracerebroventricular infusion of tin protoporphyrin IX reduced the neuroprotective effect of CDDO-Im against global and focal ischemia.

Conclusions—CDDO-Im confers neuroprotection against ischemic injury by upregulating HO-1, suggesting that enhance of HO-1 expression may be a legitimate strategy for therapeutic intervention of stroke. (Stroke. 2012;43:00-00.)

Key Words: CDDO ■ cytoprotective ■ Nrf2 ■ stroke

Apart from its role in hemoglobin and myoglobin, heme is also a key component of several cytoplasmic and mitochondrial enzyme complexes such as reduced nicotinamide-adenine dinucleotide phosphate oxidase, cytochrome c oxidase, and xanthine oxidase. Heme contains an iron and plays an important role in the electron transfer mediated by these enzymes. When released under pathological conditions such as cellular stresses and ischemia, free heme may still be functional and act as a source of free radicals.1 Cells have therefore evolved a system to degrade heme, a system composed of inducible heme oxygenases 1 (HO-1) and constitutive HO-2. The end products of the degradation include cytoprotective biliverdin and carbon monoxide; as a result, heme oxygenases are potentially neuroprotective against ischemic brain injury.2,3 HO-1 is especially attractive because of its characteristic inducibility.

HO-1 expression is controlled by a transcription factor, nuclear factor (erythroid-derived 2)-like 2 (Nrf2). Nrf2 is silent under physiological conditions, because it is bound by kelch-like ECH-associated protein 1 (Keap1). This association to Keap1 facilitates its degradation through the ubiquitin–proteasome pathway. However, when Nrf2 is dissociated from Keap1, it translocates to the nucleus and initiates transcription of Phase 2 enzyme target genes with subsequent antioxidative and cytoprotective effects. Several compounds exert neuroprotective effects against strokes through activation of Nrf2 and HO-1 such as sulforaphane,4–6 Ginkgo biloba,7,8 and polyphenols.9–11 However, the efficacy of these...
compounds is low and high doses are required to achieve neuroprotection. Thus, there remains a critical need to find potent compounds that can activate Nrf2 and HO-1 at lower doses to avoid potential side effects.

To address this critical need, a group of triterpenoids that demonstrate extremely potent effects in activating Nrf2 have been recently designed. Among them, 2-cyano-3,12 dioxooleana-1,9 dien-28-oyl imidazoline (CDDO-Im) is the most potent because it exerts effects in the picomolar to nanomolar range, is 100 times more effective than sulforaphane, and 5000 times more effective than oltipraz. CDDO-Im increases Nrf2 activity by 1.5-fold in the intact mouse brain. CDDO-Im therefore has the potential to be able to across the blood–brain barrier (BBB), because oral administration of CDDO-Im increases Nrf2 activity by 1.5-fold in the intact mouse brain. CDDO-Im therefore has the potential to provide us with a potent method of inducing Nrf2 and HO-1 in injured neurons and may thereby protect them against ischemic events. The purpose of this study was to test the hypothesis that CDDO-Im protects the brain from ischemic injury through the activation of Nrf2 and upregulation of HO-1 at a low nanomolar dose. If this potent compound is found to be neuroprotective in multiple rodent animal models, this would open the door for experiments in larger species and show promise for future clinical studies.

**Methods**

All experimental procedures were approved by the Institutional Animal Use and Care Committee of the University of Pittsburgh, and all animals were randomly allocated into control and treatment groups. CDDO-Im was dissolved in dimethyl sulfoxide at a concentration of 10 mmol/L as the stock solution. Oxygen–glucose deprivation (60 minutes) was induced in rat primary cortical neuronal cultures, transient global cerebral ischemia (12 minutes) was induced in rats, and transient focal cerebral ischemia (60 minutes) was induced in mice. Detailed methods are available in the online-only Data Supplement (http://stroke.ahajournals.org).

**Results**

CDDO-Im Upregulates HO-1 Expression in Primary Neurons

A previous report showed that CDDO-Im was a strong inducer of HO-1 in several lines of nonneural cells. To determine if CDDO-Im has a similar effect on neurons, we treated primary neurons with CDDO-Im and detected HO-1 levels using Western blot. HO-1 levels were barely detectable in the control group and at 1 hour after the CDDO-Im treatment (data not shown). However, HO-1 was increased 8-fold after 6-hour treatments with 50, 100, 200, and 300 nmol/L CDDO-Im (Figure 1A), supporting a previous report that CDDO derivatives can activate Nrf2 pathway. HO-1 levels were further increased after overnight incubation. We noticed that not all Phase 2 enzymes were upregulated after CDDO-Im treatment.1 Example was the modifier subunit of glutamate cysteine ligase (Figure 1A), suggesting HO-1 upregulation is somewhat selective under these conditions. We decided on the 100 nmol/L concentration of CDDO for follow-up experiments, because it is sufficient to induce HO1, and higher CCDO-Im concentrations such as 300 nmol/L may kill neurons.

To detect if CDDO-Im activates Nrf2 signaling, we incubated neurons with CDDO-Im for 2 or 6 hours and then extracted nuclear fractions. As shown in Figure 1B, CDDO-Im treatment increased Nrf2 levels in nuclei at both 2 hours and 6 hours, which was accompanied by increased expression of HO-1. To determine if Nrf2 is necessary for HO-1 upregulation after CDDO-Im treatment, we transfected neurons with lentiviral particles containing either rat Nrf2 shRNA or scrambled shRNAs and then treated them with CDDO-Im 3 days later. Figure 1C shows that Nrf2 levels were decreased after Nrf2 shRNA silencing and CDDO-induced HO-1 upregulation was blocked, whereas the scrambled shRNAs failed to suppress either Nrf2 or HO-1.
tively, our findings reveal that CDDO-Im activates Nrf2 signaling and upregulates HO-1 in primary neurons in both a concentration-dependent and a time-dependent manner.

**CDDO-Im Protects Primary Neurons Against Oxygen–Glucose Deprivation Through Nrf2 and HO-1**

We next determined whether CDDO-Im pretreatment protected neurons from ischemic injury induced by oxygen–glucose deprivation and if HO-1 and Nrf2 played a critical role in the protection. Cultures were treated with CDDO-Im overnight followed by 60 minutes oxygen–glucose deprivation and then subjected to lactic dehydrogenase release and Alamar blue assays 24 hours later. Compared with vehicle, CDDO-Im treatment significantly reduced lactic dehydrogenase release (Figure 2A) and maintained Alamar blue fluorescence (Figure 2B), indicating that CDDO-Im attenuated neuronal injury. This was further confirmed by Hoechst staining and cell counting (Figure 2C). The protective effects of CDDO-Im were partially blocked when cultures were transfected with Nrf2 shRNA lenti particles or cotreated with tin protoporphyrin IX (Sn-PPIX), a competitive inhibitor of HO-1 activity (Figure 2), indicating a critical role for both HO-1 and Nrf2 in CDDO-Im-mediated neuroprotection against injury.

**CDDO-Im Upregulates HO-1 Expression in Hippocampal CA1**

We next investigated whether CDDO-Im can upregulate HO-1 expression in the rat brain and render similar neuroprotection in vivo. To bypass the BBB and avoid systemic effects of CDDO-Im, we injected 0.5 g CDDO-Im into intracerebroventricular, harvested cortex, striatum, and hippocampus at the indicated times. Like in culture, HO-1 was barely detectable in vehicle-infused brains, and little HO-1 upregulation was observed at 1 hour after the injection of CDDO-Im (data not shown). At 4 hours after the injection, HO-1 was only upregulated in hippocampus; at 24 hours, HO-1 levels were significantly increased in all regions with the highest level in the hippocampus (Figure 3A). The smallest effect was seen in the cortex, which may simply reflect the longer distance of cortex from the injection center than the striatum and hippocampus. We next assessed the dose responsiveness of HO-1 to CDDO-Im in the hippocampus. As shown in Figure 3B, higher doses (1.0 and 1.5 g) of CDDO-Im led to increased levels of HO-1 24 hours after the injection, indicating that CDDO-Im induced HO-1 expression in hippocampus in both a time-dependent and a dose-dependent manner. In hippocampal CA1, 1 of the vulnerable structures to global ischemia, HO-1 level peaked at 48 hours...
after the injection and remained elevated for at least 3 days (Figure 3C). Finally, we studied the cellular distribution of HO-1 in the CA1 region. With occasional exceptions, HO-1 staining surrounded the nuclear neuronal nuclei signal within CA1 neurons (Figure 3D), indicating a predominantly neuronal distribution of HO-1 after CDDO-Im treatment.

**Time Course and Cellular Distribution of HO-1 in CA1 After Global Ischemia in Rats**

HO-1 was barely detectable in sham-operated rats (Figure 4A). After ischemia, HO-1 was slightly upregulated at 1 hour, further increased at 4 hours and thereafter, and peaked at 72 hours after ischemia. Immunostaining validated the Western data that global ischemia stimulated HO-1 expression in hippocampal CA1 (Figure 4B). Double-labeling studies revealed that HO-1 was primarily colocalized with neuronal nuclei-positive cells in CA1 24 hours after ischemia, a time point when CA1 neurons were still alive, indicating a neuronal expression of HO-1 (Figure 4C, upper panel). HO-1 occasionally colocalized with Iba1-positive cells (Figure 4C, lower panel) but seldom with glial fibrillary acidic protein-positive cells at 24 hours (Figure 4C, middle panel). HO-1 distribution was dramatically altered 3 days after ischemia, however. Although HO-1 was still detectable in dead or injured neurons in the pyramidal layer, it was now also detected in astrocytes and microglia in the pyramidal layer (Figure 4D, upper). The strongest HO-1 signal appeared in the radian layer of hippocampal CA1 (Figure 4D, lower), the white matter that contains septal and commissural fibers. This HO-1 signal localized in glial fibrillary acidic protein- and Iba1-positive cells, indicating a shift of HO-1 expression from a neuronal to a glial distribution 3 days after ischemia, when CA1 neurons had died.

**CDDO-Im Pretreatment Attenuates CA1 Neuronal Death After Global Ischemia in Rats**

We then investigated if CDDO-Im could protect CA1 neurons from ischemic injury induced by global ischemia. Electroencephalographic isoelectricity immediately after the occlusion of common carotid arteries verified the success of ischemia (Figure 5A). In vehicle-treated rats, ischemia killed approximately 90% of CA1 neurons; CDDO-Im treatment clearly attenuated neuronal injuries, indicated by an increased number of viable neurons (Figure 5B) and a decreased number of apoptotic neurons (Figure 5C). To determine the role of HO-1 in the protection, we compared the time courses of HO-1 expression in CA1 between

![Figure 3](http://stroke.ahajournals.org/)

**Figure 3.** CDDO-Im induces HO-1 expression in rat brains. A, Western blots show that ICV injection of CDDO-Im (0.5 μg) induced strong HO-1 expression in the hippocampus and striatum but modest expression in cortex. n=3, *P<0.05, **P<0.01 versus vehicle groups. B, Western blots show that CDDO-Im upregulated CA1 HO-1 in a dose-dependent manner. n=3, *P<0.05, **P<0.01 versus vehicle groups. C, Time course of HO-1 expression in CA1 after a single injection of CDDO-Im. D, Immunohistochemical staining for HO-1 expression in CA1 at 24 hours after CDDO-Im (1.0 μg) ICV injection. HO-1 was expressed predominantly in neurons. Bar=50 μm. CDDO-Im indicates 2-cyano-3,12 dioxoleana-1,9 dien-28-oyl imidazoline; HO-1, heme oxygenase-1; ICV, intracerebroventricular.
vehicle and CDDO-Im groups early after ischemia and found differences between these 2 groups with a high level of pre-existing HO-1 in the CDDO-Im group (Figure 5D). Administration of Sn-PPIX (30 μg per mouse) partially but significantly blocked the protective effects of CDDO-Im against ischemic neuronal death (Figure 5B–C).

**Postischemic CDDO-Im Treatment Decreases Infarct After Focal Ischemia in Mice**

To further strengthen the clinical relevance of the CDDO-Im treatment, we tested its effects in a mouse model of focal ischemia with a postischemia treatment. CDDO-Im was injected intraperitoneally immediately after ischemia when the permeability of the BBB was increased, and ischemic outcomes were evaluated 48 hours later. Infarct volume was 35.8 mm³ in the vehicle-treated group (Figure 6A). CDDO-Im at 10 μg per mouse did not protect the brain (data not shown), whereas 25, 50 and 100 μg CDDO-Im reduced infarcts significantly with the best result in the 50-μg group, indicating that CDDO-Im protected the brain from focal ischemia in a dose-dependent manner even when it was injected after ischemia. In supportive, Fluoro-Jade B staining of brain sections at 48 hours after middle cerebral artery occlusion demonstrated that CDDO-Im attenuated neuronal death compared with vehicle group (online-only Data Supplement Figure I). The protection of CDDO-Im was also demonstrated at the behavioral level by improved neurological function (Figure 6B). To investigate the role of HO-1 in this mouse model, we injected 20 μg Sn-PPIX into intracebroventricular after ischemia and found that the protective
The effect of CDDO-Im was again partially blocked, indicated by the relapse of infarct volume (Figure 6A). Western blots showed CDDO-Im treatments resulted in increased HO-1 expression early after middle cerebral artery occlusion (Figure 6C). To confirm that HO-1 activity is changed after CDDO-Im treatment and ischemia, and that Sn-PPIX truly inhibits HO-1 activity, we performed HO-1 activity assay in brain tissues and found that HO-1 activities were increased after protein levels were upregulated and that Sn-PPIX inhibited HO-1 activity by half in CA1 and cortical tissues (Figure 6D). Ischemia by itself was a strong inducer of HO-1, suggestive of endogenous neuroprotection in the brain after a stroke and consistent with previous studies suggesting that HO-1 knockout increases infarcts.8 Taken together, our results indicate that even a low dose of CDDO-Im is neuroprotective against ischemic injury and that HO-1 mediated the protective effects of CDDO-Im in 3 models and 2 species.

**Discussion**

In this study, we demonstrated that CDDO-Im, a synthetic triterpenoid and the strongest inducer of the Nrf2 signaling pathway known so far, induced robust HO-1 expression in neuronal cultures and brains. Using 3 ischemia models, 1 in vitro and 2 in vivo, we found that CDDO-Im treatment attenuated ischemic neuronal injury at extremely low doses. The protective effect of CDDO-Im was blocked when HO-1 activity was inhibited by Sn-PPIX or when Nrf2 was knocked down with specific shRNA, indicating that HO-1 upregulation and Nrf2 activation played important roles in the neuroprotective effects of CDDO-Im.

Triterpenoids belong to a group of 5-ring compounds that are produced by many plants, including ginsengs. Natural triterpenoids have been used as alternative medicines for centuries for their mild antioxidative, anti-inflammatory, and anticancerous effects. In an effort to enhance their potency, oleanolic acid, a natural triterpenoid, has been further modified by extensive synthetic steps, and a new set of synthetic triterpenoids has been generated.12 Among them, CDDO-Im displays the strongest bioactivities, including anticancer, anti-inflammatory, and antioxidative effects.13–15,19,20

The antioxidative role of CDDO-Im is dependent on its ability to activate Nrf2, a master transcription factor that governs Phase 2 enzyme expression.14,15 Under normal conditions, Nrf2 is not active because it binds Keap1, which facilitates their proteosomal degradation, resulting in a short half-life and a low basal level of Nrf2.20 Keap1 is rich in cysteine, and Cys-151 and Cys-275 are important for Nrf2 degradation.21 A recent study shows that dihydro-CDDO-
trifluoroethyl amide, another derivative of CDDO, dissociates Keap1 from Nrf2 by interacting with Cys-151 of Keap1 through Michael addition,\textsuperscript{22} leading to upregulation of Phase 2 enzymes. CDDO-Im may function in a similar manner to upregulate Phase 2 enzymes, although direct evidence for this is currently not available.

As a Phase 2 enzyme, HO-1 is neuroprotective against stroke, because HO-1 knockout worsens infarcts\textsuperscript{8} and HO-1 overexpression reduces infarcts in mice.\textsuperscript{2} Our data support this notion. In addition to an increase in levels, HO-1 location is also likely to contribute to its neuroprotective capacity. HO-1 was primarily expressed in neurons after CDDO-Im treatment, as shown in Figures 1 and 3 and in a previous report.\textsuperscript{17} This is likely to be important for its neuroprotective role in our hands. HO-1 was upregulated in CA1 neurons during the early stage (<24 hours) after ischemia, further supporting the notion that HO-1 may contribute to protection against ischemic neuronal injury. In the late stage, HO-1 was also strongly expressed in astrocytes and microglia. The role of glial HO-1 remains unclear, although previous reports showed that astrocytic Nrf2 and HO-1 also offered neuronal protection against oxidative stress.\textsuperscript{4,23} A prior report also showed that HO-1 could be expressed in cultured astrocytes after CDDO treatment;\textsuperscript{17} however, we did not notice strong astrocytic HO-1 in vivo, probably because astrocytes were not activated after CDDO-Im injection.

The potential benefits of CDDO-Im in the nervous system are not limited to stroke. For example, CDDO-Im reduces retinal injury from photooxidation.\textsuperscript{24} Other CDDO derivatives protect against Alzheimer disease,\textsuperscript{25} Huntington and Parkinson disease,\textsuperscript{26} and amyotrophic lateral sclerosis.\textsuperscript{27} In future studies, the neuroprotective residue for CDDO-Im could be extended to other forms of acute brain injuries such as hemorrhagic stroke and traumatic brain injury. Compared with several Nrf2/HO-1 inducers such as sulforaphane\textsuperscript{4,5} and Ginkgo biloba\textsuperscript{7} that have previously shown neuroprotective effects, a clear advantage of using CDDO-Im is its low doses required to achieve neuroprotection. Thus, the strong potency of CDDO-Im with no apparent toxicity makes this drug a promising candidate for protecting the brain against human
stroke in an Nrf2/HO-1-dependent manner. Nonetheless, several issues still need to be addressed in the future. Examples include whether other Phase 2 enzymes or other signaling pathways are involved in the neuroprotective effects of CDDO-Im, because the nuclear factor kappa B, phosphatase and tensin homolog, and mammalian target of rapamycin appear to also mediate the effects of CDDO-Im in nonneuronal cells.\textsuperscript{12,28,29} Clarification of these issues may help develop new strategies for stroke treatment.

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**Disclosures**

None.

**References**

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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©. 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©.

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©. 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©. 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. 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. 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. 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 1 min 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. 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 $p \leq 0.05$ was considered statistically significant.

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).