Osteopontin Reduced Hypoxia–Ischemia Neonatal Brain Injury by Suppression of Apoptosis in a Rat Pup Model

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Background and Purpose—Osteopontin (OPN) is neuroprotective in ischemic brain injuries in adult experimental models; therefore, we hypothesized that OPN would provide neuroprotection and improve long-term neurological function in the immature brain after hypoxic–ischemic (HI) injury.

Methods—HI was induced by unilateral ligation of the right carotid artery followed by hypoxia (8% O2 for 2 hours) in postnatal Day 7 rats. OPN (0.03 μg or 0.1 μg) was injected intracerebroventricularly at 1 hour post-HI. Temporal expression of endogenous OPN was evaluated in the normal rat brain at the age of 0, 4, 7, 11, 14, and 21 days and in the ipsilateral hemisphere after HI. The effects of OPN were evaluated using 2-3-5-triphenyl tetrazolium chloride staining, apoptotic cell death assay, and cleaved caspase-3 expression. Neurological function was assessed by the Morris water maze test.

Results—Endogenous OPN expression in the brain was the highest at the age of 0 day with continuous reduction until the age of 21 days during development. After HI injury, endogenous OPN expression was increased and peaked at 48 hours. Exogenous OPN decreased infarct volume and improved neurological outcomes 7 weeks after HI injury. OPN-induced neuroprotection was blocked by an integrin antagonist.

Conclusions—OPN-induced neuroprotection was associated with cleaved-caspase-3 inhibition and antiapoptotic cell death. OPN treatment improved long-term neurological function against neonatal HI brain injury. (Stroke. 2011; 42:764-769.)

Key Words: hypoxic/ischemic ■ neonatal ■ neuroprotection ■ osteopontin

Hypoxia–ischemia (HI) brain injury in the preterm infant impairs normal development and results in long-term neurological deficits.1 Previous studies suggest that apoptotic cell death is prominent in the neonatal brain after HI insults;2 and it is more common in the immature brain than adult brain.3 Caspase-3 cleavage and activation has been shown as a major cause of brain injury after neonatal stroke.4 To date, however, there are no effective pharmacological strategies available for neonatal brain neuroprotection after injury.

Osteopontin (OPN) is a secreted glycosylated phosphoprotein that exists in all the body fluids and is involved in multiple biological functions, including inflammation, cell migration, and antiapoptotic processes.5 OPN is widely over-expressed in various cancer diseases5,6 in accordance with the increased cell survival.7 Other than in vitro study, a protective role of OPN in ischemia has also been suggested in the kidney and brain in the adult animal.8,9 The present study was designed to investigate the effect of OPN in the neonatal brain after HI insult.

Materials and Methods

Animal Model
The Institutional Animal Care and Use Committee at Loma Linda University approved all protocols. A modified Rice-Vannucci model10 was used as previously described.11 Briefly, 7-day-old rat pups (Harlan Laboratories, Indianapolis, IN) were anesthetized with 3% isoflurane. The right common carotid artery of each pup was exposed without ligation and hypoxia.

Drug Administration
Pups were randomly assigned to 1 of the following groups: sham+phosphate-buffered saline (PBS), sham+OPN-0.1 (0.1 μg OPN injection), HI+PBS, HI+OPN-0.03 (0.03 μg OPN treatment), or HI+OPN-0.1 (0.1 μg OPN treatment). OPN (Calbiochem, CA) was prepared followed as others described with modification.12 OPN

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was dissolved in PBS (0.03 μg/μL or 0.1 μg/μL) and total volume of 1.0 μL was administered intracerebroventricularly at 0.03 μg or 0.1 μg per animal 1 hour post-HI. Briefly, 7-day-old rat pups were fixed on a stereotaxic apparatus (Stoeling, Wood Dale, IL) under isoflurane inhalation (2%). A scalp incision was made on the skull surface and the bregma was exposed. OPN was injected with a 10-μL syringe (Hamilton, NV) at the location of 1.0 mm posterior and 1.0 mm lateral to the bregma and 2.0 mm deep to the skull surface at the contralateral hemisphere. The control rats were injected with sterile PBS. The injection was completed in 5 minutes and the needle was kept in the injection position for an additional 2 minutes. Then the needle was removed slowly out of the brain and the wound was sutured. After recovery from the anesthesia, the pups were returned to their dams. To investigate whether integrin receptor is involved, an additional group was administered 1 μL GRGDS (5 μmol/L; Sigma-Aldrich) intracerebroventricularly 15 minutes before OPN treatment (0.1 μg).

**Infarct Volume Evaluation**

2,3,5-triphenyltetrazolium chloride monohydrate (TTC; Sigma-Aldrich) staining was used to measure infarct volume as previously described. Primary antibodies (osteopontin; Santa Cruz Biotechnology; cleaved caspase-3; Chemicon) and secondary antibody were used.

**Immunohistochemistry**

At 48 hours after HI, animals were anesthetized and 10-μm-thick coronal brain sections were cut using the cryostat (CM3050S; Leica Microsystems) as previously described. Brain sections were incubated with primary antibody glial fibrillary acidic protein (Dako), mitogen-activated protein-2 (Santa Cruz Biotechnology), or Iba-1 (Dako) overnight at 4°C. Fluorescein isothiocyanate- or Texas red-conjugated secondary antibodies (Jackson Immunoresearch) were used. The sections were then visualized using a fluorescent microscope (Olympus BX51; Olympus Optical Co Ltd) and pictures were recorded and analyzed (Magnafire SP 2.1B software).

**Cell Death Assay**

Apoptosis induced by HI at 24 hours in the ipsilateral hemisphere was evaluated by quantitation of DNA fragmentation using a Cell Death Detection ELISA kit (Roche Applied Science) in accordance with the manufacturer’s specification, as we previously described. Western blot analysis was performed with the manufacturer’s specification, as we previously described.

**Western Blotting**

Normal animals were euthanized at the age of 0, 4, 7, 11, 14, and 21 days (n=4 for each group) for endogenous OPN measurement. To evaluate endogenous OPN expression after HI injury, rat pups were euthanized at 3, 24, 48 hours 4, 7, and 14 days post-HI. The level of cleaved caspase-3 in the ipsilateral hemisphere was measured at 24 hours post-HI for each group. Western blot analysis was performed as described previously. Primary antibodies (osteopontin; Santa Cruz Biotechnology; cleaved caspase-3; Chemicon) and secondary antibody were used.

**Morris Water Maze Test**

The water maze test is used to evaluate the ability to learn spatial locations and memory. It was performed at 7 weeks after HI injury as previously described. Briefly, the rats need to find a visualized (cued test) or submerged (special test) platform in a pool of water with visual cues in the room. The water maze consisted of a pool (118 cm diameter) filled with water and a platform (22 cm diameter) that rats could step on to escape the water. All trials last a maximum of 60 seconds, at which point the rats were manually guided to be placed on the platform. All the activities were recorded and the animals’ swimming paths were measured for quantification of distance, latency, and swimming speed by the Video Tracking System SMART-2000 (San Diego Instruments Inc).

**Statistical Analysis**

Data were presented as mean±SEM. Statistical differences between >2 groups were analyzed by using 1-way analysis of variance followed by the Tukey multiple comparison procedure. Water maze data were analyzed using the general linear models repeated-measures analysis of variance (Statistica 6.0). Probability value <0.05 was considered statistically significant.

**Results**

**Endogenous OPN Expression in the Developing Brain**

First, the temporal profile of endogenous OPN expression in the normal rat brain was measured by Western blot at the age of 0, 4, 7, 11, 14, and 21 days (Figure 1A). The level of OPN in the brain was the highest at the age of 0 day and then significantly decreased by 29.1% because the age of 4 days with continuous reduction until the age of 21 days during development, which was 96.7% less than the level at Day 0. Next, we evaluated the changes of endogenous OPN expression in the brain after HI injury in the 7-day-old rat pups (Figure 1B). Endogenous OPN expression in the ipsilateral hemisphere...
hemisphere started to significantly increase from 3 hours post-HI and gradually peaked at 48 hours after HI injury (730.5 ± 86.4, \( P = 0.009 \) versus 0 hours, ipsilateral hemisphere, analysis of variance). OPN expression started to decrease 4 days post-HI compared with 48 hours post-HI; however, the level of OPN in the ipsilateral hemisphere is still significantly higher than in the contralateral hemisphere 7 days after HI injury.

To determine the cell type and sources of OPN expression, double staining for glial fibrillary acidic protein/OPN, mitogen-activated protein-2/OPN, and Iba-1/OPN was performed on the brain section of P7 sham-operated animals and the ipsilateral brain section at 48 hours after HI injury. The sham group showed positive expression of OPN in the neurons and macrophage (Figure 2A, iii and ix) but negative expression in the astrocytes (Figure 2A, vi). However, in the frontal cortex region of the ipsilateral hemisphere at 48 hours after HI injury, the OPN expression was colocalized with neurons, astrocytes, and macrophage (Figure 2B, iii, vi, and ix).

### Effect of OPN on Body Weight and Infarction Volume

Body weight of rat pups was monitored after HI injury as an indicator of their general health. Pups’ body weight was significantly reduced at both 24 and 48 hours post-HI compared with the sham + PBS group (\(^*P<0.001\), analysis of variance; Figure 3A). OPN treatment with a high dosage (0.1 μg) significantly improved the rat pups’ body weight at 48 hours after HI injury (\(^*P=0.01\) versus OPN + PBS, analysis of variance; Figure 3A). Moreover, there is a significantly increase of body weight in the OPN-treated sham group (0.1 μg) compared with the sham + PBS group at 48 hours post-HI (\(#P<0.001\) versus OPN + PBS, analysis of variance; Figure 3A).

OPN treatment resulted in a significantly decrease in brain infarct volume 48 hours after HI injury. Quantitative assessment of TTC-stained sections indicated that using both low and high dosage of OPN: 0.03 μg (22% ± 2.0%) and 1 μg (19.2% ± 1.8%) significantly reduced infarct volume as compared with the vehicle-treated group (29.4% ± 2.2%; \(^*P<0.05\), mean ± SEM; Figure 3B). There was no difference between low- and high-dose OPN treatment.

### Long-Term Effects of OPN Treatment

First, OPN improves functional recovery 7 weeks after neonatal HI injury. The Morris water maze test was performed at 7 weeks post-HI. The swimming distance (from releasing point to reach the platform on the cued and spatial maze) is shown in Figure 4A–B. There is no significant difference in swimming distance among all the groups on the cued and spatial maze test, the swimming distance in the HI + PBS group was significantly increased, whereas OPN treatment (0.1 μg) significantly reduced the swimming distance (Figure 4B; \(^*P<0.001\), analysis of variance). There is no significant difference in the swimming distance between the sham + PBS and sham + OPN groups (Figure 4B; \(P>0.05\), repeated-measures analysis of variance).

To determine the cell type and sources of OPN expression, double staining for glial fibrillary acidic protein/OPN, mitogen-activated protein-2/OPN, and Iba-1/OPN was performed on the brain section of P7 sham-operated animals and the ipsilateral brain section at 48 hours after HI injury. The sham group showed positive expression of OPN in the neurons and macrophage (Figure 2A, iii and ix) but negative expression in the astrocytes (Figure 2A, vi). However, in the frontal cortex region of the ipsilateral hemisphere at 48 hours after HI injury, the OPN expression was colocalized with neurons, astrocytes, and macrophage (Figure 2B, iii, vi, and ix).

![Figure 2](http://stroke.ahajournals.org/)

Figure 2. Endogenous OPN expression in the P7 sham-operated group (A) and at 48 hours after HI (B). Double fluorescent labeling for OPN with mitogen-activated protein-2 (MAP-2; i–iii), glial fibrillary acidic protein (GFAP; iv–vi), and Iba-1 (vii–ix) in the cortex of the ipsilateral hemisphere is shown (scale bar represents 50 μm). In the brain of sham-operated P7 rats, OPN expression was colocalized with the neurons and macrophage (A, iii and ix, as indicated by arrowhead) but not with the astrocytes (2A, vi, as indicated by arrowhead). At 48 hours after HI, OPN expression is colocalized with neurons, astrocytes, and macrophage (2B, iii, vi, and ix, as indicated by arrowhead). Insets in the right corner of i–ix are higher magnification (scale bar represents 20 μm).

The probe trial was carried after the spatial maze training. The frequency of the crossing target quadrant was recorded. Animals in sham + PBS and sham + OPN groups had approximately 4 times crossing frequency, and there is no significant difference between the 2 groups (Figure 4C). The crossing frequency was reduced to approximately 2 times in the HI group (HI + PBS versus sham + PBS = 2.3 ± 0.3 versus 4.6 ± 0.4, \(^*P<0.05\), analysis of variance), and OPN treatment significantly recovered the crossing frequency (3.6 ± 0.4 \(P<0.05\), analysis of variance).

The brain atrophy was measured after the water maze test as previously described.\(^{11}\) There is a trend of the recovery of brain tissue loss after OPN treatment; however, it is not
significantly different between the HI group and OPN-treated group (data not shown).

Caspase-3 Cleavage Inhibition Is Involved in OPN-Induced Neuroprotection

Significantly higher apoptotic cell death was detected at 24 hours in the HI/PBS group compared with both sham and sham+OPN (0.1 μg) groups (*P<0.05 versus sham+PBS and sham+OPN, respectively, analysis of variance; Figure 5A). It was significantly attenuated by both low-dose and high-dose OPN treatment (*P<0.01, versus HI+PBS). Apoptotic marker, cleaved caspase-3, expression was evaluated by Western blotting analyses. Cleaved caspase-3 expression was dramatically increased in the vehicle-treated group compared with both sham and sham+OPN (0.1 μg) groups, respectively (*P<0.05, analysis of variance; Figure 5B). It was significantly reversed by both low-dose and high-dose OPN treatment (*P<0.01, versus HI+PBS; Figure 5B).

OPN-Induced Neuroprotection Is Blocked by an Integrin Antagonist

To determine whether OPN-induced neuroprotection after neonatal HI brain injury involves an interaction with an integrin receptor, a RGD-containing peptide, which binds to various integrin receptors, was used. Quantitative analysis of TTC-stained sections indicated that GRGDSP peptide blocked OPN-induced neuroprotection at 48 hours post-HI (*P<0.01, versus HI+OPN-0.1; Figure 6). The infarct volume in the GRGDSP-treated group had no significant difference compared with the HI+PBS group (29.8±2.9 versus 30.3±2.2, P>0.05, analysis of variance). It suggests the integrin receptor is involved in OPN-induced neuroprotection.

Discussion

Neonatal brain injury is an important cause of mortality and long-term morbidity. To date, no pharmacological or therapeutic interventions have been proven effective in improving...
neurological outcome of infants. In this study, we have investigated the potential neuroprotection of OPN in the developing brain after HI injury. Intracerebroventricular administration of OPN significantly reduced infarct volume, ameliorated body weight loss, and improved long-term neurological impairment. OPN reduced apoptotic cell death and cleaved caspase-3 activity, possibly by activation of integrin receptors.

We first investigated the level of endogenous OPN in the brain at different developmental stages and its expression in response to HI. The level of OPN was highest at Day 0 and decreased dramatically in the normal brain during maturation. After HI, an upregulation of OPN started from 3 hours post-HI and persisted until 7 days after HI injury. Our data are consistent with published results in adult animals that elevated endogenous OPN expression has been observed in various cell types in response to inflammation, growth factor, ischemia, etc. In acute ischemia, OPN is neuroprotective, and OPN has also been implicated in tissue remodeling processes such as angiogenesis, endothelial cell migration, and vascular endothelial growth factor upregulation. The fast and prolonged OPN upregulation in response to HI injury as observed in the present study in neurons, astrocytes, and macroglial cells may indicate that OPN plays a role at both acute and delayed stage after HI brain injury. More interestingly, the role of endogenous OPN in the astrocytes post-HI needs to be examined in the future, because there is no OPN expression in the astrocytes in the normal neonatal brain.

The neuroprotection of OPN administration has been suggested in the adult rat after focal ischemic brain injury. Compared with the adult, the primary mechanisms of neuronal cell death after injury are different in the immature brain. The adult brain usually demonstrates necrotic cell death with early cytoplasmic and organelle swelling when responding to most acute insults such as HI and excitotoxicity. However, the normal developing brain retains a part of the programmed cell death for natural neuronal elimination; for instance, during the development of the vertebrate nervous system, up to 50% of many types of developing neurons are eliminated by apoptosis. Thus, the immature brain is more sensitive to HI injury because the apoptotic machinery is more easily

Figure 5. OPN inhibits apoptotic cell death through the caspase-3-dependent pathway. Significantly increased apoptotic cell death (A) and caspase-3 activation (B) was observed in the HI+PBS group compared with both sham and sham+OPN-0.1 μg groups at 24 hours after HI. Both high-dose (0.1 μg) and low-dose (0.03 μg) OPN significantly decreased apoptotic cell death (A) and reduced caspase-3 activation (B). *P<0.05 versus sham+PBS and sham+OPN-0.1 groups, respectively; †P<0.05 versus HI+PBS group; n=6 in each group.

Figure 6. OPN-induced neuroprotection is blocked by an integrin receptor analog. Rat pups were treated with GRGDS (5 μmol/L, 1 μL) 15 minutes before OPN treatment. TTC staining at 48 hours post-HI revealed that OPN-induced neuroprotection is blocked by integrin receptor analog treatment (P<0.05, versus HI+OPN-0.1). Vertical bars indicate SEM. N=10 in HI+PBS group; n=11 in HI+OPN-0.1 group; n=7 in HI+OPN-0.1+GRGDS group.
engaged and activated in response to injury in the immature brain. Previous studies demonstrated that OPN promotes cell survival through Akt activation, which might be associated with integrin-linked kinase and the P13K pathway. The effects of OPN silence were accompanied by the activation of mitochondria-related apoptosis pathway involving cytochrome c, cleaved caspase-3, and Bcl-2/Bax. In accordance with previous data, our results suggest that apoptotic cell death was significantly reduced after OPN administration after neonatal HI injury. Moreover, caspase-3 cleavage was significantly blocked in the OPN-treated group. Other than antiapoptotic effects, OPN was shown to prevent cytotoxicity through inhibition of matrix metalloproteinase-9 and nuclear factor κB activity or nitric oxide synthesis. OPN is also involved in cell migration through CD44 signaling and FAK/Src kinase induction. The other biological function of OPN against neonatal HI needs to be tested in the future. We also showed that OPN treatment in the developing brain improved long-term neurological function at 7 weeks after HI injury, although we did not see improvement of the brain atrophy. The performance of the Water maze test was determined by both motor and cognitive function. The swimming distance in the cued maze test did not show any differences among all the groups. Analysis of the swimming distance on the hidden maze test showed that the performance by rats in the HI group was significantly worse compared with sham+PBS and sham+OPN groups, which could be associated with the motor deficit after HI injury. OPN treatment significantly reduced the swimming distance on hidden maze test. However, repeated-measures analysis showed no difference among all the groups, which suggested that the learning curve is not different among all the groups. In addition, all the behavioral tests have no difference between sham+PBS and sham+OPN groups, which implies that the OPN treatment in the developing brain had no significant effects on the normal brain function. However, it is interesting to note that the body weight of the OPN-treated sham group was significantly higher than the vehicle-treated sham group at 48 hours post-HI. The related mechanism is not clear and will need to be explored in the future.

The OPN signaling pathway might be dependent on specific receptors. OPN can act on neurons, astrocytes, endothelial cells, and macrophages through integrin and CD44 receptors and trigger various signal transduction pathways. Our data suggested that the effect of OPN in the developing brain might be through integrin receptors because an integrin antagonist attenuated the neuroprotective effect of OPN. However, whether CD44 receptors are involved in OPN-induced neuroprotection after neonatal HI needs to be further investigated.

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Disclosures
None.

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**Supplemental Methods**

**Brain Atrophy**

Pups were euthanized and the brains were removed at 7 weeks post HI, after the Morris water maze test was done. The ipsilateral and contralateral hemisphere were separated by a midline incision and then weighed on a high precision balance (sensitivity ± 0.001g). Brain atrophy was expressed as the mass ratio of the ipsilateral hemisphere compared to the contralateral hemisphere.

**Supplemental Figures**

**Supplemental Figure S1.** Brain atrophy at 7 weeks post HI. There was a significant tissue loss in the ipsilateral hemisphere in HI+PBS and HI+OPN-0.1 group ($^\# P < 0.05$ vs. sham and sham+OPN-0.1). Brain atrophy in ipsilateral hemisphere was not attenuated after OPN treatment (ns = no significance). The data was expressed as the ratio of ipsilateral and contralateral hemisphere tissue mass. N = 18 animals for sham+PBS group. N = 18 animals for sham+OPN-0.1 group. N = 24 animals for HI+PBS group. N = 18 animals for HI+OPN-0.1 group. Vertical bars indicate SEM.
Supplemental Figure S2. OPN treatment didn’t change the level of 32 kD pro-caspase-3 and 29 kD caspase-3 at 24 h post HI. (A) The level of pro-caspase-3 (32 kD) was not significantly different between all the groups (ns = no significance). (B) The level of 29 kD caspase-3 was significantly increased after HI injury (#P < 0.05 vs. sham and sham+OPN-0.1). OPN treatment didn’t significantly change the level of 29 kD caspase-3 expression compared with the HI+PBS group.