Ischemic Preconditioning Reduces Neurovascular Damage After Hypoxia-Ischemia Via the Cellular Inhibitor of Apoptosis 1 in Neonatal Brain

Wan-Ying Lin, MS; Ying-Chao Chang, MD, PhD; Chien-Jung Ho, BS; Chao-Ching Huang, MD

Background and Purpose—The neurovascular unit is a major target of hypoxia-ischemia (HI) injury in the neonatal brain. Although neurons are the cellular target of ischemic preconditioning (IP), vessel tolerance also contributes greatly to protection. Nerves and vessels cross-talk and use common signals during development. Cellular inhibitor of apoptosis 1 (cIAP1) is an important regulator that inhibits apoptosis. This study hypothesized that cIAP1 is a shared molecule underlying IP-mediated neurovascular protection against HI in the neonatal brain.

Methods—In vivo IP was induced by 2-hour reversible occlusion of right carotid artery 24 hours before HI on postpartum day 7 in rat pups. In vitro oxygen–glucose deprivation (OGD) preconditioning was established in SH-SY5Y neuronal cells and in human microvascular endothelial cell-1 vascular endothelial cells. cIAP1 expression was inhibited by cIAP1 small interfering RNA in vivo or by lentivirus-mediated short hairpin RNA in vitro, or was upregulated by the lentiviral expression system.

Results—IP reduced apoptosis, selectively increased cIAP1 in neurons and vascular endothelial cells, and provided long-term neuroprotection against HI. Intracerebroventricular delivery of cIAP1 small interfering RNA significantly attenuated IP-mediated cIAP1 upregulation and neuroprotection in vivo. In vitro, OGD preconditioning induced cIAP1 and protected against OGD cell death in SH-SY5Y neuronal and human microvascular endothelial cells-1. Knockdown of cIAP1 by lentivirus-mediated short hairpin RNA decreased the protective effect of OGD preconditioning in SH-SY5Y and human microvascular endothelial cell-1, whereas overexpression of cIAP1 by lentivirus protected against OGD in these cells.

Conclusions—cIAP1 is a shared molecule underlying IP-mediated protection in neurons and vascular endothelial cells against HI in the neonatal brain. (Stroke. 2013;44:162-169.)

Key Words: cellular inhibitor of apoptosis 1 ■ hypoxia-ischemia ■ ischemic preconditioning ■ neonatal brain ■ neurovascular unit

Hypoxia-ischemia (HI) is a major cause of mortality and neurologic disability in newborns. The neurovascular unit, mainly composed of neurons and microvessels, is a major target of HI injury. Tolerance, attained by tissue preconditioning to brief, sublethal stress, mobilizes intrinsic protective mechanisms against subsequent lethal insult. Although neurons are the cellular target for preconditioning, vessel tolerance also contributes greatly to neuroprotection. During development, nerves and vessels cross-talk using common signals to determine cell fate. Thus, ischemic preconditioning (IP) may induce neuronal and vascular protection against HI via shared mechanism.

Apoptosis is an important mechanism of cell death after HI in the immature brain. The anti-apoptotic brake is raised by IP to inhibit the pro-apoptotic machinery after HI and to promote neuronal survival. The inhibitor of apoptosis (IAP) family is an important regulator of apoptotic cascades that blocks caspase activities and inhibits subsequent extrinsic and intrinsic apoptosis. The IAP family mainly includes cellular IAP1 (cIAP1), cIAP2, X-linked IAP (XIAP), and survivin. An in vivo global cerebral ischemia study has shown that IP inhibits caspase-3 activation by preserving cIAP2 in the hippocampal CA1 neurons. In vitro research also reveals that hypoxic preconditioning protects against oxygen–glucose deprivation (OGD) in human endothelial cells via survivin. However, whether IAP is the shared molecule underlying IP-induced neurovascular protection against HI in neonatal brain remains unknown.

Our previous study showed that IP induced by reversible occlusion of the carotid artery 22 hours before HI provided long-term neuroprotection in neonatal brain. We used this in vivo IP model and established in vitro OGD preconditioning models in neuronal and endothelial cells to test the hypotheses that: (1) IP upregulates cIAP1 expression in neurons and vascular endothelial cells; (2) cIAP1 is required for preconditioning-mediated protection against HI in vivo and against
OGD cell death in vitro in neurons and endothelial cells; and (3) cIAP1 is sufficient to protect against OGD in neurons and endothelial cells.

Methods

IP in Rat Pups
This study was approved by the Animal Care Committee of National Cheng Kung University. Postpartum (P) day 6 Sprague-Dawley male rat pups were anesthetized, randomized either to undergo sham operation or to have the right carotid artery occluded. A total of 98 pups were used in this study. The right common carotid artery was reversibly occluded for 2 hours using a microvascular clamp (Tuttlingen), followed by a 22-hour reperfusion before HI. On P7, the right common carotid artery was permanently ligated. One hour later, the pups were placed in air-tight 500-mL containers with 8% oxygen flow rate of 3 L/min for 2 hours.11 Pups with no IP underwent sham operation 24 hours before HI, whereas control pups were sham-operated without IP. The preconditioning experiments were performed by a laboratory technician, whereas the quantitative measurement was performed by the investigators who were blinded to the grouping during the assessment. The treatment paradigm and the time point of work-ups are shown in Supplemental Figure I.

Intracerebroventricular Delivery of cIAP1 Small Interfering RNA
The preconditioned pups were intracerebroventricularly infused with cIAP1 siSTABLE small interfering RNA (siRNA) or control siRNA in the right cerebral hemisphere as described (online-only Data Supplement).6 The siRNA were infused at a concentration of 0.2 or 0.6 mmol/L on postpartum day 3 (0.4 or 1.2 nmol) and at 30 minutes before IP on P6 (0.4 or 1.2 nmol). The cortices were collected 24 hours post-HI, and brain damage was determined on P14.

Pathological Outcome
On P14 (for cIAP1 siRNA effect) or P42 (for IP long-term outcome), the brain sections (40-μm-thick) were stained with cresyl violet. The areas of the cortex and hippocampus in the 3 reference planes (plates 27, 31, and 39) of a rat atlas12 were assessed. The percentage of area loss in the cortex and hippocampus was determined.6

Immunohistochemistry
The pups were perfused for cryo-sections (14-μm-thick) 24-hour post-HI. The sections were incubated with primary antibody against cIAP1 (1:500; Abnova).

Immunofluorescence
Brain sections were incubated with primary antibodies: Neuronal Nuclei (NeuN) (1:100; Chemicon), rat endothelial cell antigen-1 (1:100; Abcam), glial fibrillary acidic protein (1:100; Chemicon), or cIAP1 (1:500; Abnova). The sections were incubated with Alexa Fluor 488 anti-rabbit and Alexa Fluor 594 anti-mouse (Invitrogen) secondary antibodies.

Quantitative Analysis of Neurons and Vessels
The NeuN-positive neurons and rat endothelial cell antigen-1-positive vessels were counted in the corresponding planes (plates 18, 31, and 39) of a rat atlas.22 NeuN-positive cells were measured in 3 visual fields (each visual field: 220 μm × 166 μm) in each section, and the cell number was expressed as the average number of NeuN-positive cells per visual field. Rat endothelial cell antigen-1-positive blood vessels were measured in 3 visual fields (one visual field: 890 μm × 670 μm) in each section. Blood vessels were analyzed, and blood vessels per visual field were put in binary form at an average between the minimum and maximum intensity of the neighborhood.13

Western Blot Analysis
The cortex was homogenized and blotted electrophoretically to polyvinylidene fluoride membranes.15 The primary antibodies used included anti-caspase-8 (1:1000; Calbiochem), anti-caspase-9, anti-caspase-3, anti-poly (ADP-ribose) polymerase, anti-XIAP (all 1:1000; Cell Signaling), anti-survivin (1:1000; NOVUS), anti-cIAP2 (1:100; Millipore), anti-β-actin (1:10000; Sigma-Alrich), and anti-cIAP1 (1:800; Santa Cruz) for tissue lysates, and anti-human cIAP1 (0.5 μg/mL; R&D) was used for cell lysates.14

Oxygen-Glucose Deprivation
Human neuroblastoma cells (SH-SY5Y), human microvascular endothelial cells-1 (HMEC-1), and human glioblastoma cell line (U87) were maintained in their respective culture medium.15 The SH-SY5Y cells (1.5×10⁵ cells/well), HMEC-1 cells (3×10⁵ cells/well), and U87 cells (3×10⁵ cells/well) were plated, respectively, in 6-well plates at 37°C for 24 hours (online-only Data Supplement).

OGD Cell Death
The SH-SY5Y, HMEC-1 cells, and U87 cells were washed with their respective OGD medium before being placed in hypoxia chambers8 and maintained under 95% N₂/5% CO₂ at 37°C at 3 L/min for the designated duration.

OGD Preconditioning
SH-SY5Y, HMEC-1 cells, and U87 cells were grown for 24 hours in complete medium and washed with OGD medium or buffer before switching to OGD medium. OGD preconditioning was performed for 8 hours for SH-SY5Y, 7 hours for HMEC-1 cells, and 4 hours for U87 cells. The OGD-preconditioned and cells without preconditioning were maintained in complete medium under normoxia for 24 hours before being exposed to OGD.

Lactate Dehydrogenase Measurement
Cytotoxicity was performed using lactate dehydrogenase (LDH) release assay (CytoTox96, Promega) 24 hours post-OGD. The percentage of cytotoxicity was calculated as: (experimental value − culture medium background) × 100/(target cell maximum LDH release − culture medium background) using an ELISA plate reader (absorbance at 490 nm).

Lentivirus-Mediated Silencing of cIAP1
Lentivirus-mediated hairpin RNA targeting cIAP1 mRNA (LV-sh-cIAP1; sc-29848-V; Santa Cruz) and nontarget shRNA control lentiviral particles encoding a scrambled shRNA sequence (LV-sh-scramble control; sc-108080) were used. The SH-SY5Y and HMEC-1 cells were transduced using lentiviral particles for mRNA silencing by generating stable LV-sh-cIAP1 cell lines.

Lentivirus-Mediated cIAP1 Overexpression
To establish HMEC-1 cells stably expressing cIAP1, the cells were infected with recombinant lentiviruses encoding cIAP1 (LV-cIAP1) for 24 hours. To overexpress cIAP1 in the SH-SY5Y cells, the cells were infected with LV-cIAP1 for 24 hours and allowed to grow in the complete medium for another 24 hours (online-only Data Supplement).

Statistics
In a 1-way ANOVA analysis of the primary outcomes, the effect size 1.0, the smallest based on our preliminary data, was chosen for power analysis. The sample size of 4 for each group achieved at least 80% power to detect the effect size with a 0.05 significance level. Normality of the data was assessed using Shapiro-Wilk test, which indicated the data that followed a normal distribution. The 1-way ANOVA was used to evaluate the protective effect of IP, densitometry data, and LDH levels, whereas Tukey significant difference method was used for post-hoc comparisons. Continuous data were means±SEM unless indicated otherwise. P<0.05 was considered statistically significant, and all probabilities were 2-tailed.
Results

IP Protected Neurons and Vascular Endothelial Cells Against HI

The mortality during hypoxic-ischemia was 0% in the sham control group (n=14), 11.5% in the no IP group (n=26), and 0% in the IP group (n=23). Compared with the no IP group, the IP group had markedly increased NeuN-positive neurons (P<0.001) in the ipsilateral cortex 24-hour post-HI (Figure 1A). Rat endothelial cell antigen-1 staining also revealed significantly increased vascular density and total vascular length in the IP group (Figure 1B) (both P<0.001). Long-term outcome assessment on P42 showed that the IP group had significantly less damage in the cortex and hippocampus compared with the no IP group (P<0.001) (Figure 1C).

IP Reduced Apoptosis After HI

Western blot revealed that the no IP but not the IP group markedly increased the cleaved levels of caspase-8 (P<0.05), caspase-9 (P<0.01), caspase-3 (P<0.01), and poly (ADP-ribose) polymerase (all P<0.05) 24-hour post-HI compared with the control group. The IP group had significantly decreased levels of cleaved caspase-8, caspase-9, caspase-3, and anti-poly (ADP-ribose) polymerase compared with the no IP group 24-hour post-HI (all P<0.05) (Figure 2A).

IP Increased cIAP1 in Neurons and Endothelial Cells After HI

Compared with the control, both the IP and no IP groups had unchanged cIAP2, XIAP, and survivin levels post-HI. The no IP group had markedly decreased cIAP1 levels (3 hours, 62%; 24 hours, 37%; 72 hours, 83% of control), whereas the IP group showed progressively increased cIAP1 (3 hours, 114%; 24 hours, 150%; 72 hours, 264% of control) after HI. The IP group had significantly higher cIAP1 levels at 3-, 24-, and 72-hour post-HI (all P<0.05) (Figure 2B) compared with the no IP group. Immunohistochemistry confirmed that compared with the control, cIAP1 was markedly decreased in the no IP group but increased in the IP group 24 hours after HI. Increased cIAP1 in the IP group was mainly expressed in the vascular and non-vascular cells (Figure 3A). Further immunofluorescence in the

Figure 1. Immunofluorescence showed ischemic preconditioning (IP) group had significantly increased (A) NeuN-positive neurons, and (B) vascular area (top) and total vascular length (bottom) in the ipsilateral cortex 24-hour post-hypoxia-ischemia (HI) compared with no IP group. n=4 per group. Scale bar, 50 μm (A) and 200 μm (B). C, At postpartum day 42, the IP group showed significantly less brain area loss than the no IP group. n=6–7 per group. #P<0.001.
IP group revealed that cIAP1 was mainly expressed in the rat endothelial cell antigen-1-positive vascular endothelial cells and NeuN-positive neurons, but not in astrocytes (Figure 3B).

cIAP1 siRNA Attenuated IP-Induced cIAP1 and Neuroprotection

The IP group pretreated with control siRNA had significantly increased cIAP1 (≈151% of control) in the cortex 24 hours post-HI compared with the control (P<0.05). In contrast, compared with control siRNA, cIAP1 siRNA (1.2 but not 0.4 nmol) delivery significantly decreased cIAP1 expression in the IP group (≈107% of control) (Figure 3C). On P14, compared with the no IP group pretreated with control siRNA, the IP group pretreated with control siRNA had significantly less damage in the cortex and hippocampus (both P<0.01) (Figure 3D). In the IP group, the pups pretreated with cIAP1 siRNA (1.2 nmol) had significantly more damage in the cortex and hippocampus than those pretreated with control siRNA (both P<0.05).

Figure 2. A, The no ischemic preconditioning (IP) group had significantly increased cleaved caspase-8, caspase-9, caspase-3, and poly(ADP-ribose) polymerase (PARP) 24 hours post-hypoxia-ischemia (HI) compared with the IP group. B, The IP group showed significantly increased cellular inhibitor of apoptosis 1 (cIAP1) but not cIAP2, X-linked IAP (XIAP), or survivin. Data from 4 different experiments; *P<0.05.

Figure 3. A, Immunohistochemistry showed that cellular inhibitor of apoptosis 1 (cIAP1) was markedly decreased in the group with no ischemic preconditioning (IP) but increased in the IP group 24-hour post-hypoxia-ischemia (HI). In the IP group, cIAP1 was mainly expressed in the vascular (arrows) and nonvascular cells. Scale bar, 100 μm. B, Immunofluorescence confirmed that cIAP1 expressed mainly in the rat endothelial cell antigen-1-positive endothelial cells and NeuN-positive neurons, but not in astrocytes (glial fibrillary acidic protein [GFAP]), in IP group. C, Compared with control, cIAP1 was significantly upregulated in IP group pretreated with control small interfering RNA (siRNA) but not in that pretreated with cIAP1 siRNA. n=4 per group. D, In IP group, the pups pretreated with cIAP1 siRNA had significantly more brain damage than those with control siRNA. n=7 per group. Scale bar, 20 μm. *P<0.05; **P<0.01; #P<0.001.
cIAP1 Was Required for Preconditioning-Mediated Protection Against OGD in Neurons and Endothelial Cells

In vitro OGD preconditioning was established in SH-SY5Y neuronal cells, HMEC-1 vascular endothelial cells, and U87 astrocyte cells.

Cytotoxicity in SH-SY5Y cells showed that neuronal cell death was not noted until after 12-hour OGD, and 55% of maximum LDH was released after 15-hour OGD, compared with 20% after normoxia (Figure 4A). Eight-hour OGD preconditioning before 15-hour OGD significantly decreased cytotoxicity from 58% to 30% (P<0.001) (Figure 4A). Compared with controls, the group without preconditioning had significantly decreased cIAP1 at 1-, 6-, and 24-hour post-OGD (all P<0.05). The preconditioned group had significantly increased cIAP1 levels at 1-, 6-, and 24-hour post-OGD compared with the group without preconditioning (all P<0.05) (Figure 4B).

LV-shRNA was used to knockdown cIAP1 in SH-SY5Y cells. LV-sh-cIAP1 cells had 25% of the cIAP1 levels of LV-sh-scramble cells (Figure 4C). LV-sh-scramble cells and LV-sh-cIAP1 cells had comparable cytotoxicity under normoxia. Compared with the nonpreconditioned LV-sh-scramble, preconditioned LV-sh-scramble cells had significantly decreased cell death post-OGD (from 55% to 33%) (P<0.001) (Figure 4D). In contrast, preconditioned LV-sh-cIAP1 cells showed significantly increased cytotoxicity compared with preconditioned LV-sh-scramble cells (from 33% to 50%) (P<0.001). Preconditioned LV-sh-cIAP1 cells showed no significant difference compared with nonpreconditioned LV-sh-scramble cells.

Cytotoxicity was increased progressively in HMEC-1 endothelial cells when OGD duration was increased up to 15 hours, and 65% of the maximum LDH was released after 15-hour OGD, compared with 10% after normoxia (Figure 5A). Seven-hour OGD preconditioning before 15-hour OGD significantly decreased cytotoxicity from 65% to 45% (P<0.001) (Figure 5A). Compared with controls, the nonpreconditioned group, but not the preconditioned group, had significantly decreased cIAP1 levels at 24- and 48-hour post-OGD (both P<0.05). Preconditioned cells had significantly increased cIAP1 levels at 24- and 48-hour post-OGD than nonpreconditioned cells (both P<0.05) (Figure 5B).

The LV-sh-cIAP1 HMEC-1 cells had 45% of cIAP1 levels of LV-sh-scramble cells (Figure 5C). Cytotoxicity was comparable between LV-sh-cIAP1 and LV-sh-scramble cells after normoxia. Preconditioned LV-sh-scramble cells had significantly decreased cytotoxicity post-OGD compared with nonpreconditioned LV-sh-scramble cells (from 70% to 50%) (P<0.001). Preconditioned LV-sh-cIAP1 cells showed significantly increased cytotoxicity compared with preconditioned LV-sh-scramble cells (from 50% to 65%) (P<0.001) (Figure 5D). Preconditioned LV-sh-cIAP1 cells also showed significantly decreased cytotoxicity post-OGD than nonpreconditioned LV-sh-scramble cells (P<0.05).

Cytotoxicity was increased progressively in U87 astrocytic cells when OGD duration was increased up to 12 hours, and...
57% of the maximum LDH was released after 12-hour OGD compared with 2% after normoxia (Supplemental Figure IIA).

Four-hour OGD preconditioning before 12-hour OGD significantly decreased cytotoxicity from 57% to 30% \((P < 0.001)\). Compared with controls, both preconditioned and nonpreconditioned groups had significantly decreased cIAP1 levels at 24-hour post-OGD. The preconditioned and nonpreconditioned cells showed comparable levels of cIAP1 post-OGD (Supplemental Figure IIB).

cIAP1 Overexpression in Neurons and Endothelial Cells Protected Against OGD

cIAP1 overexpression in SH-SY5Y cells showed that LV-cIAP1 cells had 30% increased cIAP1 than LV-control cells (Figure 6A). LV-cIAP1 and LV-control cells had similar cytotoxicity under normoxia. After 15-hour OGD, LV-cIAP1 cells had significantly decreased cytotoxicity (53% vs 43%; \(P < 0.01\)) than LV-control cells (Figure 6B). Preconditioned LV-control cells had significantly decreased cytotoxicity (30% vs 43%) compared with LV-cIAP1 cells \((P < 0.001)\).

cIAP1 overexpression in HMEC-1 cells showed that LV-cIAP1 cells had 75% increased cIAP1 than LV-control cells (Figure 6C). LV-cIAP1 and LV-control cells had comparable cytotoxicity under normoxia. LV-cIAP1 cells had significantly decreased cytotoxicity (70% vs 55%; \(P < 0.01\)) than LV-control cells after 15-hour OGD, and showed comparable cytotoxicity with preconditioned LV-control cells (Figure 6D).

Discussion

Our study presents evidence that IP upregulates cIAP1 in neurons and vascular endothelial cells and downregulates apoptosis after HI, and it provides long-term neuroprotection in neonatal brain. Intracerebroventricular delivery of cIAP1 siRNA significantly attenuated IP-mediated cIAP1 upregulation and neuroprotection. In vitro studies showed that cIAP1 is required for OGD preconditioning-mediated neuronal and endothelial cell protection, and it also provides neuronal and endothelial cell protection against OGD cell death. Thus, both in vivo and in vitro evidence reveal that cIAP1 is a critical shared molecule underlying IP-induced neuronal and vascular protection against HI.

Both apoptotic and anti-apoptotic pathways are activated after HI. Suppressing apoptosis and increasing survival protein expression are proposed as neuroprotective mechanisms against neonatal HI. Although studies have shown the neuroprotective effects of IAP proteins like XIAP and survivin, very few studies have addressed the neuroprotective role of cIAP1 in IP. Furthermore, none has focused on the effect of cIAP1 in IP-mediated neurovascular protection. Studies have shown that IP protected against cerebral ischemia by inhibiting caspase-3 and preserving cIAP2 in hippocampus, and that hypoxic preconditioning protected the human brain endothelium from ischemic apoptosis by Akt-dependent survivin activation. We found that IP markedly increased cIAP1, but not cIAP2, XIAP, or survivin, in the neurovascular unit post-HI, and that cIAP1 siRNA delivery significantly attenuated IP-mediated neuroprotection. Furthermore,
lentivirus-mediated short hairpin RNA targeting on cIAP1 also significantly attenuated OGD preconditioning-mediated neuronal and endothelial protection. These evidences suggest cIAP1 is required for IP-mediated neurovascular protection.

cIAP1 is the key inhibitor of caspases in neurons and endothelial cells.18–20 cIAP1 protected against tumor necrosis factor–related apoptosis-inducing ligand-mediated apoptosis in human fetal neural progenitor cells,18 and also was essential for maintaining endothelial cell survival and vessel homeostasis during vascular development.19 A zebrafish null mutant for cIAP1 showed severe hemorrhage and vascular regression attributed to endothelial cell defects and apoptosis.19 Vascular shear stress also prevented endothelial apoptosis by increasing cIAP1 but not XIAP or survivin in human umbilical vein endothelial cells.20 We showed that HI or OGD markedly decreased cIAP1 expression in neurons and endothelial cells, and that overexpression of cIAP1 was sufficient to protect against OGD cell death in these cells.

In the present study, IP significantly increased cIAP1 and decreased cleavage of caspases and poly (ADP-ribose) polymerase post-HI, suggesting that cIAP1 protected the neonatal brain by inhibiting active caspases and poly (ADP-ribose) polymerase. Accumulating evidences indicate that cIAP1 inhibits apoptosis through nuclear factor-xB activation.21 cIAP1 possesses a RING domain that may function as an ubiquitin E3 ligase that degrades caspases and the second mitochondria-derived activator of caspases.22,23 cIAP1 expression may be regulated by ubiquitination, degradation, or mRNA destabilization.24 The mechanism of cIAP1 upregulation in neurons and endothelial cells after IP remains unclear. Our previous study showed that IP-induced cyclic AMP response element binding protein activation in neonatal brain.11 Cyclic AMP can upregulate cIAP1 via cyclic AMP response element binding protein binding in the promoter region of cIAP1.25 Whether the cyclic AMP response element binding protein–cIAP1 axis is involved in IP-mediated neurovascular protection against HI in neonatal brain warrants further investigation.

For translation application and feasibility of transfection manipulation, we chose SH-SY5Y for neuronal preconditioning and HMEC-1 for endothelial cell preconditioning experiments in this study. SH-SY5Y has been commonly used to study the neural cell survival in the developing brain.26 HMEC-1 that displays the same morphologic, phenotypic, and functional characteristics as the normal HMECs also has been used as an in vitro model for blood–brain barrier damage.27 Although primary neuronal and endothelial cells may have a limited lifespan, further study using these cells should be performed to examine the role of cIAP1 in preconditioning-mediated neurovascular protection against OGD.

We are the first to show cIAP1 acts as a common molecule underlying IP-mediated neuronal and vascular protection against HI in the neonatal brain. cIAP1 is a critical endogenous anti-apoptotic brake induced by IP to protect against neurovascular injury in neonatal brain. Its upregulation in the neurovascular unit may be the potential link between IP and neuroprotection. Elucidating the pathway leading to cIAP1 upregulation after IP may yield neurovascular protective drugs that mimic the beneficial effects of IP for treating high-risk newborns with HI.
Acknowledgments

Supported by grants from the Taiwan National Science Council (NSC-98-2626-B006-001-MY3) and the National Health Research Institute (NHRI-EX99-9916NI).

Disclosures

None.

References

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Stroke. 2013;44:162-169; originally published online November 27, 2012;
doi: 10.1161/STROKEAHA.112.677617
Stroke is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0039-2499. Online ISSN: 1524-4628

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://stroke.ahajournals.org/content/44/1/162

An erratum has been published regarding this article. Please see the attached page for:
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The version of the article, “Ischemic Preconditioning Reduces Neurovascular Damage After Hypoxia-Ischemia Via the Cellular Inhibitor of Apoptosis 1 in Neonatal Brain” by Lin et al that published online ahead-of-print on November 27, 2012, and appears in the January issue (Stroke. 2013;44:162–169) contained an error in Ying-Chao Chang’s affiliation. The correct affiliation is Department of Pediatrics, Kaohsiung Chang Gung Memorial Hospital and Chang Gung University College of Medicine, Kaohsiung, Taiwan. The authors regret the error.

This correction has been made to the online version of the article, which is available at http://stroke.ahajournals.org/content/44/1/162.
SUPPLEMENTAL MATERIAL

Supplemental Methods

Based on the mRNA sequence for the rat cIAP1 (Genebank accession number NM_021752), the siRNA sequence matched with the rat cIAP1 mRNA sequence, but the control siRNA showed no homology to all predicted reference mRNA sequences. OGD buffer was composed of 0.4 g KCl, 6.8 g NaCl, 2.2 g NaHCO₃, 0.14 g NaH₂PO₄·H₂O, 0.48 g HEPES, and 0.2 g CaCl₂ in 1 L ddH₂O, with pH of 7.4.

**Intracerebroventricular delivery of cIAP1 siRNA.** The cIAP1 siSTABLE siRNA (Dharmacon) had the sequence: 5’- GCUUUAUGUCAUAUUGUAU -3’; and control siSTABLE siRNA : 5’- GAUCAUACGUGCGAUCAGA -3’. The preconditioned pups were intracerebroventricularly infused with cIAP1 siSTABLE siRNA or control siRNA in the right cerebral hemisphere using a 30-gauge needle on a 10-μL Hamilton syringe at a rate of 1 μL/minute. The location of injection in relation to the bregma was 2.0 mm posterior to, 1.5 mm lateral to, and 2.0 mm beneath the skull surface. The siRNA were infused at a concentration of 0.2 or 0.6 nmol/μl on P3 (0.4 or 1.2 nmol) and at 30 minutes before IP on P6 (0.4 or 1.2 nmol). To determine the effect of siRNA, the cortices were collected 24 hours post-HI, and brain damage determined on P14.

**Pathological outcome.** On P14 (for cIAP1 siRNA effect) or P42 (for IP long-term outcome), the brains were cryoprotected, coronally sectioned (40-μm thick), and stained with cresyl violet. The areas of the cortex and hippocampus in the three reference planes (plates 27, 31, and 39) of a rat atlas were assessed using Image-Pro Plus 4.5. The percentage of area loss in the cortex and hippocampus in the right hemisphere versus that in the left hemisphere was determined.

**Quantitative analysis of neurons and vessels.** The NeuN-positive neurons and
RECA-1-positive vessels were counted in the ipsilateral cortex of the sections corresponding to the planes (plates 18, 31, and 39) of a rat atlas. NeuN-positive cells were measured in three visual fields (each visual field: 220 μm×166 μm) within the cortex in each section and photographed, while the cell number was expressed as the average number of NeuN-positive cells per visual field. RECA-1 positive blood vessels were measured in three visual fields (one visual field: 890 μm×670 μm) within the cortex in each section. Blood vessels were analyzed using the NIH ImageJ, and blood vessels per visual field were put in binary form at an average between the minimum and maximum intensity of the neighborhood, thus segmenting vessels close to half-height.

**Oxygen-glucose deprivation.** Human neuroblastoma cells SH-SY5Y were cultured in Modified Eagle's Medium (MEM) and Ham’s F12 media (Invitrogen) supplemented with 10% (v/v) fetal bovine serum (FBS, Invitrogen) and 1% penicillin/streptomycin (Thermo Scientific) (complete medium). Human microvascular endothelial cell line-1 (HMEC-1) cells were maintained in medium 200 (Invitrogen) composed of Low Serum Growth Supplement (LSGS) (complete medium). The human glioblastoma U87 cell line was maintained in Dulbecco's modified Eagle's medium (DMEM, Invitrogen) with 10% FBS and 1% penicillin/streptomycin (complete medium). The SH-SY5Y cells (1.5×10⁵ cells/well), HMEC-1 cells (3×10⁵ cells/well) and U87 cells (3×10⁵ cells/well) were plated, respectively, in 6-well plates at 37°C with 5% CO₂ for 24 hours.

**OGD cell death.** The SH-SY5Y cells were washed twice in MEM/F12 (1:1) medium without glucose (OGD medium). The HMEC-1 cells were washed twice in OGD buffer and the U87 cells were washed twice with glucose-free DMEM medium (OGD medium) (Invitrogen) before being placed in hypoxia chambers (NexBiOxy,
Taiwan), maintained under a gas mixture of 95% N₂/5% CO₂ at 37°C at 3 L/minute for the designated duration (1% O₂ for SH-SY5Y cells and HMEC-1 cells, 0% for U87 cells).

**OGD preconditioning.** SH-SY5Y, HMEC-1 cells and U87 cells were grown for 24 hours in complete medium. The SH-SY5Y cells and U87 cells were washed with OGD medium before switching to OGD medium, while the HMEC-1 cells were washed with OGD buffer before switching to OGD buffer. OGD preconditioning was performed for 8 hours for SH-SY5Y, 7 hours for HMEC-1 cells, and 4 hours for U87 cells. The OGD-preconditioned and non-preconditioned cells were maintained in complete medium under normoxia for 24 hours before being exposed to 15-hour OGD (SH-SY5Y cells and HMEC-1 cells) and 12-hour OGD (U87 cells). Culture medium background was assay medium to correct for LDH activity contributed by serum and phenol red in the culture medium. Target cell maximum LDH release was assay medium (plus 2% Triton X-100) plus cells to determine 100% release of LDH. All assays were performed in triplicate.

The lentiviral expression system was provided by the National RNAi Core Facility, Academia Sinica, Taiwan. To produce recombinant lentiviruses expressing cIAP1, HEK293T cells were co-transfected with pLKO_AS2.neo-cIAP1 carrying a cytomegalovirus (CMV) promoter together with the packaging vector pCMV-ΔR8.91 and envelope vector pMD.G.

**Lentivirus-mediated cIAP1 over-expression.** To construct pLKO-AS2.neo-cIAP1, the DNA fragment encoding cIAP1 was amplified by polymerase chain reaction (PCR) with forward primer 5’-GGGGGCTAGCATGCACAAAACTGCCTCC-3’ and reverse primer 5’-GGGGGAATTCTTAAGAGAGAAATGTACGAACAGT-3’ and subsequently cloned into the Nhe I and EcoR I site of the lentiviral vector.
pLKO-AS2.neo. To establish HMEC-1 cells stably expressing cIAP1, the cells were infected with recombinant lentiviruses encoding cIAP1 (LV-cIAP1) for 24 hours. To over-express cIAP1 in the SH-SY5Y cells, the cells were infected with LV-cIAP1 for 24 hours and allowed to grow in the complete medium for another 24 hours.

Supplemental Figure 1

<table>
<thead>
<tr>
<th>P3</th>
<th>P6</th>
<th>P7</th>
<th>P14</th>
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<tr>
<td>IP</td>
<td>H1</td>
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-24h 0h +3h +24h +72h +7d +35d

siRNA WB WB WB Outcome Outcome IF, IHC

IP: Ischemic preconditioning
H1: Hypoxia-Ischemia
WB: Western blotting for apoptotic and anti-apoptotic markers
IF: Immunofluorescence for NeuN+ and RECA+ cells
IHC: Immunohistochemistry for cIAP1 expression
Outcome: Pathology at P14 (for siRNA effect) and P42 (for IP outcome)
Supplemental Figure 2

A

![Graph showing LDH cytotoxicity in U87 astrocytic cells and OGD duration](image)

B

![Graph showing cIAP1 levels in U87 astrocytic cells and OGD duration](image)

Supplemental Figure legend

**Figure 1.** The summary of the treatment paradigm and time points of work-ups in the IP study.

**Figure 2.** (A) Left panel: Cytotoxicity increased progressively in U87 astrocytic cells when OGD duration was increased up to 12 hours. Right panel: Four-hour OGD preconditioning before 12-hour OGD significantly decreased cytotoxicity in U87 cells. (B) The no-preconditioned and the preconditioned group had similar levels of cIAP1 post-OGD. All data were from 4 different experiments. *p<0.001*