Preconditioning Suppresses Inflammation in Neonatal Hypoxic Ischemia Via Akt Activation

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Background and Purpose—Hypoxic preconditioning (PC) confers robust neuroprotection against neonatal hypoxic-ischemic brain injury (H-I), yet the underlying mechanism is poorly understood. In the adult brain, neuronal survival after ischemia is associated with the activation of the phosphatidylinositol 3-kinase (PI3-K)/Akt signaling pathway. Suppression of inflammation is a newly identified direct consequence of PI3-K/Akt signaling. We therefore investigated whether PI3-K/Akt suppresses inflammation and contributes to PC-induced neuroprotection.

Methods—Postnatal day 7 rats were exposed for 3 hours to either ambient air or 8% oxygen, which induces hypoxic PC. H-I was produced 24 hours later by unilateral carotid artery ligation followed by 2.5 hours of hypoxia. Animals were euthanized 0 to 24 hours later for detecting Akt and glycogen synthetase kinase-3β phosphorylation (p-Akt, p-GSK-3β), 24 hours later for assessing cytokine expression and inflammatory markers, and 7 days later for measuring brain tissue loss. In addition, LY294002 was injected intracerebroventricularly to inhibit PI3-K/Akt.

Results—Brains with H-I without PC showed delayed but sustained reduction in p-Akt. PC restored the levels of p-Akt and the Akt substrate GSK-3β, reduced proinflammatory markers (NF-κB, COX-2, CD68, myeloperoxidase, and microglial activation), and markedly ameliorated H-I-induced brain tissue loss. Inhibition of PI3-K/Akt using LY294002 attenuated PC neuroprotection and promoted the expression of NF-κB, COX-2, and CD68. Proteomic microarray analysis revealed that PC inhibited expression of proinflammatory cytokines induced by H-I or a dose of lipopolysaccharide that resulted in minimal tissue damage.

Conclusions—Suppression of inflammatory responses may contribute to PC neuroprotection against neonatal H-I brain injury. This effect is mediated in part via upregulating PI3-K/Akt activity. (Stroke. 2007;38:1017-1024.)

Key Words: hypoxia-ischemia • inflammation • neonatal • p-Akt • preconditioning

Perinatal hypoxic-ischemic brain injury (H-I) is a major cause of permanent neurological dysfunction in neonatal children. Emerging evidence suggests that acute inflammation contributes considerably to the pathogenesis of neonatal H-I brain injury.1 Inflammatory reactions to ischemia involve the release of pro-inflammatory cytokines, increased expression of endothelial adhesion molecules, activation of microglia and macrophages, and leukocyte infiltration.2,3 The synergistic actions of inflammatory events exacerbate brain injury, leading to deterioration of neurological outcomes.3 Thus, from a clinical perspective, suppressing inflammation represents a legitimate strategy to reduce neonatal H-I brain injury.

Tolerance to otherwise lethal ischemic events can be induced in neonatal brain when a preconditioning event (PC), such as sublethal ischemia and/or hypoxia, precedes H-I.4 Although the mechanisms controlling PC-induced neuroprotection are not completely understood, it has been suggested that hypoxic PC in neonatal rat brain enhances intracellular pro-survival signaling pathways via altering gene expression and/or posttranslational modulation of signaling molecules.5 Understanding of the precise mechanisms underlying PC may help identify new targets for therapeutic intervention in H-I brain injury.6

Phosphatidylinositol 3-kinase (PI3-K)/Akt is one of the pathways activated by PC in adult brain, likely playing a critical role in promoting neuronal survival after ischemia.7,8 Classically, the neuroprotective role of PI3-K/Akt has been attributable almost entirely to its anti-apoptotic actions. However, recently it has been found that activation of the PI3-K/Akt pathway in human monocytes can directly inhibit lipopolysaccharide-induced inflammation.9

Despite of the established neuroprotective role of PI3-K/Akt in adult brain ischemia, evidence supporting this role in
neonatal PC and neuroprotection against H-I is rather sparing and indirect. Therefore, in this study, we investigated whether hypoxic PC activates PI3-K/Akt and modulates the inflammatory response in neonatal rat brain. The effects of inhibiting the PI3-K pathway on PC-induced Akt activation, cerebral protection, and expression of inflammatory markers were also determined.

Materials and Methods

Model of Neonatal H-I Injury and Hypoxic Preconditioning

The Institutional Animal Care and Use Committee of the University of Pittsburgh approved the animal protocol used in this study. Sprague-Dawley rat pups (Charles River Laboratory, Wilmington, Mass) were used at either postnatal day 6 or 7. Hypoxic PC was induced in postnatal day 6 rats 24 hours before H-I by exposure for 3 hours to a humidified gas mixture of 8% oxygen/92% nitrogen (37°C) or ambient air (control animals), respectively. On postnatal day 7, H-I was produced according to the modification of Levine method. Pups were anesthetized with 3% isoflurane mixed with ambient air under spontaneous inhalation, and the left common carotid artery was ligated. After a 1.5-hour recovery period, the pups were placed in glass chambers containing a humidified atmosphere of 8% oxygen/92% nitrogen and submerged in a 37°C water bath. After 2.5 hours of hypoxia, the pups were returned to their dam. Control animals were sham-operated, allowed to recover for 1.5 hours, and then exposed to ambient air while submerged in the 37°C water bath for 2.5 hours.

Intracerebroventricular Injection

The pups were anesthetized and mounted in a stereotactic frame and intracerebroventricular injections using a 27-gauge needle and a 5-μL Hamilton syringe were made at the following coordinates: 2.0 mm rostral, 1.5 mm lateral, and 2.0 mm deep in relation to the lambda. LY294002 (0.2 mol/L; Calbiochem) was injected 1 hour before PC, whereas lipopolysaccharide (LPS) serotype 055:B5 (5 μg; Sigma) was used in place of H-I.

Assessment of Brain Damage

Seven days after H-I, the brains were removed, paraffin-embedded, and cut into 4-μm-thick coronal sections. Sections through the corpus callosum and dorsal hippocampus were stained with hematoxylin and eosin. The extent of tissue damage was determined by calculating the amount of surviving tissue in each section. Briefly, the cross-sectional areas of the striatum, cortex, and hippocampus in 8 equally spaced sections were assessed using the MCID image analysis system (St. Catharine’s, Ontario, Canada). The volume of each brain region and the percent volume loss in the lesioned versus unlesioned hemisphere was determined for each animal by an investigator blinded to the experimental conditions using the following equation: (volume of unlesioned hemisphere−volume of lesioned hemisphere/volume of unlesioned hemisphere).

Detection of Hypoxic Cells with EF5

To confirm the hypoxic state of brain tissues during H-I, we used an oxygen-sensitive immunofluorescence method based on the ability of the pentfluorinated derivative of etanidazole (EF5) (a gift from Dr C. J. Koch, University of Pennsylvania, Philadelphia, Pa) to form stable adducts with intracellular macromolecules at low or anoxic oxygen levels. Pups received an intraperitoneal injection of either 0.01 mol/L EF5 (in 0.9% sodium chloride, pH 7.4) or an equivalent volume of vehicle 30 minutes before H-I. Immediately after H-I, the pups were decapitated and their brains quickly frozen at −70°C. EF5 binding was detected using the Cy3-conjugated anti-ELK3–51 antibody as described previously on coronal sections (16 μm) fixed in 4% paraformaldehyde. Raw fluorescent levels were integrated and measured using MCID. The pyramidal cell layer values were adjusted using the cell-poor molecular layer as background level, and the contralateral hippocampus was used as control.

Western Blots

Cortical protein extraction and Western blot analyses were performed as previously described. The blots were semi-quantified using gel densitometry and MCID. The primary antibodies used in this study were anti-phospho-Akt (Ser473), total Akt, rabbit anti-phospho-enzyme glycogen synthase kinase-3β (GSK-3β) (Ser9), GSK-3β (Upstate Cell Signaling), mouse anti-anti–NF-κB, p65 subunit monoclonal antibody (Chemicon International), and mouse anti–rat CD68 (Serotec).

Immunohistochemistry and TUNEL Staining

Immunofluorescent staining was performed on paraffin-embedded coronal sections from pups euthanized 24 hours after H-I as described previously, using anti-p-Akt (Ser473), anti-NeuN, anti-GFAP (Chemicon International), Texas Red or biotinylated Tomato Lectin (Vector Laboratories), anti-myeloperoxidase (MPO) (Chemicon International), and anti-myelin basic protein (Covance). Terminal transferase-mediated dUTP nick-end labeling (TUNEL) staining was performed according to the manufacturer’s instructions (Roche).

Proteomic Analysis

Protein arrays of System-Chemiarray rat cytokine (Chemicon) antibodies were used to measure cytokines in cerebral cortical extracts. Signal intensity was quantified by densitometry using MCID. For each spot, the net gray level density was determined by subtracting the background gray levels from the total raw levels. A positive control was used to normalize the results from different membranes. The relative fold-difference in cytokine amount was determined in reference to the amount present on the control membrane.

Statistical Analysis

All data are reported as mean±SEM. Significant differences between means were assessed by ANOVA and post hoc Scheffe tests for multiple comparisons. P<0.05 was considered statistically significant.

Results

Hypoxic PC Is Protective Against Neonatal H-I

Figure 1A illustrates the timeframe of the study and the time points for each assay performed after H-I. The induction of hypoxic PC 24 hours before H-I in postnatal day 7 rats reduced lesion size, protected against both neuronal and white matter injury (white matter injury is demonstrated by the reduced immunoreactivity of myelin basic protein) (Figure 1B). Measurement of tissue damage in the cortex, hippocampus, and striatum revealed that PC significantly attenuated brain loss volume (Figure 1C). To determine whether the neuroprotective effects of PC were caused by altered oxygenation of the neuropil, relative oxygen levels were determined using EF5 and anti-ELK3–51/Cy3 staining (Figure 1D). Fluorescent signals were elevated in both the H-I (16.17±1.35-fold, ipsilateral side; 10.08±3.19-fold, contralateral side) and PC plus H-I (15.10±2.34-fold, ipsilateral side; 9.72±1.23-fold, contralateral side) hippocampi as compared with non–H-I controls (P<0.05, n=3 animals per condition). There was no significant difference between the H-I group and PC plus H-I group (P>0.05). Similar results were obtained for cortex and striatum (data not shown).
PC Protects Neonatal Brain Via PI3-K and Activation of Akt

Phospho-Akt (p-Akt) levels were examined in the cortex using Western blot analysis (n=4 per condition). H-I caused an immediate decrease followed by an abrupt increase (1 hour after H-I) in p-Akt. Thereafter, p-Akt levels subsided until they were again significantly below control levels at 16 and 24 hours after H-I (Figure 2A). Decreases in p-Akt were found consistently at 24 hours after H-I, and this time point was used for subsequent experiments.

To determine whether PC could activate Akt in neonatal rats, p-Akt levels were analyzed in pups that underwent PC alone (n=4 per condition). Compared with control animals, PC induced a significant increase in cortical p-Akt after H-I (Figure 2C). Augmentation of p-Akt in the PC+H-I animals was also dependent on PI3-K activity, because LY294002 significantly reduced its formation (Figure 2C). Consistent with the role of PI3-K/Akt in neuroprotection, LY294002 significantly, although partially, abolished the protective effect of PC on tissue loss 7 days after H-I (Figure 2D).

Double-label immunofluorescent staining was performed at 24 hours after H-I to examine p-Akt expression and DNA fragmentation (TUNEL) at cellular levels. As represented in the cortex (Figure 3A), the number of p-Akt–positive cells was decreased while the number of TUNEL-positive cells was increased after H-I as compared with the non–H-I controls. These changes were largely reversed in H-I brains preceded with PC. In all cases, no double-stained cells were seen, suggesting that p-Akt was expressed mainly in surviving cells.

To examine which type of cells expressed p-Akt in H-I brains with or without PC, immunofluorescent double staining was performed at 24 hours after H-I (n=5 per experi-
mental condition) to determine whether p-Akt colocalizes with the neuronal marker NeuN, the astroglial marker GFAP, or the microglial marker tomato lectin. We found that p-Akt was expressed mainly in neurons and microglia, but not in astrocytes, after H-I with PC treatment (Figure 3B). In H-I brains without PC treatment, the double-staining pattern for p-Akt was similar except that the numbers of neurons and microglial cells expressing p-Akt were substantially fewer than H-I brains with PC treatment (data not shown).

PC Inactivates (Increased Phosphorylation) GSK-3β
GSK-3β is a transducer of both pro-apoptotic and pro-inflammatory signals, and is negatively modulated through phosphorylation at Ser9 by p-Akt. The levels of p-GSK-3β (Ser9) were significantly decreased immediately after H-I and 24 hours after H-I (n=4 per condition), indicating the activation of GSK-3β (Figure 4A, 4C). When rats were exposed to PC alone or PC preceding H-I, a significant increase in p-GSK-3β (Ser9) levels were found compared with non–H-I controls or H-I animals (Figure 4B, 4D).

PC Decreases Inflammatory Markers After H-I
To determine the effect of PC on H-I–induced inflammation, several inflammatory markers were measured 24 hours after H-I. This time point was chosen in this model, because inflammatory responses can be reliably detected and irreversible brain damage has yet to occur. NF-κB, which regulates proinflammatory cytokines, was activated (increased p65 subunit) in the H-I cortex. PC returned NF-κB activation to

Figure 2. Activation of Akt in neonatal brain contributes to PC neuroprotection. A, Representative Western blot of activated Akt (p-Akt) in cortex from control (Ctrl) and after various time points after H-I. There was no change in total Akt or β-actin. B, Western blot of p-Akt in control or 24 hours after PC with or without LY294002 (LY). C, Representative Western blot of p-Akt in control, 24 hours after H-I treatment, or H-I preceded by PC (PC+H-I) in the absence or presence of the PI3-K inhibitor LY294002 (LY). The graphs (A, B, C) illustrate the semi-quantitative results from 4 independent experiments. D, Tissue loss assayed in cortex, striatum and hippocampus 7 days after H-I alone or with PC and PC plus LY294002. N=15 animals per group. *P<0.05 vs control, or between bars indicated by brackets.
control levels, and inhibition of PI3-K using LY294002 abolished the reduction (Figure 5A, 5B). Cyclooxygenase-2 (Cox-2), an inducible enzyme downstream of NF-κB and implicated in neuroinflammation, followed the same pattern of expression in cortical tissue as activated NF-κB (Figure 5A, 5B). Microglial activation in the cortex was measured using a marker for activated microglia, CD68. After H-I, a significant increase in CD68 expression was found, which was reversed by PC (Figure 5A, 5B).

Immunohistochemical examination of microglia and macrophages in cortical sections showed that lectin-positive cells were small and ramified in non–H-I animals. After H-I, however, lectin-positive cells became stout and rounded (Figure 5C), indicating the activation of microglia and macrophages. Neutrophils in the brain were examined by immunoreactivity to MPO. A marked increase in MPO expression in the choroids plexus of the lateral ventricles was found at 24 hours after H-I, similar to results reported by Hudome et al.19 Microglial and neutrophil activation subsided in H-I animals receiving PC (Figure 5C, 5D). The inhibitory effect of PC on microglial activation was abolished by LY294002 (data not shown).

**PC Suppresses Inflammatory Cytokines**

Protein antibody arrays were used to determine whether PC altered pro-inflammatory cytokines 24 hours after H-I. Out of 19 cytokines measured, a total of 9 were significantly increased after H-I (based on 4 different sets of experiments), all of which were returned at or below control levels by PC (Figure 6A).
To determine whether PC has any direct inhibitory effects on cytokine expression, brain inflammation was induced using LPS, an established model that induces negligible amount of neuronal death in the brain. LPS significantly increased the levels of 6 cytokines (based on 3 different sets of experiments), all of which were significantly reduced by PC 24 hours before LPS injection (Figure 6B).

**Discussion**

Using a well-established rat model of neonatal H-I, we investigated one of the mechanisms underlying the neuroprotective effect of hypoxic PC. The results suggest that activation of the PI3-K/Akt signaling pathway and suppression of inflammation, 2 potentially interrelated processes, may contribute to PC-induced tolerance against H-I brain injury. Our
findings support the recent speculation of inhibiting cytokine signaling in ischemic tolerance induced in the adult brain.21

As recently shown, the inhibitory effect of Akt on inflammation is dependent on its downstream substrate, GSK-3β.9 Akt phosphorylates and inactivates GSK-3β at its N-terminus (at Ser9). Under conditions where Akt activity is decreased, such as after H-I, GSK-3β can thus be activated. Functionally, active GSK-3β can enhance the operation of various pro-inflammatory signaling molecules, including NF-κB,22 IL-6, and MCP-1,23 and reduce anti-inflammatory CREB DNA-binding activity.24 In our study, hypoxic PC lowered the levels of active GSK-3β after H-I, thereby decreasing the ability of GSK-3β to augment the inflammatory response. In addition, PC decreased the active form of NF-κB and diminished COX-2 expression, and lessened the capacity of the downstream effectors of GSK-3β to promote inflammation. Although not determined here, PC could also upregulate the feedback inhibitors of inflammation.21 Whether the mechanism by which hypoxic PC suppresses H-I–induced inflammation occurs solely by decreasing the pro-inflammatory response or by triggering an endogenous anti-inflammatory reaction will need to be determined by further investigation.

As determined using double-label immunofluorescent staining, expression of p-Akt was primarily in neurons and microglia in H-I preceded with PC. Given that microglial activation plays an important role in postischemic inflammation, p-Akt may negatively modulate pro-inflammatory actions within microglia via the GSK-3β signaling pathway. However, how could p-Akt expressed in neurons regulate the inflammatory response after H-I? Although this inquiry is outside of the scope of the current study, several recent studies based on neuron/microglia cocultures have suggested that sublethally stimulated neurons are capable of initiating pro-inflammatory signals to microglia via active transport and release of cytokines or cytokine-like factors.25 Thus, it is plausible that H-I–induced inflammation can be opposed by the PC-mediated increase in p-Akt expression, directly decreasing release of inflammatory factors from neurons, and diminishing the activation of microglia and neutrophils. More detailed examinations are warranted to explore the precise role of neuronal versus microglial expression of p-Akt in regulating H-I–induced inflammation.

A potential confounding factor that may influence data interpretation is whether the observed decreases in inflammation after PC was a secondary effect caused by the reduced tissue damage itself or by a diminished effect of an inflammatory response. To partially address this issue, we investigated LPS-induced cytokine expression in brains with or without PC. The data revealed that PC was capable of suppressing cytokine release after LPS induction. Because the dose of LPS used in this study induces brain inflammation without gross brain damage or neuronal loss,20 these results suggest that the inflammation-suppressing effect of PC in H-I brain may be attributable, at least in part, to its direct interference with the signaling processes governing inflammation regulation.

The data presented here suggest that the PI3-K–dependent activation of Akt is critical for PC-induced neuroprotection against H-I brain injury. However, other mechanisms may also be contributing, as the PI3-K inhibitor LY294002 failed to completely block the PC-induced neuroprotection against tissue loss in the present study. Previous studies have shown that hypoxia-induced tolerance is correlated with the induction of the transcription factor hypoxia-inducible factor 1, an important signaling molecule upstream of the PI3-K/Akt pathway.2 Hypoxia-inducible factor 1 increases, among other
pathways, PI3-K/Akt activity via enhanced production of the endogenous PI3-K activator, erythropoietin. Other transcription factors enhanced by hypoxic PC include CREB. We also found a marked elevation of phospho-CREB in the cortex of PC-treated animals (Yin, unpublished data, 2006), suggesting that Akt may act in concert with other neuroprotective pathways, including CREB and hypoxia-inducible factor 1. Our data further suggest that suppression of inflammatory responses may be an important mechanism by which Akt, and possibly other signaling pathways, protects the neonatal brain from H-I insults. Whether sex differences have been involved in this particular mechanism remains unclear. It has been reported that although the extent of hypoxic ischemic damage does not appear to differ between males and females during the neonatal period, different apoptotic mechanisms may be activated in male and female brains after neonatal hypoxic ischemia. Thus, the potential gender difference for the current mechanism should be tested in future studies.

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Disclosures
None.

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