The Akt-Endothelial Nitric Oxide Synthase Pathway in Lipopolysaccharide Preconditioning-Induced Hypoxic–Ischemic Tolerance in the Neonatal Rat Brain

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Background and Purpose—Low-dose lipopolysaccharide (LPS) preconditioning provides neonatal rats long-term neuroprotection against hypoxic ischemia (HI). Upregulating endothelial nitric oxide synthase (eNOS) protects against cerebral ischemia; however, whether eNOS is required for LPS preconditioning-induced protection in neonatal rats is unknown. We hypothesized that Akt activation, which upregulates eNOS in neurons and endothelial cells, is required for LPS preconditioning-induced tolerance against HI in the neonatal brain.

Methods—Six-day-old rat pups were intraperitoneally injected with LPS (0.05 mg/kg) or normal saline 24 hours before HI. Immunoblotting and immunohistochemistry were used to determine the phospho-Akt (pAkt Ser473), phospho-eNOS (peNOS Ser1177), and eNOS levels and immunofluorescence to determine the cellular distribution of eNOS and pAkt Ser473. Pharmacological and genetic approaches were used to regulate Akt and eNOS, and the weight loss of cerebral hemispheres on postnatal Day 21 was used to assess outcomes.

Results—eNOS, peNOS (Ser1177), and pAkt (Ser473) levels were significantly higher in LPS- than in normal saline-treated rats 24 hours postinjection. LPS-induced eNOS was expressed primarily in neurons and vascular endothelial cells. N-omega-(H9275)-nitro-L-arginine and antisense oligodeoxynucleotide treatment significantly reduced eNOS expression in neurons and endothelial cells and inhibited LPS-induced protection against HI in rat pups. L-arginine and adenovirus eNOS transfection upregulated eNOS and protected the rat pups against HI. Wortmannin treatment before LPS preconditioning significantly reduced eNOS expression in neurons and endothelial cells, which inhibited LPS-induced protection against HI.

Conclusions—Akt-mediated eNOS upregulation in neurons and vascular endothelial cells is required for LPS-induced tolerance against HI in the neonatal rat brain. (Stroke. 2010;41:1543-1551.)

Key Words: Akt ■ eNOS ■ LPS ■ neonatal ischemia ■ preconditioning

Hypoxic ischemia (HI) is a major cause of neonatal mortality and subsequent neurodevelopmental morbidity in surviving infants. A new approach to studying treatment for HI brain injury is investigating the state of tolerance.1 Tolerance is attained by preconditioning tissue to sublethal stress, which augments endogenous defense mechanisms against subsequent lethal insults. Clarifying the mechanisms of preconditioning may lead to new pharmacological therapies against HI in neonates.1,2

Endothelial nitric oxide synthase (eNOS) is found primarily in the endothelium of cerebral blood vessels. Nitric oxide generated by eNOS is crucial for vascular function and homeostasis. Using L-arginine, statins, or physical activity to upregulate eNOS increases cerebral blood flow and protects against cerebral ischemia.3 Lipopolysaccharide (LPS) preconditioning provides tolerance against ischemia in adult and neonatal rats.4–6 Systemic LPS injections upregulated eNOS rather than inducible NOS (iNOS) in astrocytes and blood vessels.7,8 However, whether eNOS is required for LPS preconditioning-induced HI tolerance in neonatal brain remains unclear. If eNOS is required, then the preconditioning-activated upstream pathway that leads to eNOS upregulation warrants investigation because the pathway will be an attractive therapeutic target for HI.

Akt, a pivotal molecule for cell survival and vascular homeostasis, is a key mediator of eNOS activation because it phosphorylates eNOS on Ser1177.3 Hypoxic preconditioning suppressed neuroinflammation in neonatal HI through Akt activation.9 Akt activation accounted for eNOS upregulation in vascular endothelial cells10 and in ischemic tolerance of gerbil hippocampus CA1.11 Our previous study showed that low-dose LPS (0.05 mg/kg) preconditioning provided long-term neuroprotection in neonatal rats.5 Whether Akt-eNOS signaling is critical in LPS preconditioning-induced HI toler-
ance in the neonatal brain remains undescribed. A shared signaling pathway may underlie preconditioning-induced protection in neurons and vascular endothelial cells. Therefore, we hypothesized that Akt-mediated eNOS upregulation in neurons and endothelial cells is required for LPS-induced HI tolerance in the neonatal brain.

Materials and Methods

Low-Dose LPS Preconditioning

This study was approved by the Animal Care Committee of National Cheng Kung University. Postnatal (P) Day 6 Sprague-Dawley female rat pups (purchased from BioLASCO Taiwan Co, Ltd) were intraperitoneally injected with LPS (0.05 mg/kg; *Escherichia coli* 0111:B4; Sigma-Aldrich) or pyrogen-free normal saline (NS) 24 hours before HI on P7. On P7, the rats were anesthetized (2.5% halothane), and then the right common carotid artery was permanently ligated. After they had been allowed to recover for 1 hour, the rats were placed in airtight 500-mL containers with humidified 8% oxygen flow rate of 3 L/min for 2 hours. The pups were sorted into 4 groups: NS (NS without HI), LPS (LPS without HI), NS-HI (NS 24 hours before HI), and LPS-HI (LPS 24 hours before HI).

Outcome Measure

On P21, the rat pups’ brains were sectioned at the midline, and the left and right hemispheres were weighed. The percentage of hemispheric weight loss, measured as [(left hemisphere weight – right hemisphere weight)/left hemisphere weight], was used as the measure of cerebral HI injury.

Immunohistochemistry and Analysis

Immunohistochemistry for eNOS and pAkt (Ser473) was done 24 hours after LPS or NS injection. The brains were coronally sectioned (14-μm thick). The primary antibodies used were anti-eNOS (1:100; BD Transduction) and antiAkt (Ser473; 1:200; Cell Signaling). Biotinylated antirabbit and antimus antibodies were the secondary antibodies (all 1:200). eNOS and pAkt expression was analyzed using integrated optical density in the cross-sectional areas of the cortex in 2 reference planes corresponding to plates 31 and 39 in a rat brain atlas. The analysis used imaging software (ImagePro Plus 6.0; Media Cybernetics) at 400× magnification per visual field (one visual field=0.096 mm²). Three visual fields in the cortex of the hemisphere per section and 2 sections per brain were analyzed and averaged.

Immunofluorescence

The sections were incubated overnight with a mixture of 2 of the following primary antibodies: anti-eNOS (1:1000), anti-pAkt (1:1000), antineuronal nuclear antigen (NeuN) (neurons; 1:100; Chemicon), antiendothelial cell antigen (RECA) (endothelial cells; 1:100; Abcam), antilglial fibrillary acidic protein (GFAP) (1:100; Chemicon), or terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling (TdT-fluorescein-FragEL DNA fragmentation detection kit; Calbiochem). The sections were then washed and incubated with fluorescein isothiocyanate-conjugated antimouse IgG antibody (1:500; Invitrogen). The fluorescence signals were detected and the results recorded (Eclipse E400 microscope; Nikon) at excitation–emission wavelengths of 596 to 615 nm (Texas Red, red) and 470 to 505 nm (fluorescein isothiocyanate, green).

Immunoblotting

The cortex was homogenized, resolved using 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and blotted electrophoretically to polyvinylidene fluoride membranes. The membranes were incubated with primary antibodies: anti-eNOS (1:1000), antiphospho-eNOS (peNOS, Ser177; 1:1000; Cell Signaling), antineuronal NOS (nNOS; 1:1000; BD Transduction), anti-iNOS (1:800; Santa Cruz), anti-Akt (1:1000; Cell Signaling), antiphotidinositol 3-Kinase/Akt Inhibition

Pharmacological Activation

Twenty-four and 16 hours before HI, the pups were intraperitoneally injected with L-arginine (200 mg/kg), an eNOS agonist, or D-arginine (200 mg/kg; Sigma-Aldrich). The cortices for immunoblotting and the brain sections for immunohistochemistry were collected 16 hours after the second arginine injection.

Adenoviral eNOS Gene Transfer

The replication-deficient recombinant adenoviruses nuclear-targeted β-galactosidase, and eNOS were kindly provided by Professor S.K. Shyu (Institute of Biomedical Sciences, Academia Sinica, Taiwan). The constructed adenoviruses were replicated in 293 cells and harvested using CsCl gradient ultracentrifugation. The viral particles collected were dialyzed and viral titers determined using a plaque-assay method. These adenoviruses (1×10⁹ plaque-forming units per injection) were infused into the right hemisphere. The pups were given 2 consecutive injections; the first injection was 2.0 mm posterior to and 1.5 mm lateral to the bregma and 2.0 mm beneath the skull surface, and the second injection was 2.0 mm posterior to and 2.0 mm lateral to the first injection site and 2.0 mm beneath the skull surface. These transfers occurred on P2, P4, and P6, respectively, and the cortices were collected on P7.

Phosphotidylinositol 3-Kinase/Akt Inhibition

A specific phosphotidylinositol 3-kinase (PI3K) inhibitor, Wortmannin (2 mmol/L in 2% dimethyl sulfoxide; Sigma-Aldrich) or 2% dimethyl sulfoxide was injected into the right cerebral hemisphere 30 minutes before and 16 hours after LPS and NS injection. The cortices for immunoblotting and brain sections for immunohistochemistry were collected 8 hours after the last injection.
Figure 1. A, Hemispheric weight reduction was significantly less in the LPS-HI than in the NS-HI group on P21. B, Immunofluorescence 24 hours post-HI showed many TUNEL(+) cells in the cortex of NS-HI group but very few in the LPS-HI group. Most TUNEL(+) cells in the NS-HI group were neurons (short arrows) and endothelial cells (long arrows). C, LPS induced eNOS, but not iNOS or nNOS, in the cortex 12 and 24 hours postinjection (immunoblotting). D, LPS significantly increased eNOS mRNA 12 and 24 hours postinjection (reverse transcription–polymerase chain reaction). E, Representative brain sections marking the visual fields in the cortex (square marks) that were used for eNOS and pAkt immunohistogram analysis. F, eNOS was significantly upregulated in the cortex in LPS-preconditioned pups 24 hours after LPS compared with the weak eNOS immunoreactivity in NS-treated pups (immunohistochemistry). The LPS-induced eNOS was expressed primarily in neurons and vascular cells. For B, C, D, F, and G, N=4 to 5. G, Most LPS-induced eNOS(+) cells were neurons (short arrows) and endothelial cells (long arrows) but not astrocytes. *P<0.05, **P<0.01, §P<0.001.
Semiquantitative Reverse Transcription Polymerase Chain Reaction

Total RNA was extracted from the cortex using a reagent (Trizol; Invitrogen). Five micrograms of total RNA and 1.5 μg of oligo-dT primer were mixed and preincubated at 70°C for 10 minutes. This mixture was mixed with reverse transcription (RT) reagent containing 25 U of MMLV-reverse transcriptase, 10 μL of reaction buffer, and 0.5 mmol/L of dNTP (Promega). Each polymerase chain reaction mixture contained 5 μL of RT product, 1 U of Taq DNA polymerase (Viogene), 2 μL of reaction buffer plus MgCl2, 0.2 μmol/L of dNTP, 0.5 μmol/L of eNOS specific primers (forward primer sequence: 5′GAGAATTCCACCTCACTGTAGCTGTGCTGGCA3′ and reverse primer sequence: 5′TCGATTTCCCAGGGCACTGCGCCCCGCAACTG3′; product sizes: 395 bp), and actin (forward primer sequence: 5′ACCCAGATCATGTTTGAGAC3′ and reverse primer sequence: 5′TCTCTTGCTCGAAGTCTAGG3′; product sizes: 220 bp). The amplified reaction was done using a thermocycler for a single 3-minute initial denaturation at 94°C and then 28 cycles for eNOS or 22 cycles for actin under the polymerase chain reaction conditions.

Statistical Analysis

Statistical significance (P<0.05) was determined using 1-way analysis of variance. Tukey significant difference method was used for post hoc comparisons in 1-way analysis of variance. Continuous data were means±SEM unless indicated otherwise.

Results

Low-Dose LPS Upregulated eNOS

The LPS-HI group had a lower mortality rate (3% versus 16%) during HI in P7 and significantly less brain injury in P21 (P<0.001, Figure 1A) than the NS-HI group. Immunofluorescence at 24 hours after HI showed many terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling (TUNEL) cells in the ipsilateral (I) but not in the contralateral (C) cortex after intracerebroventricular infusions of antisense ODN (immunoblotting). D, eNOS antisense ODN markedly attenuated LPS-induced eNOS expression in neurons and vascular cells (immunohistochemistry). For C and D, N=4. E, LPS-induced protection was significantly lower in eNOS antisense- compared with scrambled ODN-infused rat pups. *P<0.05, **P<0.01, §P<0.001.
Semiquantitative reverse transcription–polymerase chain reaction analysis revealed that LPS significantly upregulated eNOS mRNA at 12 (\(P < 0.01\)) and 24 hours (\(P < 0.05\)) postinjection (Figure 1D). The representative pictures showed the locations of cortical areas used for the quantitative measurement of eNOS and pAkt immunoreactivities (Figure 1E). Immunohistochemistry revealed weak eNOS immunoreactivity in the NS group but significantly higher eNOS in the LPS group (\(P < 0.01\)). The LPS-induced eNOS expression was located primarily in neurons and vascular cells (Figure 1F). Immunofluorescence confirmed that most eNOS(+) cells in LPS-preconditioned pups were neurons and vascular endothelial cells but not astrocytes (Figure 1G).

**eNOS Was Required for LPS Preconditioning-Induced Tolerance**

LPS preconditioning-induced protection was significantly lower in pups pretreated with N(ω)-nitro-L-arginine (\(P < 0.01\)), but...
not in pups pretreated with AG or 7-NI before LPS (Figure 2A). In contrast to the irreversible and long-acting property of Nω-nitro-l-arginine, both 7-NI and AG have short-acting properties. To further delineate the effect of nNOS and iNOS on both the early and delayed phase of LPS preconditioning, we next administrated 7-NI (40 mg/kg) or AG (400 mg/kg) 30 minutes before and 12 hours after LPS injection in rat pups. We found that LPS preconditioning-induced protection was still not lower in pups treated with AG or 7-NI before and after LPS (Figure 2B). Immunoblotting showed that eNOS antisense ODN significantly reduced eNOS in the ipsilateral cortex more than in the contralateral cortex 12 hours after the second ODN injection ($P<0.001$). Scrambled ODN had no significant effect on eNOS expression between the 2 hemispheres (Figure 2C). Immunohistochemistry showed that eNOS antisense markedly reduced eNOS expression ($P<0.001$) in neurons and vascular cells in LPS-preconditioned pups (Figure 2D). LPS-HI pups pretreated with eNOS antisense had significantly more brain damage than those with scrambled ODN ($P<0.05$; Figure 2E). LPS-HI pups pretreated with eNOS antisense had similar level of brain damage as NS-HI pups.

**eNOS Upregulation by LPS Protected Against HI**

Immunoblotting showed that eNOS expression was significantly higher in l-arginine- than in d-arginine- and NS-treated pups 24 hours postinjection (both $P<0.01$; Figure 3A). Immunohistochemistry revealed that eNOS expression was significantly higher in l-arginine- than d-arginine- and NS-treated pups (both $P<0.01$) and l-arginine-induced eNOS was localized in neurons and vascular cells (Figure 3B). L-arginine- ($P<0.01$) but not d-arginine-treated pups had significantly less brain injury compared with NS-treated pups (Figure 3C).

Immunoblotting showed that eNOS levels were significantly higher in the cerebral cortex 3 and 5 days after adenosviruses nuclear-targeted eNOS than after adenosviruses nuclear-targeted β-galactosidase (all $P<0.05$) or NS treatment (5 days, $P<0.01$; 3 days, $P<0.05$; Figure 4A). Pups pretreated with adenosviruses nuclear-targeted eNOS 5 days before HI had significantly less brain injury than pups pretreated with adenosviruses nuclear-targeted β-galactosidase or NS (all $P<0.001$; Figure 4B).

**LPS Preconditioning Activated Akt**

eNOS levels were significantly higher 12 ($P<0.05$) and 24 hours ($P<0.01$) after LPS injection, and peNOS (Ser1177) levels were significantly higher at 1 and 24 hours (both $P<0.01$) postinjection (Figure 5A). eNOS and peNOS levels were significantly higher 24 hours after LPS preconditioning. Compared with NS, LPS significantly upregulated pAkt (Ser473) at 0.5 ($P<0.05$), 1 ($P<0.01$), 3 ($P<0.01$), and 24 hours ($P<0.05$) postinjection. Immunohistochemistry confirmed that LPS preconditioning significantly induced pAkt ($P<0.01$) and that LPS-induced pAkt was found primarily in neuronal and vascular cells (Figure 5B). Immunofluorescence revealed that most LPS-induced pAkt(+) cells were neurons and endothelial cells (Figure 5C). In addition, the pAkt(+) neurons and endothelial cells coexpressed eNOS.

**Akt-eNOS Activation Was Required for LPS-Induced Tolerance**

The intracerebroventricular infusion of Wortmannin 30 minutes before and 16 hours after LPS significantly reduced pAkt compared with the infusion of dimethyl sulfoxide ($P<0.01$; Figure 6A). Wortmannin also significantly downregulated peNOS (Ser1177; $P<0.01$) and eNOS ($P<0.05$) levels. In LPS-preconditioned pups, Wortmannin significantly downregulated eNOS in neurons and endothelial cells ($P<0.01$; Figure 6B). In rat pups without preconditioning, brain injury was not significantly different in rat pups pretreated with Wortmannin and dimethyl sulfoxide 24 hours before HI. In contrast, in rat pups with LPS preconditioning, brain injury was significantly higher in pups pretreated with Wortmannin ($P<0.05$) than in pups pretreated with dimethyl sulfoxide (Figure 6C).

**Discussion**

The present study provides evidence that low-dose LPS preconditioning in rat pups confers neuroprotection and vasculoprotection against HI injury by activating both Akt and eNOS in neurons and endothelial cells. We found that low-dose LPS increased eNOS, but not iNOS or nNOS, expression. The LPS-induced eNOS expression was localized primarily in neurons and endothelial cells. Inhibiting eNOS expression by Nω-nitro-l-arginine or antisense ODN showed that eNOS was required for LPS-preconditioning-induced HI tolerance. We also found that Akt activation was necessary to upregulate eNOS in neurons and endothelial cells as well as to provide protection against HI in LPS preconditioning. Furthermore, we showed that pharmacologically and genetically upregulating eNOS protected rat pups against HI. Taken together, these findings show that upregu-
lating eNOS by activating Akt in the neonatal brain is critical for low-dose LPS preconditioning-induced survival.

The 3 isoforms of NOS that are distributed over the brain have distinct biological functions in neuropathological conditions and are selectively induced in different cell types by systemic LPS injection. In the mature brain, high-dose LPS (5 to 50 mg/kg) predominantly upregulated iNOS and downregulated eNOS in blood vessels and glial cells.17 In contrast, medium-dose LPS (2.5 to 10 mg/kg) induced eNOS in astrocytes and blood vessels, but not in neurons.7 Here, we provided evidence that low-dose LPS (0.05 mg/kg) selectively upregulated eNOS, but not iNOS or nNOS, in neurons.

Figure 5. A, LPS significantly increased eNOS expression at 12 and 24 hours, peNOS (Ser1177) at 1 and 24 hours, and pAkt (Ser473) at 0.5, 1, 3, and 24 postinjection (immunoblotting). B, LPS also induced pAkt expression in neurons and vascular cells (immunohistochemistry). C, Most LPS-induced pAkt(+) cells were neurons and endothelial cells (immunofluorescence). pAkt(+) neurons and endothelial cells also coexpressed eNOS. For A, B, and C, N=3 to 4. *P<0.05, **P<0.01.
and endothelial cells in the neonatal brain. The mechanisms for upregulating and downregulating different isoforms of NOS in different neural cell types after LPS remain unknown. They may be related to the ontogenic brain (immature versus mature brain) responses and to the LPS dose administered. Our finding that low-dose LPS selectively upregulated eNOS expression in neurons and endothelial cells in the neonatal brain may be unique and distinct from LPS preconditioning in the mature brain, in which the upregulated expression of both iNOS and eNOS in glial and endothelial cells has been reported.4,7,8,18

eNOS is important in vascular signaling and tone regulation and leukocyte–endothelial interactions. eNOS phosphorylation at serine residue 1177 upregulates eNOS enzymatic activity and NO production.19 eNOS-deficient mice are hypertensive and have an increased infarct volume after a stroke.3,19 In contrast, eNOS transgenic mice in which eNOS is overexpressed in endothelial cells are resistant to stress-induced hypotension, inflammation, and stroke.3,20 In vitro studies also show that eNOS, activated by tumor necrosis factor α through Akt activation in neurons is cytoprotective against neurotoxicity.21 These findings suggest that eNOS expressed in neurons and endothelium is protective and that the mechanisms may involve antiapoptosis.3,21 The interaction among astrocytes, neurons, and endothelial cells may also play a role in the protective effect of preconditioning; further in vitro study is needed to examine whether the increased eNOS expression in neurons and endothelial cells per se is protective against HI.

PI3K/Akt activation is common after LPS treatment.22 LPS-induced PI3K/Akt is antiapoptotic in endothelial cells.23 Hypoxic preconditioning protected against HI by upregulating PI3K/Akt activity in the neonatal rat brain.9 PI3K/Akt-dependent eNOS phosphorylation is important in the antiapoptotic effect of insulin, estrogen, and statins in stroke.3 PI3K/Akt signaling leading to eNOS activation in endothelial cells, but not in neurons, contributed to the ischemic tolerance of the gerbil hippocampus.11 Our study showed that Akt activation was required for eNOS upregulation in neurons and endothelial cells and for the acquisition of LPS-induced HI tolerance. We hypothesize that activating Akt-eNOS signaling in neurons and endothelial cells using low-dose LPS is both neuroprotective and vasculoprotective in the neonatal brain.

Both nontranscription and transcription mechanisms may be involved in regulating eNOS expression.3 LPS preconditioning is a delayed type of HI tolerance.4–6 Akt-mediated cell survival through eNOS activation and regulation may involve transcriptional regulation and posttranscription modulation mechanisms. Although eNOS phosphorylation was observed as early as 1 hour after LPS, transcriptional regulation of eNOS may be more related to LPS preconditioning-induced tolerance than nontranscriptional mechanisms. The underlying mechanisms on how low-dose LPS activates Akt, and how Akt activation leads to eNOS expression in neurons and vascular endothelial cells, requires additional study.

Combining neuroprotection and vasculoprotection by activating Akt-eNOS may be a viable strategy in treating HI brain injury.12,18 We found that in low-dose LPS preconditioning of the developing rat brain, Akt was protective by upregulating eNOS expression in neurons and endothelial cells. Our study leads us to hypothesize that drugs that mimic the beneficial effects of LPS preconditioning by targeting Akt-eNOS signaling are powerful therapeutics for treating HI injury in the neonatal brain.
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Disclosures
None.

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