ORIGINAL ARTICLE

Endothelial Nitric Oxide Synthase Protects Neurons against Ischemic Injury through Regulation of Brain-Derived Neurotrophic Factor Expression

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Keywords

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SUMMARY

Aims: Several lines of evidence demonstrated that endothelial nitric oxide synthase (eNOS) confers protective effects during cerebral ischemia. In this study, we explored the underlying cellular and molecular mechanisms of neuroprotection by eNOS. **Methods:** A series of *in vivo* and *in vitro* ischemic models were employed to study the role of eNOS in maintaining neuronal survival and to identify the downstream factors. **Results:** The current data showed that pretreatment with a specific eNOS inhibitor, L-N5-(1-iminoethyl) ornithine (L-NIO), aggravated the neuronal loss in the rat cerebral ischemic model, accompanied by reduction in brain-derived neurotrophic factor (BDNF) level, which was consistent with the findings in an oxygen-glucose deprivation model (OGD) with two neuronal cells: primary rat cortical neurons and human neuroblastoma SH-SY5Y cells. Furthermore, the extensive neuronal loss induced by L-NIO was totally abolished by exogenous BDNF in both *in vitro* and *in vivo* models. On the other hand, eNOS overexpression through an adenoviral vector exerted a prominent protective effect on the neuronal cells subject to OGD, and the protective effect was totally abrogated by a neutralizing anti-BDNF antibody. **Conclusion:** Collectively, our results indicate that the neuroprotection of neuron-derived eNOS against the cerebral ischemia was mediated through the regulation of BDNF secretion. In conclusion, our discovery provides a novel explanation for the neuroprotective effect of eNOS under pathological ischemic conditions such as stroke.

Introduction

Ischemic stroke is the third leading cause of death and a major cause of long-term disability throughout the world. Following thrombosis or embolism in cerebral arteries, damage to brain tissue is initiated by cerebral ischemia with depletion of tissue energy supplies followed by secondary cascades [1]. Cerebral ischemia is characterized by a reduced blood supply to the brain tissue, thus leading to neuronal cell death. Mechanisms of cell damage include cell depolarization and swelling, release of excitatory amino acids (e.g., glutamate) in the extracellular space, increase in intracellular Ca^{2+} levels, production of free radicals and activation of inflammatory mediators, including cytokines, adhesion molecules and nitric oxide (NO) [1,2].

Nitric oxide is a putative neurotransmitter in the brain and peripheral nervous system [3]. It is generated by three different

types of NO synthase (NOS), the constitutive calcium/calmodulin-dependent neuronal (nNOS) and endothelial (eNOS) isoforms and the inducible calcium-independent isoform (iNOS). Accumulating evidence suggests that both nNOS and iNOS have detrimental effects on neurons in the ischemic brain [4,5] and our recent finding demonstrated that the neurotoxic effects of nNOS and iNOS were mediated by c-Jun N-terminal kinase 1/2 (JNK1/2) [6]. In contrast, eNOS confers beneficial effects during cerebral ischemia, as indicated by increased infarct size in the eNOS^{-/-} mice after middle cerebral artery (MCA) occlusion [7]. It has also been suggested that the neuroprotective effects of eNOS are mediated through maintenance of vascular homeostasis and promoting angiogenesis [7–9]. In infarcted areas, increase in eNOS protein levels after transient focal ischemia and global cerebral ischemia has been reported [10–12], and the cellular localization of eNOS proteins was not limited in endothelial cells, but also in neurons

and astrocytes [13,14]. However, it remains unclear whether eNOS expressed by neurons affects neuron survival in the ischemic brain.

Brain-derived neurotrophic factor (BDNF) is known to regulate the differentiation and survival of central nervous system (CNS) neurons [15]. Recently, it has been reported that BDNF expression is regulated by NO, derived from eNOS in the mammalian brain [16,17]. These observations highlight the potentially important role of neuron-derived eNOS in modulating BDNF secretion and the need to investigate the potential impact of eNOS on neuron survival in the brain after stroke.

In this study, we investigated the hypothesis that neuron-derived eNOS has a direct impact on neuron survival in cerebral ischemia. To elucidate the precise cellular mechanism of neuroprotection by eNOS, we simulated ischemia *in vitro* by oxygen-glucose deprivation (OGD) in both primary rat cortical neurons and SH-SY5Y, a human-derived neuroblastoma cell line in parallel, in which effects of eNOS overexpression and eNOS antagonism by a pharmacological tool, L-N⁵-(1-iminoethyl) ornithine (L-NIO) on neuron apoptosis were evaluated. We provide strong evidence that the neuroprotection by eNOS may be attributed to the regulation of BDNF secretion in neurons.

Materials and Methods

Antibodies and Reagents

The following primary antibodies were used: rabbit monoclonal anti-caspase-3, rabbit monoclonal anti-eNOS were purchased from Cell Signaling Technology (Boston, MA, USA) and the mouse polyclonal anti-Actin antibodies were purchased from Sigma (St Louis, MO, USA). The secondary antibodies used in our experiment were goat anti-mouse IgG and goat anti-rabbit IgG and were purchased from Cell Signaling Technology. 7-nitroindazole (7-NI) and 2-Amino-5, 6-dihydro-6-methyl-4H-1, 3-thiazine (AMT), and L-N⁵-(1-iminoethyl) ornithine (L-NIO) were purchased from Sigma. We obtained recombinant human BDNF, the neutralizing anti-BDNF antibody and the isotype control antibody from Millipore.

Drug Treatment

7-NI (25 mg/kg) was administered to rats by intraperitoneal injection 20 min before ischemia. AMT (0.65 mg/kg) dissolved in 1% DMSO or L-NIO (1 mg/kg) dissolved in 1% DMSO was administered intracerebroventricularly (10 μ L, i.c.v., bregma: 1.5 mm lateral, 0.8 mm posterior, 3.5 mm deep) to the rats 20 min before ischemia (N = 6/group). For intracerebroventricular administration, rats were anesthetized, the bregma was identified. Artificial cerebrospinal fluid (aCSF: 0.166 g/L CaCl₂, 7.014 g/L NaCl, 0.298 g/L KCl, 0.203 g/L MgCl₂/6H₂O and 2.10 g/L NaHCO₃) was used as the vehicle for intracerebroventricular administration. Animals in the vehicle group received the vehicle containing 1% DMSO through i.c.v. For administration of BDNF, BDNF (25 μ g/animal, i.c.v., 3 μ L/shot) and/or L-NIO (1 mg/kg, i.c.v) was injected into the lateral ventricle. The animals were pretreated with BDNF and/or L-NIO 20 min before ischemia.

Animal Surgical Procedures

Adult male Sprague–Dawley rats (Shanghai Experimental Animal Center, Chinese Academy of Science) weighing 200–350 g were used. The experimental procedures were in compliance with the local legislation for ethics of experiments on animals and were approved by Animal Care and Use Committee of Xinhua Hospital, Shanghai Jiaotong University. Under guidelines and the terms of all relevant local legislation, surgical procedures were conducted. Efforts were made to minimize the number of animals used and their suffering. Transient brain ischemia (15 min) was induced by four-vessel occlusion (4-VO) method, as described previously [18]. Briefly, under anesthesia with chloral hydrate (350 mg/kg, i.p. Sigma Aldrich), vertebral arteries were electrocauterized, and common carotid arteries were exposed. Ischemia was induced by occluding the common arteries with aneurysm clips. Rats that lost their righting reflex within 30 seconds and those whose pupils were dilated and unresponsive to light were selected for the experiments. Besides the response to light, the responsiveness of individual rats to the nonaversive tail pinch and toe pinch was tested as well. Lack of response to the tail and toe pinch indicates a deep anesthesia adequate for the following neurosurgery. During ischemia (15 min) and the first 2 h of reperfusion, rectal temperature was maintained at about 37°C. Sham control rats received the same surgical procedures except that the carotid arteries were not occluded. After the surgery, the overwhelming majority of rats in our experiment recovered very well from the neurosurgery, with normal appetite and defecation.

Histology

Rats were perfusion-fixed with 4% paraformaldehyde under anesthesia after 5 days of reperfusion. Paraffin sections (5 μ m) were prepared and stained with cresyl violet. An initial dissector frame was positioned randomly in the CA1 region and cells in every 10th section throughout the entire hippocampus. The cell numbers in the CA1 region were assessed by means of previously published unbiased stereological techniques. In brief, cell counts were performed at 400 magnifications with the use of an Olympus BH-2 microscope connected to a Sony chargecoupled device video camera, a motorized stage system, and commercial stereology software. The optical dissector technique was used to avoid double counting of cells [19,20].

Cell Culture

Primary mouse cortical neuron culture was performed as previously described [21]. Briefly, primary rat cortical neurons were isolated from fetal rats (gestational age of 17–18 days, Sprague–Dawley rats, Shanghai Experimental Animal Center). Neurons from each embryo cortex were isolated and seeded into multiple well plates with equal cell numbers (1.5×10^5 cells/well in 24-well plates, 6×10^5 cells/well in a 6-well plate). The neurons were maintained in Neurobasal medium (NBM) with 3% B27 and 0.3 mM glutamine (Invitrogen Corporation, Carlsbad, CA, USA). Medium was half changed in each of 3–4 days. The purity of neurons is more than 90%, judged by NeuN immunostaining.

The SH-SY5Y human neuroblastoma cells were cultured in Dulbecco's modified Eagle medium/Ham's F-12 nutrient mixture (1:1) containing 10% fetal bovine serum. The culture medium was changed every 2 days. The cells were kept in a 95% air/5% CO₂ humidified incubator at 37°C.

Oxygen-Glucose Deprivation Model

The cells were subjected to OGD as described earlier [22,23]. Briefly, a glucose-free buffer containing 154 mM NaCl, 5.6 mM KCl, 5.0 mM HEPES, 3.6 mM NaHCO₃, and 2.3 mM CaCl₂ (pH 7.4) was bubbled with 95% N₂–5% CO₂ for 2 h at 37°C. After the culture medium that contained 4.5 g/L glucose was replaced with this oxygen-glucose-deprived buffer, the culture plates were then put into an airtight chamber (~5 L in volume) gassed with 95% N₂/5% CO₂ (preheated to 36°C) at 4 L/min for 5 min. The chamber was then sealed and placed in an incubator at 37°C for 4 h (primary neurons) or 5 h (SH-SY5Y cells). The chamber was opened, and the cells were returned to their respective normal culture conditions for 20 h before they were used for cell viability analysis. For compound treatment, cells were preconditioned with 7-NI (3 μ M), AMT (0.1 μ M), or L-NIO (3 μ M) in oxygen-glucose-deprived buffer for 15 min prior to anoxia exposure. The compounds were dissolved in DMSO, and DMSO was used as vehicle control. For cotreatment with BDNF, BDNF (50 ng/mL) was added into media together with L-NIO.

Adenovirus Infection

Recombinant adenoviruses expressing human eNOS cDNA were prepared as described previously using pAdEasy™ vector system (Qbiogene Corporation, Carlsbad, CA, USA). Briefly, human eNOS cDNA cloning was carried out by PCR, and then it was subcloned into a shuttle vector pTrack-CMV (Qbiogene, USA). The pAdTrack-CMV-eNOS and pAdEasy-1 (Qbiogene, USA) expressing RFP gene were homologously recombinant in bacteria BJ5183. The pAdEasy-1 and recombinant plasmid pAd-eNOS were propagated in HEK293 cells. The propagated recombinant adenoviruses in the HEK293 cells were purified, and the titer of virus was measured by plaque assays. One day after cell passage, viral infection was carried out at 37°C and 5% CO₂ at MOIs of 50 for 2 h. It was found that most of the cells (>80%) expressed RFP 24 h after the transfection. The empty adenovector Ad RFP was used as negative control. Twenty-four hours after viral infection, primary neurons or SH-SY5Y cells were then exposed to OGD treatment.

For cotreatment with anti-BDNF antibody, infected cells were first treated with the neutralizing anti-BDNF antibody or the isotype control antibody then exposed to OGD.

Cell Viability

Cell viability was quantified using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay kit. Absorbance was measured at 570 nm with the reference wavelength of 650 nm using a microplate reader (Bio-Rad Laboratories, Hercules, CA, USA). In each experiment, the average of the MTT measurements from the control group was set as 100%. The results of other groups were then expressed as a percentage of the control group.

Enzyme Linked Immunosorbent Assay (ELISA)

The supernatants from treated and untreated cell cultures were collected at the end of reoxygenation and were then concentrated to fourfold using centrifugal filter devices (Amicon Ultra-4 Ultra-cel-10k; Millipore Corporation, Billerica, MA, USA). The protein concentration of the samples was adjusted to the same levels before 100 μ L of each sample was applied into each well (Maxisorp 96-well plate, Nunc) for the immunoassay. The protein concentration of BDNF was measured using BDNF Emax immunoAssay System (Promega Corporation, Madison, WI, USA) and the procedures performed following manufacturers' instruction. The average of the BDNF measurements from the control without any treatment (sham control) was set as 100%. The results of other groups were then expressed as a percentage of the sham control.

Brain extracts were obtained from the ischemic border identified visually at 3 days after ischemia (N = 6/group). Tissue blocks were dissected on ice, and wet weight was rapidly measured. The brain extracts were divided into 200 μ L triplicate samples. Using ELISA kit (Promega) BDNF, ELISAs were performed. The average level of the vehicle group was set as 100%. The results of each individual mouse were then expressed as a percentage of the control.

Western Blot Analysis

Following reoxygenation, the cells were washed with ice-cold phosphate-buffered saline (PBS) and lysed in ice-cold lysis buffer. The protein concentration was determined with the Bio-Rad DC Protein assay, and 10–15 μ g of protein was separated on an SDS-PAGE gel, transferred to PVDF membranes, and blotted with antibodies specific for GAPDH, cleaved and full-length caspase-3. GAPDH was used as a loading control. Signals were detected with enhanced chemiluminescence and quantified by densitometry using GeneGenius BioImaging System (Syngene, Synoptics Ltd., UK).

Caspase 3 Activity

Caspase 3 protease activity was detected following 1 h reoxygenation using CaspACE™ assay system (Promega Corporation), which is based on the colorimetric detection of the chromophore p-nitroanilide (pNA) after cleavage from the labeled substrate DEVD-pNA at 405 nm. Following 1 h of reoxygenation, the cells were washed with 2 mL PBS then were lysed with lysis buffer provided in the kit. Assays were performed according to the manufacturer's instructions.

Terminal Deoxynucleotidyl Transferase dUTP Nick End Labeling (TUNEL) Assays

Identification of cell apoptosis was performed with *In Situ* Cell Death Detection Kit (Roche Corporation, Mannheim, Germany). The primary rat cortical neurons or SH-SY5Y cells plated on Lab-Tek chamber glass slides were washed with PBS and fixed in 1% paraformaldehyde for 10 min and postfixed in precooled ethanol-acetic acid (2:1) for another 5 min at 220°C. After being washed with PBS, the cells were incubated with a TUNEL reaction buffer

for 1 h at 37°C in a humidified chamber. As a positive control, cells were treated with DNase I (1.0 mg/mL, Sigma) for 10 min to introduce nicks in the genomic DNA. The percentage of cells with DNA nick end labeling was determined by counting cells exhibiting green fluorescence among 1000 nuclei in triplicate samples. The fluorescent images were captured using an Olympus BX60 microscope and a Zeiss LSM confocal microscope.

Statistical Analysis

All data are given as means $\pm$ standard deviation (SD). Statistical analysis was performed using an unpaired Student's *t*-test for all experiments. Statistical significance was inferred if $P < 0.05$.

Results

Effects of Pretreatment with NOS Specific Inhibitors on Hippocampal Neuron Loss in a Cerebral Ischemia Model

To investigate the roles of different NOS subtypes in cerebral ischemia, the effects of nNOS inhibitor 7-NI, iNOS inhibitor AMT and eNOS inhibitor L-NIO [24] on the neuronal survival of CA1 pyramidal neurons in rat hippocampus were compared in an *in vivo* model of cerebral ischemia. Transient brain ischemia (15 min) was induced by the four-vessel occlusion method (4-VO) and then followed by 5 days of reperfusion. Animals were pretreated for 20 min with nNOS inhibitor 7-NI (25 mg/kg, intraperitoneal injection), iNOS inhibitor AMT (0.65 mg/kg, intracerebroventricular injection) or eNOS inhibitor L-NIO (1 mg/kg, intracerebroventricular injection). Cresyl violet staining was used to examine the surviving cells in CA1 region after 5 days of reperfusion. Normal cells showed round and pale stained nuclei. The shrunken cells with pyknotic nuclei were counted as dead cells. As shown in Figure 1, transient cerebral ischemia followed by 5 days of reperfusion induced severe cell death; pretreatment with 7-NI and AMT markedly ameliorated the neuronal loss; on the other hand, administration of eNOS inhibitor L-NIO significantly reduced the survival rate of CA1 pyramidal neurons. The results indicate that eNOS protected neurons while nNOS or iNOS was neurotoxic in cerebral ischemia, which is consistent with the previous reports [4,5,25].

Effect of eNOS Inhibitor, L-NIO on Cell Survival in a Cellular Model of OGD

To further dissect the precise cellular mechanism underlying the neuroprotective role of eNOS, first, we set up an OGD model with the SH-SY5Y cells to mimic cerebral ischemia, in which cells were exposed to OGD for 5 h followed by 20 h of reoxygenation to induce apoptosis. The effects of OGD on cell viability were assessed using the MTT assay to measure total cell viability and two other quantitative readouts to distinguish apoptotic and necrotic forms of cell death of SH-SY5Y cells: the TUNEL staining and the measurement of caspase-3 activity. Based on the morphologic change and TUNEL staining, the OGD protocol caused ~12% apoptotic cell (Figure 2A and Figure S2A). The selective NOS inhibitors, 7-NI (3 μ M), AMT (0.1 μ M), and L-NIO (3 μ M), were then tested in the cellular model and treatment with specific eNOS

Figure 1 Treatment with L-NIO deteriorated ischemia/Reperfusion (I/R)-induced neuronal loss in hippocampal CA1 region. Representative images of cresyl violet-stained sections of the hippocampus in sham operation, rats subjected to 5 days of reperfusion after 15 min of ischemia, pretreatment with vehicle, 7-NI, AMT, or L-NIO (20 min before ischemia). Data were obtained from six independent animals. Boxed areas in left column are shown at higher magnification in right column. Scale bars: (left panel) = 200 μ m; (right panel) = 10 μ m. The quantitative analysis of the total number of live neurons in hippocampal CA1 region was performed at 5 days of reperfusion after ischemia. Data are normalized by the absolute number of the sham group and expressed as mean $\pm$ standard deviation (SD, $n = 6$), * I/R versus sham, ** $P < 0.01$; # compound versus vehicle, # $P < 0.05$.

inhibitor, L-NIO significantly worsened cell apoptosis, measured by TUNEL staining, caspase-3 activity, and MTT assay; in contrast, inhibitors against nNOS and iNOS demonstrated protective effects (Figure 2A–D). Together with the *in vivo* finding, these results

Figure 2 Pretreatment with endothelial nitric oxide synthase (eNOS) inhibitor, L-NIO aggravated neuronal cell apoptosis induced by oxygen-glucose deprivation (OGD). **(A)** Quantitative analysis of percentage of TUNEL+ cells in SH-SY5Y cells after pretreated with vehicle, 7-NI (3 μ M), AMT (0.1 μ M), or 7-NIO (3 μ M), then exposed to OGD. The cells in normoxia were referred as sham control. The proportion of apoptotic populations (TUNEL+) was calculated by counting total of 1000 nuclei in each slide under a microscope from triplicate samples. Scale bar, 100 μ m. **(B)** Total cell viability measured by MTT assay. Results were expressed as percentage of the viability of sham control. The normalized results represented average of three independent experiments. **(C)** Caspase-3 cleavage was analyzed by western blot (upper panel) and shown as the normalized densitometric results averaged over three independent experiments (lower panel). Data are expressed as fold changes relative to full-length caspase-3. Values represent mean $\pm$ SD from triplicate samples for each treatment. **(D)** Activity of caspase-3 was determined using a caspase colorimetric protease assay kit. **(E)** Quantitative analysis of percentage of TUNEL+ cells in primary neurons after pretreated with vehicle or 7-NIO (3 μ M), then exposed to OGD. **(F)** Total cell viability of primary neurons after OGD and reoxygenation measured by MTT assay. **(G)** Expression profile of eNOS during OGD and reoxygenation: left panel, typical images of western blot; right panel, normalized densitometric results averaged over three independent experiments. Values represent mean $\pm$ SD from triplicate samples for each treatment. *OGD versus Sham (* P < 0.05, ** P < 0.01); #compound versus vehicle (* P < 0.05, ** P < 0.01).

demonstrated a neuroprotective role of eNOS in the neuronal cells exposed to ischemic injury.

To further validate the protective effect of eNOS on neuronal cells against ischemic challenge, we employed the primary rat cortical neurons to repeat the experiment of L-NIO. The OGD model was modified to accommodate characteristics of neurons and 4 h OGD followed by 20 h of reoxygenation induced 24.4% apoptotic neurons (Figure 2E and Figure S2B, TUNEL staining). Pretreat-

ment with L-NIO significantly exacerbated neuron viability, as indicated by TUNEL staining (from 24.4% to 30.7%, apoptotic cells) and cell viability assay (from 48.7% to 28.5%, percentage of sham, Figure 2F), which is consistent with the finding in SH-SY5Y. Further, we measured expression profile of eNOS in the setting of OGD/reoxygenation and observed that expression level of eNOS was significantly upregulated during OGD then diminished in reoxygenation (Figure 2G). This finding further

underpins the proposed mechanism that eNOS plays a critical role in neuroprotection against ischemic challenge.

Neuroprotection against OGD by Adenovirus-Mediated eNOS Overexpression

To complement the pharmacological results and further address role of eNOS in neuroprotection against the ischemic injury, full length of eNOS cDNA was forced into SH-SY5Y cells via an adenoviral vector, pAdEasyTM (Ad-eNOS), which carried red fluorescent protein (RFP), and the empty adenoviral vector without an insert was used as control (Ad RFP). One day after infection, forced eNOS expression was validated through western blot and fluorescence imaging, showing that >80% of cells expressed RFP (Figure S3A,B).

As compared with the empty vector, eNOS overexpression significantly reduced the percentage of TUNEL+ cells in OGD model

(Figure 3A and Figure S3B), maintained the cell viability (Figure 3B). Caspase-3 cleavage was employed here as an early apoptotic marker and eNOS overexpression reversed cleavage of caspase-3 induced by OGD (Figure 3C,D), but had no obvious effect in the corresponding sham groups.

To further confirm that the mechanism of eNOS is not peculiar to the neuroblastoma cells, the primary cortical neurons were subject to eNOS overexpression then OGD challenge. More than 80% cells were infected by the adenovirus and expressed high level of eNOS (RFP expression, Figure S3A,C). TUNEL staining was used to label apoptotic neurons, and we noted that because TUNEL is a late apoptotic marker, the TUNEL+ cells demonstrated a shrunken and round morphology and lost expression of microtubule-associated protein (MAP-2) expression (Figure S3C). Consistently, pAdEasyTM (Ad-eNOS), but not the empty vector, significantly reduced the percentage of TUNEL+ cells and ameliorated cell viability after OGD/reoxygenation (Figure 3E,F, and Figure S3C).

Figure 3 Adenovirus-mediated endothelial nitric oxide synthase (eNOS) overexpression (Ad-eNOS) conferred the neuroprotective effect against oxygen-glucose deprivation (OGD). **(A)** Quantitative analysis of percentage of apoptotic cells in RFP+ population in virus-infected SH-SY5Y cells. The proportion of apoptotic populations was calculated by counting total of 1000 RFP+ cells in each slide under a microscope from triplicate samples. The cells cultured in normoxia and normal glucose level were referred as sham control. **(B)** Total cell viability measured by MTT assay. Results were expressed as percentage of the viability of sham control. The normalized results represented average of three independent experiments. **(C)** Caspase-3 cleavage was analyzed by western blot (upper panel) and shown as the normalized densitometric results averaged over three independent experiments (lower panel). Data are expressed as fold changes relative to full-length caspase-3. Values represent means $\pm$ SD from triplicate samples for each treatment. **(D)** Activity of caspase-3 was determined using the caspase colorimetric protease assay kit. **(E)** Quantitative analysis of percentage of apoptotic cells (TUNEL+) in RFP+ population in virus-infected primary neurons. **(F)** Total cell viability of primary neurons measured by MTT assay. Values represent means $\pm$ SD from triplicate samples for each treatment. *OGD versus Sham ($P < 0.05$, ** $P < 0.01$); #Ad-eNOS versus Ad RFP ($P < 0.05$).

Figure 4 Brain-derived neurotrophic factor (BDNF) ameliorated the detrimental effect of L-NIO on neuronal cell apoptosis induced by oxygen-glucose deprivation (OGD). **(A)** Effects of OGD and/or L-NIO on BDNF level in SH-SY5Y cells. Relative amounts are shown. The amounts of BDNF secreted to the medium were analyzed by enzyme linked immunosorbent assay and the cells in normoxia were referred as sham control. *OGD versus Sham, ** $P < 0.01$; [#]L-NIO versus Vehicle, ^{*} $P < 0.05$, ^{###} $P < 0.01$. **(B)** Quantitative analysis of percentage of SH-SY5Y cells undergoing apoptosis after treated with L-NIO (3 μ M) and/or BDNF (50 ng/mL), then exposed to OGD. * L-NIO versus Vehicle, $P < 0.05$; [#]without BDNF versus with BDNF, $P < 0.05$, ^{###} $P < 0.01$. **(C)** Total cell viability measured by MTT assay. Results were expressed as percentage of the viability of OGD-treated vehicle group. The normalized results represented average of three independent experiments. *L-NIO versus Vehicle, $P < 0.05$; [#]without BDNF versus with BDNF, $P < 0.05$; Not significant, N.S. **(D)** Caspase-3 cleavage was analyzed by western blot (upper panel) and shown as the normalized densitometric results averaged over three independent experiments (lower panel). Data are expressed as fold changes relative to full-length caspase-3. *L-NIO versus Vehicle, ** $P < 0.01$; [#]without BDNF versus with BDNF, ^{###} $P < 0.01$. Values represent mean $\pm$ SD from triplicate samples for each treatment. **(E)** Activity of caspase-3 was determined using the caspase colorimetric protease assay kit. **(F)** Quantification of percentage of apoptotic cells (TUNEL+) in primary neurons pretreated with L-NIO (3 μ M) and/or BDNF (50 ng/mL), then exposed to OGD. **(G)** Total cell viability of primary neurons measured by MTT assay. Values represent mean $\pm$ SD from triplicate samples for each treatment. *L-NIO versus Vehicle, $P < 0.05$. [#]without BDNF versus with BDNF, ^{###} $P < 0.01$.

Collectively, results of the above assays, in which two neuronal cell types were adopted and multiple readouts regarding cell apoptosis were measured, indicated that overexpressed eNOS is sufficient to confer protective effect on neuronal cells against the OGD challenge, which aligns very well with the results of the pharmacological experiments.

Molecular Mechanism Underlying the eNOS-Mediated Neuroprotection against Ischemic Injury

Chen et al. [26] reported that brain BDNF level was decreased in the eNOS^{-/-} mice, compared with the wild type mice, in the

cerebral ischemia model; this finding together with other publications [16,17] implied a connection between eNOS and BDNF expression in the brain. Several cell types of the CNS could be engaged in the regulatory mechanism between eNOS and BDNF, for example, endothelial cells, astrocytes, and neurons.

We suspected that BDNF derived from neuron itself may play an important role in the neuroprotection conferred by eNOS. We first tested this hypothesis in the *in vitro* OGD/reoxygenation system of SH-SY5Y cells with the pharmacological tool. In this model, L-NIO treatment markedly reduced BDNF secretion in the SH-SY5Y cells (Figure 4A). To determine whether the ischemic neuron cell death caused by eNOS inhibition was mediated through the regulation of BDNF level, exogenous BDNF was added into the SH-SY5Y cell culture together with L-NIO and BDNF (50 ng/mL) showed robust neuroprotective effect against the ischemic insult and totally overcame the destructive effect of L-NIO (Figure 4B–E and Figure S4A). This mechanism was further validated with the primary neuron culture, in which the primary neurons were treated with the same scenario, and we observed that exogenous BDNF efficiently abolished extra cell death induced by L-NIO (Figure 4F,G and Figure S4B). These findings suggest that eNOS protected neurons from ischemic insults through regulating autocrine/paracrine BDNF secretion from neurons.

Furthermore, we determined *in vivo* BDNF level by ELISA in the ischemic brain treated with NOS selective inhibitors at day 3 after ischemia. Consistently, it was found that treatment with L-NIO, significantly reduced BDNF secretion in the ischemic brain, compared with the vehicle control (Figure 5A). The involvement of BDNF was further confirmed by intracerebroventricular administration of BDNF in the *in vivo* ischemic model, in which exogenous

BDNF reversed the excessive neuronal loss induced by L-NIO in the hippocampus (Figure 5B). Together these data suggest that severe neuronal loss induced by L-NIO treatment is attributed to decreased BDNF expression by eNOS inhibition.

In contrast, an elevated BDNF secretion was detected in the cells infected with Ad-eNOS (Figure 6A). Taken together, the results of pharmacological tool and genetic manipulation suggest that eNOS is both necessary and sufficient to trigger BDNF secretion from the neuronal cells. To further confirm the involvement of BDNF in eNOS-mediated neuroprotection, we employed a neutralizing antibody against BDNF to block BDNF signals in the OGD model, and the same set of quantitative readouts of cell apoptosis were used to assess the functional consequence of the BDNF antibody in both SH-SY5Y cells and primary neurons. In the presence of the neutralizing BDNF antibody, but not the isotype control, the protective effect of eNOS overexpression was totally abolished, as evident by the prominent decrease in total cell viability and the concomitant increase in TUNEL+ cells (Figure 6B,C and Figure S5A) and cleaved caspase-3 (Figure 6D,E) in the SH-SY5Y cells. In the primary neurons treated with BDNF antibody, two readouts, TUNEL staining and cell viability, demonstrated the key role of BDNF in the eNOS-mediated neuroprotection as well (Figure 6F, G and Figure S5B). These findings provide compelling evidence validating that the mechanism of eNOS-mediated neuroprotection involves regulation of BDNF in the neuronal cells.

Discussion

Increased NO level by eNOS leads to endothelial cell proliferation and migration *in vivo* and *in vitro* [27,28] and eNOS, which is

Figure 5 Administration of brain-derived neurotrophic factor (BDNF) reversed the detrimental effect of L-NIO in the ischemia/Reperfusion (I/R) model. **(A)** BDNF level of ischemic brain after treated with L-NIO. Enzyme linked immunosorbent assay of BDNF was performed with the ischemic brain samples 3 days after stroke. **(B)** Left panel: Representative images of cresyl violet-stained sections of the hippocampus of rats. Right panel: The quantitative analysis of the total number of live neurons in hippocampal CA region. The survival count of cells in hippocampal was determined 5 days after ischemia. Scale bars: (left panel) = 200 μ m; (right panel) = 10 μ m. Data are normalized by the absolute number of the sham group and expressed as mean $\pm$ SD ($n = 6$), *L-NIO versus vehicle, $P < 0.05$; #without BDNF versus with BDNF, $^{\#}P < 0.05$.

Figure 6 Neutralization of brain-derived neurotrophic factor (BDNF) abrogated neuroprotective effect of endothelial nitric oxide synthase (eNOS) overexpression. **(A)** BDNF secretion by SH-SY5Y cells with eNOS overexpression. Results were expressed as percentage of the level of sham control (Ad RFP). *Sham versus oxygen-glucose deprivation (OGD), $P < 0.05$; #Ad RFP versus Ad-eNOS, $P < 0.05$. **(B)** Quantification of percentage of apoptotic cells in RFP+ population of SH-SY5Y cells, after pretreated with Ad-eNOS and/or anti-BDNF antibody, then exposed to OGD. The proportion of apoptotic populations was calculated by counting total of 1000 RFP+ cells in each slide under a microscope from triplicate samples. #Ad RFP versus Ad-eNOS, $P < 0.05$; *anti-BDNF versus isotype control, $P < 0.05$. **(C)** Total cell viability measured by MTT assay. Results were expressed as percentage of the viability of OGD-treated Ad RFP group. The normalized results represented average of three independent experiments. #Ad RFP versus Ad-eNOS, $P < 0.05$; *anti-BDNF versus isotype control, $P < 0.05$. **(D)** Caspase-3 cleavage was analyzed by western blot (upper panel) and shown as the normalized densitometric results averaged over three independent experiments (lower panel). Data are expressed as fold changes relative to full-length caspase-3. Values represent means $\pm$ SD from triplicate samples for each treatment. **(E)** Activity of caspase-3 was determined using the caspase colorimetric protease assay kit. **(F)** Quantification of percentage of apoptotic cells (TUNEL+) in primary neurons after pretreated with Ad-eNOS and/or anti-BDNF antibody, then exposed to OGD. **(G)** Total cell viability of primary neurons measured by MTT assay. Values represent means $\pm$ SD from triplicate samples for each treatment. #Ad RFP versus Ad-eNOS, $P < 0.05$; *anti-BDNF versus isotype control, $P < 0.05$.

upregulated after cerebral ischemia, plays a critical role in the regulation of vascular function and structure and is a downstream mediator of the angiogenic response to numerous vascular growth factors [29]. Therefore, the neuroprotective effect of eNOS was mainly attributed to the benefits on vasculogenesis and angiogenesis. This study is the first to demonstrate a novel role for neuron-derived eNOS in protecting neurons against apoptosis. With the OGD model in two neuronal cell types, SH-SY5Y cells and primary neurons, we provide compelling evidence that the activity of

eNOS is central to neuron viability in the ischemic condition, which accounts for the remarkable neuroprotective effect of eNOS in the *in vivo* ischemic model. Our findings suggest that eNOS expressed by neurons functions as a transducer of survival signals, which is upregulated and activated by ischemic stimulation.

Another important aspect in this study is the elucidation of the precise signaling mechanism involved in the anti-apoptotic effect of eNOS. Here, it was suggested that reduced BDNF level correlated with the decreased survival rate of neurons by eNOS inhibitor

L-NIO both *in vitro* and *in vivo*. Exogenous BDNF reversed the excessive neuronal loss by L-NIO treatment and these results further supported the involvement of BDNF in the neuroprotection of eNOS. On the other hand, forced eNOS overexpression alone induced higher BDNF secretion from the SH-SY5Y cells and elevated BDNF accounted for the protection exerted by exogenous eNOS. Together, our findings have identified eNOS as a key regulator, which counteracted ischemia-induced neuron death through stimulating BDNF secretion from neurons. It is noted that BDNF is not only involved in eNOS pathway but also in other pathways, like downstream of calcium and cAMP, which are also turned on/off in ischemia. We cannot rule out the possibility that addition of BDNF would introduce a comprehensive protective effect, more than compensating the inhibition on eNOS. Therefore, a further investigation on the mediator between eNOS and BDNF is required to address this question; gene profiling analyses in our laboratory on L-NIO-treated neurons, and neurons with eNOS overexpression have revealed several downstream genes of eNOS, which may be involved in the regulation of BDNF. The detailed characterization is currently under investigation.

To address the neuroprotective effect of eNOS *in vivo*, L-NIO was employed in the rat ischemic model. We fully appreciate that although L-NIO is the most specific tool inhibiting eNOS ($IC_{50\text{eNOS}} = 0.5 \mu\text{M}$), the selectivity is still relative. Therefore, we deliberately selected the *in vivo* dose according to the potency and a pilot pharmacokinetic study. Furthermore, to confirm that the effect of L-NIO was mediated by eNOS, a series of eNOS overexpression experiments were performed to consolidate the *in vivo* findings. Together, the role of eNOS in neuroprotection was validated by the solid data package in this article.

It is difficult to define the precise cellular mechanisms by which eNOS exert neuroprotection during ischemic injury, because

there are many variables of *in vivo* ischemia/reperfusion models, including the potential effect of angiogenesis and vascular remodeling, heterogeneity of cell types, influence of neurohormonal systems, and the exacerbation of tissue damage caused by inflammation. Therefore, in this study, to identify the main cell source of BDNF regulated by eNOS and rule out the influence of other cell types, we chose SH-SY5Y cells and primary cortical neurons to investigate the direct influence of eNOS on neuron apoptosis due to OGD, an *in vitro* model of cerebral ischemia. In this context, the *in vitro* culture systems provide advantages that overcome the embedded complications of *in vivo* models and complement the *in vivo* findings; the results of two neuronal cell types aligned very well, suggesting the mechanistic regulations on eNOS/BDNF and the pathway of ischemia-induced apoptosis are largely shared in neuronal cells. Another complexity involves the difficulty of distinguishing neuronal apoptosis from necrosis in the *in vivo* models. For this reason, a combination of TUNEL assay, caspase-3 cleavage, and MTT assay were employed here to evaluate neuron apoptosis, by which we cautiously confirmed that apoptosis is a predominant mode of cell death during OGD in our model, and eNOS plays a critical role in protecting neurons against ischemia-induced apoptosis.

In conclusion, our discovery provides a novel explanation for the neuroprotective effect of eNOS under pathological ischemic conditions such as stroke. The evidence presented here strongly supports eNOS as an important target gene for new therapeutic strategies in brain ischemia.

Conflict of Interest

The authors declare no conflict of interest.

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Supporting Information

The following supplementary material is available for this article:

Figure S1. Effects of oxygen-glucose deprivation (OGD) and/or L-NIO on brain-derived neurotrophic factor (BDNF) level secreted by primary cortical neurons. The amounts of BDNF secreted to the medium were analyzed by ELISA and the cells in normoxia were referred as sham control. *OGD versus Sham, $P < 0.05$; #L-NIO versus Vehicle, $P < 0.05$.

Figure S2. Pretreatment with endothelial nitric oxide synthase inhibitor, L-NIO aggravated neuronal cell apoptosis induced by oxygen-glucose deprivation (OGD). (A) Representative images of SH-SY5Y cells pretreated with Vehicle, 7-NI (3 μ M), AMT (0.1 μ M), or 7-NIO (3 μ M) then exposed to OGD. Nuclei staining (Hoechst, blue) and TUNEL staining (green, merged). Scale bar, 100 μ m. (B) Representative images of primary neurons pretreated with Vehicle or 7-NIO (3 μ M) then exposed to OGD. Nuclei staining (Hoechst, blue), anti-microtubule-associated protein (MAP-2) staining (Red) and TUNEL staining (Green). Scale bar, 100 μ m.

Figure S3. Adenovirus-mediated endothelial nitric oxide synthase (eNOS) overexpression (Ad-eNOS) conferred the neuroprotective effect against oxygen-glucose deprivation (OGD). (A) Schematic representation of control (Ad RFP), eNOS (Ad-eNOS), upper panel; expression of eNOS detected by western blotting using an eNOS antibody 24 h postinfection, lower panel. (B) Rep-

resentative images of SH-SY5Y cells after adenovirus infection and/or OGD (RFP, red; TUNEL, green; Hoechst, blue). Scale bar, 100 μ m. (C) Representative images of primary neurons after adenovirus infection and/or OGD (RFP, red; MAP-2, white; TUNEL, green; Hoechst, blue). Scale bar, 50 μ m.

Figure S4. Brain-derived neurotrophic factor (BDNF) ameliorated the detrimental effect of L-NIO on neuronal cell apoptosis induced by oxygen-glucose deprivation (OGD). (A) Representative images of SH-SY5Y cells pretreated with L-NIO (3 μ M) and/or BDNF (50 ng/mL), then exposed to OGD. Nuclei staining (Hoechst, blue) and TUNEL staining (green, merged). Scale bar, 100 μ m. (B) Representative images of primary neurons pretreated with L-NIO (3 μ M) and/or BDNF (50 ng/mL), then exposed to OGD. Nuclei staining (Hoechst, blue), anti-microtubule-associated protein (MAP-2) staining (Red) and TUNEL staining (Green). Scale bar, 100 μ m.

Figure S5. Neutralization of brain-derived neurotrophic factor (BDNF) abrogated neuroprotective effect of endothelial nitric oxide synthase (eNOS) overexpression. (A) Representative images of SH-SY5Y cells pretreated with Ad-eNOS and/or anti-BDNF antibody, then exposed to oxygen-glucose deprivation (OGD; RFP, red; TUNEL, green; Hoechst, blue). Scale bar, 100 μ m. (B) Representative images of primary neurons pretreated with Ad-eNOS and/or anti-BDNF antibody, then exposed to OGD (RFP, red; MAP-2, white; TUNEL, green; Hoechst, blue). Scale bar, 50 μ m.