AN ANG1-TIE2-PI3K AXIS IN NEURAL PROGENITOR CELLS INITIATES SURVIVAL RESPONSES AGAINST OXYGEN AND GLUCOSE DEPRIVATION

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Abstract—Neural progenitor cells (NPCs) have the potential to survive brain ischemia and participate in neurogenesis after stroke. However, it is not clear how survival responses are initiated in NPCs. Using embryonic mouse NPCs and the in vitro oxygen and glucose deprivation (OGD) model, we found that angiopeptin-1 (Ang1) could prevent NPCs from OGD-induced apoptosis, as evidenced by terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling and annexin V labeling. Ang1 significantly elevated tunica intima endothelial kinase 2 (Tie2) autophosphorylation level, suggesting the existence of functional Tie2 receptors on NPCs. NPCs under OGD conditions exhibited reduction of Akt phosphorylation, decrease of the Bcl-2/Bax ratio, activation of caspase-3, cleavage of PARP, and downregulation of β-catenin and nestin. Ang1 reversed the above changes concomitantly with significant rising of survival rates of NPCs under OGD, but all these effects of Ang1 could be blocked by either soluble extracellular domain of Tie2 Fc fusion protein (sTie2Fc) or the phosphoinositide 3-kinase (PI3K) inhibitor 2-(4-morpholinyl)-8-phenyl-1(4H)-benzopyran-4-one (LY294002). Our findings suggest the existence of an Ang1–Tie2–PI3K signaling axis that is essential in initiation of survival responses in NPCs against cerebral ischemia and hypoxia. © 2009 IBRO. Published by Elsevier Ltd. All rights reserved.

Key words: angiopeptin-1, Akt, nestin, cerebral ischemia, apoptosis, stem cell.

Cerebral ischemia has been investigated intensively on animal models subjected to artificial artery occlusion, or on tissue and cell cultures subjected to serum starvation and/or oxygen and glucose deprivation (OGD). Accumulated evidence supports that programmed cell death or apoptosis is the central pathophysiological event leading to brain tissue damage and functional failure after stroke. Fortunately, brain cells have the potential to overcome ischemic or hypoxic insult through initiation of survival responses. Although published data suggest that activation of the phosphoinositide 3-kinase (PI3K) is critical in initiation of survival responses in brain cells (Horn et al., 2005; Valable et al., 2003), certain investigation emphasizes the neuroprotective effect of other elements rather than PI3K against OGD-induced apoptosis (Lin et al., 2008). Such discrepancy reflects the fact that the underlying mechanisms of survival signaling in cerebral ischemia are cell type and developmental stage specific. Thus, more extensive and detailed studies are demanded. Recent advances reveal that neural stem cells (NSCs) or neural progenitor cells (NPCs, it will be used as a generic term not discriminating NSCs and NPCs in this paper) participate in regeneration of damaged neural tissues after cerebral ischemia/hypoxia (Zhang et al., 2005, 2008; Kim and Szele, 2008; Felling et al., 2005). Exciting new therapeutic approaches based on NPC biology have been proposed (Imitola, 2007; Nakatomi et al., 2002). However, some basic questions concerning the fate of NPCs after ischemic or hypoxic insult remain to be answered: First, are NPCs resistant to ischemia-induced apoptosis, or can we awake their latent survival potential through pharmacological intervention? Second, what is the pivotal signaling pathway that controls the initiation of survival responses in NPCs? Finally, how does survival signaling in NPCs orchestrate with other important processes such as cell migration, adhesion, proliferation and differentiation, so that functional recovery can be realized? Although it is too early to address these questions without emergence of substantial progress, several lines of evidence are promising for finding satisfactory answers. A report on adult rat models showed that mature neurons were vulnerable to apoptosis induced by transient forebrain ischemia, but doublecortin-positive neuroblasts and nestin-positive NPCs were resistant (Chen et al., 2005). Ectopic overexpression of doublecortin in rat subventricular zone (SVZ) cells and U-87 human glioma cells significantly elevated nestin levels, and meanwhile allowed the two cells to survive severe OGD, even when it lasted 32 h (Santra et al., 2006). Nestin also served as a potent anti-apoptotic protein in immortalized ST15A precursor cells against hydrogen peroxide–induced apoptosis (Sahlgren 2005).
Tie2-PI3K activation caused increase of the Bcl-2/Bax ratio, protected NPCs from OGD-induced apoptosis. Moreover, binding of Ang1 to Tie2 activated the PI3K pathway and protected NPCs from OGD-induced apoptosis. Moreover, Tie2-PI3K activation caused increase of the Bcl-2/Bax ratio, and prevented downregulation of β-catenin and nestin in NPCs under OGD treatment. The essential roles of Ang1–Tie2–PI3K signaling in survival responses support the presence of a neurovascular niche that controls the fate of NPCs in cerebral ischemia.

**EXPERIMENTAL PROCEDURES**

**Reagents**

Recombinant human Ang1 and recombinant human Tie2/Fc chimera (soluble extracellular domain of tunica intima endothelial kinase 2 and Fc fusion protein, sTie2Fc), a fusion protein constructed from the extracellular domain of human Tie2 and the Fc region of human IgG1, were purchased from R&D Systems (Minneapolis, MN, USA). Monoclonal antibody against nestin (Rat-401), and specific antibodies against Tie2, Bax, extracellular signal-regulated kinase 1/2 (ERK1/2), phosphorylated-ERK1/2 (P-ERK1/2), ERK1/2 and β-actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Polyclonal antibodies against phosphorylated-Tie2 (Ty992P), Akt, phosphorylated-Akt (Ser473P), and the PI3K inhibitor LY294002 2-(4-morpholinyl)-8-phosphoryl-1-(4H)-benzopyran-4-one were obtained from Cell Signaling Technology (Danvers, MA, USA). ERK inhibitor U0126 was obtained from Calbiochem (Gibbstown, NJ, USA). Rabbit monoclonal antibodies against Bcl-2 (E17) and poly (ADP-ribose) polymerase (PARP)-1 p25 fragment (E51) were purchased from Epitomics (Burlingame, CA, USA). Specific antibodies against β-catenin were purchased from Neomarker (Westinghouse, CA, USA).

**Culture of adherent mouse embryonic NPCs**

Animal treatment was in accordance with the *Guide for the Care and Use of Laboratory Animals* of the National Institutes of Health, and was licensed by the Lab Animal Care Committee in Beijing. All efforts were made to minimize the number of animals used and their suffering. Culture of adherent mouse embryonic NPCs was performed according to a published report (Pollard et al., 2006) with minor modifications. Dissected telencephalons of embryonic day (E) 12.5 mouse brains were digested with Accutase solution (Sigma, St. Louis, MO, USA) for 5 min at 37 °C and mechanically dissociated using a fire-polished glass Pasteur pipette. Cells were washed once in phosphate-buffered saline (PBS), plated in plain six-well plastic plates (Iwaki, Tokyo, Japan), and then cultured in the serum-free DMEM:F12 (7:3) medium supplemented with 2% B27 (Invitrogen, Carlsbad, CA, USA), 2 mM L-glutamine (R&D Systems), 20 ng/ml epidermal growth factor (EGF) (R&D Systems) and 20 ng/ml basic fibroblast growth factor (bFGF) (R&D Systems). The cultures were maintained for 1 week at 37 °C in a CO2 incubator with a humidified atmosphere containing 5% CO2, with half-volume changes of fresh media every 2–3 days. NPC-containing neurospheres formed during the period were collected by centrifugation at 1000 × g for 30 s. The pellets were resuspended in Accutase solution and cells were dissociated by repeated pipetting. Then the cells were plated in 35-mm plastic Petri dishes coated with polyornithine (Sigma). Attachment and outgrowth occurred within 1–2 days, and the cells were expanded under the same adherent condition. NPCs prepared in our laboratory were characterized by uniform expression of nestin and conservation of neuronal- and glial-differentiation potentials after repeated passages.

**OGD**

OGD on NPCs was achieved in an anaerobic chamber (Thermo Forma Scientific, Inc., Waltham, UK) according to published reports (Yu et al., 2007) with modifications. Briefly, NPCs grown in the 35-mm culture dishes or 96-well plates were washed three times with serum- and glucose-free Earle’s balanced salt solution (Invitrogen). After being washed, cells were immersed in 1.5 ml (for 35-mm Petri dishes) or 100 µl (for 96-well plates) of the above Earle’s solution. All cultures were then wrapped with Parafilm and placed inside the anaerobic chamber saturated with N2:CO2:H2 (85:5:10) and incubated at 37 °C. Normoxic control NPC cultures were grown simultaneously at 37 °C in a CO2 incubator with the abovementioned supplemented serum-free DMEM:F12 (7:3) medium under normoxic conditions, i.e. humidified air plus 5% CO2.

**Cell viability assay**

For quantitative analysis of cell viability, NPCs were plated in 96-well plates at a density of 1×10^4 cells/well. Cells were subjected to OGD for the indicated times in the presence or not of Ang1, sTie2Fc, or LY294002, respectively. Cell Counting Kit-8 (CCK-8) was used to determine cell viability following the manufacturer’s protocol (Dojindo, Kumamoto, Japan). Briefly, after treatment, a 10 µl of CCK-8 solution was added to each well. After incubation at 37 °C for 2 h in a humidified CO2 incubator, absorbance at 540 nm was monitored with the SpectraMax Plus 384 microplate reader (Molecular Devices, Sunnyvale, CA, USA). The values were used to calculate cell viability by setting the normoxic control as 100%.

**Terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) and confocal microscopy**

Terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) was used to detect apoptotic DNA strand breaks. NPCs were maintained in OGD conditions for 12 h and apoptotic DNA breaks were detected using terminal deoxynucleotidyl transferase (TdT) to incorporate fluorescein isocyanate (FITC)–labeled dUTP onto 3'-OH ends at DNA breaks with the help of an in situ Cell Death Detection Kit (Roche, Mannheim, Germany), according to the manufacturer’s instruction. Briefly, cells were fixed with 4% paraformaldehyde in PBS (pH 7.4) for 30 min at room temperature, and then permeabilized with 0.1% Triton X-100 in 0.1% sodium citrate for 2 min on ice. Permeabilized cells were incubated with a mixture of TdT and FITC-dUTP at 37 °C for 60 min in the dark. After washing with PBS, the cells were blocked for 1 h at room temp with PBS containing 10% BSA and 2% horse serum, and then incubated with mouse monoclonal anti-nestin antibody Rat-401. After washing, cells were incubated with tetramethylrhodamine isothiocyanate–conjugated rabbit antumouse
secondary antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) at room temperature for 60 min. After washing, the cells were stained with Hoechst 33342 (1 μg/ml), treated with the Fluorescence Mounting Medium (Vector Laboratories, Burlingame, CA, USA), and then observed under a confocal laser scanning microscope (Leica, Germany). TUNEL-positive apoptotic cells were counted.

Flow cytometry analysis of apoptosis

Apoptotic cells were detected using the Apoptosis Detection Kit (Oncogene, San Diego, CA, USA) containing FITC labeled annexin V (AV), according to the manufacturer’s instruction. In brief, cells were rinsed with ice-cold PBS and then resuspended in 200 μl of binding buffer. Ten microliters of AV-FITC stock solution was added to cell suspensions and incubated for 30 min at 4 °C. The cells were then further incubated with 5 μl propidium iodide (PI) and were immediately analyzed on a FACStar Plus flow cytometer (Becton–Dickinson, Mountain View, CA, USA). Approximately 10,000–20,000 cells in the tested sample were counted by the cytometer.

Caspase-3 assay

Caspase-3 activity in NPCs was measured using a caspase Colorimetric Kit (Promega, Madison, WI, USA) under the guidance of the manufacturer’s instruction. Briefly, NPCs were rinsed with ice-cold PBS, and lysed by repeated freeze and thaw. Lysates were centrifuged at 4 °C (15,000 g), 20 min), supernatants collected, and then incubated at 37 °C overnight with the assay buffer containing 0.2 mmol/L DEVD-para-nitro aniline as substrate. Absorbance at 405 nm was measured.

Western blotting

Whole-cell lysates from NPC samples were prepared with the help of lysis buffer (50 mM Tris–HCl pH 8.0, 150 mM NaCl, 1% Nonidet P-40, 0.1% SDS, 1% Triton X-100) containing the protease inhibitor cocktail (Roche) and phosphatase inhibitor cocktail (Roche). Total protein concentration was determined by the Bradford assay. Samples containing equal amount (30 μg) of total proteins were loaded onto 10% SDS–polyacrylamide gels. After electrophoresis, protein bands were transferred to PVDF membranes. The membranes were incubated overnight at 4 °C with the primary antibodies. After extensive wash with PBS containing 0.1% Tween 20, the membranes were then incubated with the fluorescently-labeled secondary antibodies conjugated with Alexa Fluor 680 or IRDye 800 (1:5000, Rockland Immunochemicals, Gilbertville, PA, USA) at room temp. The membranes were then washed with PBS containing 0.1% Tween 20 and scanned with the Odyssey Infrared Imaging System (LI-COR Biosciences, Lincoln, NE, USA) by setting the detection channels at 700 nm (for Alexa Fluor 680) and 800 nm (for IRDye 800CW). Scanned bands were quantified using the Odyssey software.

Immunoprecipitation

Aliquots of whole-cell lysates were diluted to 500 μl with TNT buffer (50 mM Tris–HCl, 150 mM NaCl, 0.5% Triton X-100, pH 7.4) and incubated with 0.8 μg of Tie2 antibody. The mixture was incubated overnight at 4 °C, and then mixed and incubated with 20 μl of Protein G-Sepharose/Fast Flow (Amersham Biosciences, Uppsala, Sweden) for 4 h. The Sepharose beads were washed twice with the TNT buffer, and then bound proteins were denatured and eluted by boiling the beads in 20 μl of SDS-PAGE loading buffer containing dithiothreitol (DTT). Proteins were separated on 7.5% SDS-PAGE gels, transferred onto PVDF membranes, and analyzed by Western blotting.

Statistical analysis

Data were analyzed with the statistical software package SPSS 13.0 (SPSS, Chicago, IL, USA) and were expressed as mean±standard deviation (SD). One-way ANOVA test was performed, and post hoc multiple comparisons were done with the Student–Newman–Keuls test. Statistically significant differences were defined as P<0.01.

RESULTS

Ang1 protects NPCs from OGD-induced cell death

Viability of mouse embryonic NPCs decreased progressively during the course of OGD, with only about 24% of viable cells remained at 24 h (Fig. 1A), in agreement with a report on cultured mouse HN33 cells (Jin et al., 2000). Next, we tested whether Ang1 could prevent NPCs from OGD-induced cell death. Without Ang1, NPC viability was about 62% after OGD for 12 h. With the increase of Ang1 concentration, ranging from 50 to 150 ng/ml, NPC viability increased linearly (Fig. 1B). Compared with OGD alone, statistically significant increase of cell viability against OGD was observed on all the tested Ang1 doses shown in

![Fig. 1](image-url)  
**Fig. 1.** Ang1 protected NPCs from cell death caused by OGD. (A) Time course of NPC viability under OGD without Ang1. (B) Dose–response curve of Ang1 on viability of NPCs subjected to OGD for 12 h. Ang1 was added to NPC cultures at the indicated concentrations just prior to the onset of OGD. Data in A and B are mean±SD from three independent experiments and each experiment was an average of triplicate measures.
Fig. 1B. At the maximally effective concentration (200 ng/ml) of Ang1, a dramatic increase of viability to about 89% was shown. Thus, Ang1 at 200 ng/ml was used in all subsequent experiments.

Ang1 prevents OGD-induced apoptosis in NPCs

The TUNEL assay was used to detect DNA fragmentation, the biochemical hallmark of apoptosis. In the normoxic control, almost all NPCs were TUNEL-negative. However, TUNEL-positive signals appeared extensively in the nuclei of NPCs under OGD for 12 h (Fig. 2A). The TUNEL-positive rate was about 37% in OGD alone, but reduced to about 12% if Ang1 was added (Fig. 2B). To confirm the antiapoptotic activity of Ang1 was mediated specifically through binding and activation of Tie2, we simultaneously added both Ang1 and sTie2Fc (soluble extracellular fragment of Tie2 and Fc fusion protein) to the culture medium prior to the onset of OGD. Excessive sTie2Fc can block the interaction of Ang1 with cellular Tie2 (Kim et al., 2000; Makinde and Agrawal, 2008). Indeed, sTie2Fc at 1 μg/ml effectively inhibited the antiapoptotic activity of 200 ng/ml Ang1 (Fig. 2B). To confirm the neural progenitor/stem cell identity of our NPC cultures, we also detected nestin expression in the samples. As expected, intense nestin fluorescence was observed in the cytoplasm of each nucleated cell in control. Surprisingly, nestin staining decreased dramatically in NPCs under OGD treatment, and addition of Ang1 partially prevented the decrease (Fig. 2A). These observations persuaded us to investigate nestin expression in later experiments.

The antiapoptotic activity of Ang1 was further quantified with flow cytometry by combined staining of NPC samples with AV-FITC and PI (Fig. 3). Being a prominent event in early apoptosis, phosphatidylserine (PS) flips from the inner- to the outer-leaflet of cell membrane. Exposed PS on cell surfaces can be detected specifically with AV-FITC. At the late stage of apoptosis or in necrosis, cell membrane integrity is lost, allowing entry of the DNA-binding dye PI into cells. After 12 h of OGD, AV-positive apoptotic NPCs reached to 42% (the sum of AV+/PI− cells and AV−/PI− cells). AV+/PI+ necrotic cells were negligible. Addition of Ang1 significantly diminished the AV-positive population (to ~14%) in NPCs under OGD. However, sTie2Fc at 1 μg/ml completely abrogated the antiapoptotic activity of Ang1.

Ang1 stimulates phosphorylation of Tie2 and Akt in NPCs

NPCs cultured under normoxic and serum-free conditions were treated with Ang1, and the time course of Tie2 phosphorylation was studied by Western blotting. As revealed in Fig. 4A, dramatic increase of Tie2 tyrosine phosphorylation was detected after 15–30 min exposure to Ang1. Total Tie2 level was not affected within 1 h of observation. Noticeably, there was basal phosphorylation of Tie2 maintained at a low level in NPCs, probably caused by autose-
cretion of Ang1. Indeed, we have detected Ang1 activity in the media of serum-free NPC cultures (our unpublished observations). Tyrosine autophosphorylation on Tie2 is a signature of receptor activation (Jones et al., 1999). Thus, exogenously added Ang1 could activate significant amount of functional Tie2 receptors on NPCs.

To uncover the downstream events after Tie2 activation, we used antibodies specific to the phosphorylated Ser473 epitope on Akt to probe Akt activation in NPCs. Ang1 was added to NPC cultures maintained under normoxic and serum-free conditions, and whole-cell lysates harvested at indicated times shown in Fig. 4B were analyzed by Western blotting. The results manifested that Ang1 caused significant elevation of phosphorylated- but not total-Akt levels within 10 – 30 min treatment. A maximal increase of Akt phosphorylation up to 10.8-fold was observed (Fig. 4B), followed by a gradual decrease after prolonged incubation (data not shown).

Inhibition of PI3K abrogates both Ang1-induced Akt phosphorylation and survival activity of Ang1

Without supplementation of Ang1 to the culture medium, the basal level of Akt phosphorylation in NPCs decreased significantly after 12 h of OGD; meanwhile NPC viability was also decreased. Contrarily, in the OGD plus Ang1 group, there was a significant elevation of Akt phosphorylation level, which even far exceeded the basal level (up to 3.6-fold) under normoxic conditions (Fig. 5).

To elucidate the significance of PI3K activation by Ang1 in NPCs under OGD, we investigated Akt phosphorylation changes under the influence of either sTie2Fc or a specific PI3K inhibitor LY294002 (Fig. 5). First, we treated NPCs with Ang1 in the presence of sTie2Fc. As expected, sequestering of Ang1 by sTie2Fc completely blocked Akt phosphorylation; meanwhile the protective effect of Ang1 against OGD-induced NPC death was also completely abrogated by sTie2Fc. Second, when LY294002 instead of sTie2Fc was used in combination with Ang1, complete abrogation of both Akt phosphorylation and survival activity of Ang1 was also observed. These data suggest that activation of Tie2–PI3K pathway by Ang1 is essential in survival responses of NPCs under OGD.

Ang1 suppresses the activation of apoptotic pathway caused by OGD

Activated caspase-3 is considered to be the final executioner of apoptosis, since it cleaves multiple vital cellular proteins such as PARP-1, lamins, β-catenin, and vimentin (Marceau et al., 2007; Danial and Korsmeyer, 2004). Through direct assay of caspase-3 activity using DEVD-paranitro aniline as an enzymatic substrate, we demonstrated that OGD caused significant elevation of caspase-3 activity in NPCs; but such elevation could be significantly suppressed by Ang1. Moreover, both sTie2Fc and LY294002 could abrogate the suppressive effect of Ang1 on caspase-3 activation (Fig. 6).

Western blotting revealed that OGD on NPCs resulted in cleavage of PARP-1, as evidenced by significant decrease in the protein level of Bcl-2, but upregulated Bax in NPCs (Fig. 7B). Bcl-2 and Bax are con and pro elements in

Fig. 3. Antia apoptotic effect of Ang1 quantified by flow cytometry. NPCs were treated with 12 h OGD alone, OGD plus 200 ng/ml Ang1 (OGD + Ang1), or OGD plus 200 ng/ml Ang1 and 1 µg/ml sTie2Fc (OGD + Ang1 + sTie2Fc), respectively. NPCs under normoxic conditions were used as control. (A) Relative fluorescence in NPC populations double-stained with AV-FITC and PI. In each panel, viable AV+/PI- cells were in the left lower region. The AV-/PI+ fraction (upper-right) was consisted of apoptotic cells, whereas a small fraction of AV-/PI- cells (lower-right) was in early apoptosis. Necrotic cells were AV+/PI+ (upper-left). (B) Percentages of differently labeled NPC fractions were calculated from flow cytometry data in A. Each data point represents mean±SD of three independent experiments. *P<0.01 by one-way ANOVA.
apoptosis, respectively. The ratio of Bcl-2/Bax serves as a sensitive index on likelihood of apoptosis (Danial and Korsmeyer, 2004). Our data showed that OGD resulted in a 8.8-fold decrease (compared with normoxic control) of the Bcl-2/Bax ratio in NPCs, but addition of Ang1 prior to OGD increased the ratio by approximately fivefold compared with OGD alone. Both sTie2Fc and LY294002 could reverse the effect of Ang1 on the Bcl-2/Bax ratio (Fig. 7B).

Ang1 inhibits downregulation of nestin and /β-catenin in NPCs under OGD

We probed changes of nestin and /β-catenin with specific antibodies by Western blotting (Fig. 8). Intensive signals for nestin were detected in control NPCs without treatment, confirming the neural progenitor/stem cell identity of our NPC cultures. High expression of /β-catenin in control NPC cultures was also noticeable, highlighting its functional importance. Under OGD conditions, however, both nestin and /β-catenin were significantly downregulated. Ang1 significantly elevated nestin and /β-catenin levels in NPCs under OGD, and such effects could be completely eliminated either by sTie2Fc or by LY294002 (Fig. 8), suggesting the importance of Tie2-PI3K activation. Changes of nestin levels displayed here were consistent with the observations under a confocal microscope (Fig. 2). Note that nestin in NPC preparations displayed multiple fragmented bands on Western blotting, which has been reported elsewhere (Sahlgren et al., 2006). However, nestin fragmentation is not caused by caspases, due to absence of any caspase-cleavage site on nestin (Sahlgren et al., 2006). On the other hand, we failed to detect caspase-catalyzed /β-catenin fragmentation in NPCs under OGD (Fig. 8). It is likely that decrease of /β-catenin observed in NPCs under OGD was mainly mediated by glycogen synthase kinase-3β (GSK-3β) and the ubiquitin–proteosome pathway.

DISCUSSION

The existence of an Ang1–Tie2–PI3K axis that is vital to NPCs

It was reported that embryonic human NSCs expressed high levels of Tie2 mRNA and proteins, and the expressions persisted during differentiation (Parati et al., 2002). However, functional significance of these findings was not explored. Another report demonstrated that MAP-2-positive mouse neurons could coexpress Ang1, Ang2, and Tie2, and addition of Ang1 to the neural cells caused phosphorylation of Tie2 and Akt (Valable et al., 2003). The authors also provided evidence showing that Ang1 could prevent neurons from serum starvation-induced apoptosis through activation of PI3K, but whether the same signaling pathway might exist in NPCs was not explored either. In
The present study, we demonstrated that OGD in NPC cultures caused extensive cell death through apoptotic pathway activation, which was evidenced by TUNEL and AV labeling, caspase-3 activation, and PARP-1 cleavage. All these parameters of apoptosis were significantly suppressed by Ang1. To our knowledge, our data provided for the first time convincing evidence that exogenous Ang1 protected mouse embryonic NPCs from OGD-induced apoptosis, and the neuroprotective effect of Ang1 was mediated through activation of Tie2 and PI3K. We also detected basal levels of Tie2-PI3K activation and autosecretion of Ang1 in NPC cultures (not shown), suggesting that the Ang1–Tie2–PI3K pathway also operated physiologically in NPCs.

The signaling events from Ang1–Tie2 interaction to PI3K activation have been studied extensively in endothelial cell systems (Kim et al., 2000; Kanda et al., 2005; Makinde and Agrawal, 2008). For a generalized scenario, binding of Ang1 to its receptor Tie2 causes Tyr autophosphorylation of Tie2 at multiple sites that are recognized by the SH2 domains on p85, i.e., the regulatory subunit of PI3K. Recruitment and subsequent phosphorylation of p85 by Tie2 cause activation of p110, the catalytic subunit of PI3K (Jones et al., 1999). However, detailed mechanisms and possible variations governing these events in different cellular systems including NPCs need to be investigated.

It should be emphasized that our findings were based on in vitro studies on mouse embryonic NPCs. Although the OGD model could partially mimic both ischemic and hypoxic insults, in vivo investigations remain to be conducted for better understanding of the significance of Ang1–Tie2 interaction in both embryonic and adult NPCs. Robust in vivo regenerative response of NPCs to perinatal hypoxia/ischemia has been reported (Pourié et al., 2006; Felling et al., 2005). It was also reported that infusion of FGF-2 and EGF into the lateral ventricles of adult rats subjected to transient forebrain ischemia markedly promoted NPC proliferation, neuronal cell regeneration and functional recovery (Nakatomi et al., 2002). It is interesting to test whether infusion of Ang1 along or in combination with FGF/EGF could bring forth better outcomes using similar in vivo ischemia/hypoxia experimental settings.
Ang1–Tie2–PI3K signaling causes phosphorylation of Akt and activation of downstream antiapoptotic responses

Akt is a well-known major effector in cellular survival responses. Activated PI3K catalyses localized formation of the second messenger phosphatidylinositol (3,4,5)-trisphosphate or PtdIns(3,4,5)P3, which recruits inactive Akt to plasma membrane. Then phosphorylation of Akt at Thr308 and Ser473 takes place, transforming Akt into fully activated form (Toker, 2008). We found that either blocking of the activation of Tie2 by sTie2Fc, or blocking of the enzymatic activity of PI3K by LY294002, could completely abrogate Akt phosphorylation at Ser473 in NPCs subjected to OGD plus Ang1 treatment (Fig. 5). These results underscored the importance of the Ang1–Tie2–PI3K axis in survival signaling responses against OGD-induced apoptosis in NPCs.

Recently, an alternative route leading to Akt activation was proposed, i.e. phosphorylation of nuclear Akt at Ser473 by DNA-PK with the help of nuclear localized PI3K and its enzymatic product PtdIns(3,4,5)P3 (Bozulic et al., 2008; Toker, 2008). Since DNA-PK is activated by DNA damages which are generated by irradiation or genotoxic agents such as reactive oxygen species (ROS), this new route arms cells with the ability to survive genome-damaging insults. We hypothesize that the Ang1–Tie2–PI3K axis also works in NPCs in order to survive genome-damaging insults. Not only OGD in stroke-affected areas but also the ROS burst caused by reperfusion threatens the survival of brain cells (Pluta et al., 2001). Supporting this notion, the work by Kwak et al. (2000) indicated that Ang1 protected endothelial cells from irradiation-induced apoptosis in a Tie2- and PI3K-dependent way. An investigation focusing on this issue is under way.

The antiapoptotic effects of Akt are well documented in the literature (Wymann and Marone, 2005; Wang et al., 2007). Substrate proteins subject to Akt-catalyzed Ser/Thr phosphorylation involve many pro- and antiapoptotic molecules, such as caspase-9, Bad, IκB kinase (IκBK), FOXO, TSC1/2, CREB, MDM2, and GSK-3β. Among dozens of the Akt-catalyzed events, phosphorylation and inactivation of proapoptotic caspase-9 and Bad, but activation of antiapoptotic IκB kinase is a typical example. Moreover, within the Akt-signaling web, there is an interesting domain that commits to cellular control of the Bcl-2/Bax ratio. Antiapoptotic Bcl-2 (also includes Bcl-XL) inhibits the activation of proapoptotic Bax through formation of Bcl-2/Bax heterodimers. However, Bcl-2 is inactivated by another bind-
ing partner Bad. Phosphorylation of Bad by Akt releases Bcl-2 from Bcl-2/Bad, thus restoring the antiapoptotic ability of Bcl-2. Moreover, Akt phosphorylates and activates IκB, causing nuclear translocation of NF-κB that increases Bcl-2 transcription. The ratio of Bcl-2/Bax inside a cell serves as a rheostat that sets the threshold for triggering apoptosis (Danial and Korsmeyer, 2004). In this study, we have demonstrated that the Bcl-2/Bax ratio correlates well with NPC survival, supporting the notion that the Ang1–Tie2–PI3K axis serves as a signal input device to upregulate the Bcl-2/Bax threshold for triggering apoptosis in NPCs.

Reports on blood vessel endothelial cells confirmed that binding of Ang1 to Tie2 induced activation of PI3K, and phosphorylation of mitogen-activated protein kinase (MAPK)/ERK1/2 (p44/p42) (Harfouche et al., 2003; Kanda et al., 2005) and p70 S6K (Kanda et al., 2005). Also in the reported endothelial cell and myocyte systems, Ang1 selectively bound to integrins and induced PI3K-dependent phosphorylation of both Akt and MAPK/ERK1/2, so that the cells survived serum starvation (Dallabrida et al., 2005). These data inspired us to uncover possible changes of the MAPK/ERK1/2 pathway in NPCs. However, neither the total proteins nor the phosphorylation levels of MAPK/ERK1/2 were changed; regardless of whether the NPCs were treated with OGD alone, or OGD plus Ang1 (Fig. 9). Although MAPK inhibitor U0126 completely blocked ERK1/2 phosphorylation in NPCs under OGD or OGD plus Ang1, it did not abolish the protective effect of Ang1 in NPCs under OGD. Thus, contrary to other cellular systems, the roles of the MAPK pathway seem less significant in survival responses of NPCs against OGD.

**Ang1–Tie2–PI3K signaling and stabilization of β-catenin and nestin**

In NPCs, β-catenin and nestin are two unusual molecules, for their stable expressions are essential for NPCs to maintain their identity and undergo population expansion. β-Catenin and nestin are also major players in survival responses. Nevertheless, the underlying mechanisms that control the expression and stability of β-catenin and nestin in NPCs are puzzling. In the case of β-catenin, the related processes are extremely complicated, and conflicting observations have been reported: some reports favored self-renew and population expansion, but others favored differentiation, if expression of β-catenin in NPCs was stabilized (Chenn and Walsh, 2002; Castelo-Branco et al., 2004; Schuller and Rowitch, 2007; Wrobel et al., 2007). Such discrepancy probably reflects the fact that β-catenin function and its regulation in NPCs are tightly coupled with developmental stages and niches in the CNS.

Results in this paper suggested that Ang1–Tie2–PI3K activation could stabilize β-catenin in NPCs under OGD, suggesting an antiapoptotic role for β-catenin. Existing
evidence links PI3K to β-catenin stabilization through two mediators: Akt and GSK-3β. GSK-3β phosphorylates β-catenin and facilitates its rapid degradation through the ubiquitin–proteasome system. Akt can phosphorylate the Ser9 residue on GSK-3β, leading to GSK-3β inactivation and in turn upregulation of the cytosolic and nuclear β-catenin pools (Cross et al., 1995). Nuclear β-catenin associates with the LEF/TCF transcription factors; upregulates the expression of a variety of proteins such as Myc, cyclin D, BMP, Bcl-2 and survivin; and thus promotes growth, survival and proliferation (Haegele et al., 2003; Beurel and Jope, 2006). Inactivation of GSK-3β by Akt also directly stabilizes Myc and cyclin D, since these two proteins are also targeted to proteasomc degradation if phosphorylated by GSK-3β.

High expression of nestin is a prominent molecular marker of NPCs (Lendahl et al., 1990; Kawaguchi et al., 2001; Hall et al., 2006). Loss of nestin expression due to proteasomc degradation is one of the earliest phenotypic changes in NPC differentiation (Mellodew et al., 2004). Rat SVZ cells and human U-87 cells overexpressing doublecortin remained viable against severe OGD lasted 32 h, meanwhile significantly elevated nestin levels were observed in these doublecortin-transfected cells (Santra et al., 2006). Recent studies on the interactions between nestin and Cdk5 provided important clues on the roles of nestin in regulating NPC survival, proliferation and differentiation (Cicero and Herrup, 2005; Sahlgren et al., 2006). Cdk5 can stop NPC proliferation and promote differentiation. Cdk5 also has potent proapoptotic activity. Binding of the Cdk5/p35 complex to nestin has sequestering effect on Cdk5. Thus, maintaining a high nestin level can prevent both differentiation and apoptosis. Moreover, nestin may exert antiapoptotic effect by stabilizing its partner vimentin. Nestin cannot form intermediate filament (IF) by itself, but coassembles with vimentin or α-internexin to form hybrid fibers (Steinert et al., 1999). Many caspases cleave vimentin and generate fragments that can accelerate apoptosis progression (Marceau et al., 2007). Nestin may mask the cleavage sites on vimentin, since nestin itself does not have caspase cleavage site.

Data in this paper suggested that Ang1–Tie2–PI3K signaling could stabilize both β-catenin and nestin in NPCs under OGD. We speculate that a process similar to β-catenin stabilization also operates on nestin in NPCs, since it was suggested that in a differentiating NPC, downregulation of nestin operated at the proteasome level, not transcription level (Mellodew et al., 2004). Proteosomic down-regulation of nestin was also a prerequisite for oxidant-induced apoptosis of NPCs (Sahlgren et al., 2006). However, detailed molecular events that bridge the gap between Tie2-PI3K activation and nestin stabilization remain to be uncovered.

The Ang1–Tie2–PI3K axis and the neurovascular niche

The concept of neurovascular niche has been supported with increasing evidence in recent years (Zhang et al., 2005, 2008). The niche concept implies that neurogenesis is tightly coupled to angiogenesis, i.e. ischemia/hypoxia-activated endothelial cells promotes proliferation and neuronal differentiation of NPCs; while ischemia/hypoxia-activated NPCs promotes capillary tube formation (Teng et al., 2008). It has been suggested that neuro-angiogenesis coupling is mediated by VEGF and its receptor VEGFR2 (Teng et al., 2008). Accordingly, Ang1 and Tie2 are another pair of mediators for the coupling (Ohab et al., 2006). The importance of Ang1–Tie2–PI3K signaling and its relationship with cytoskeleton elements β-catenin and nestin in NPCs revealed in this paper not only support the hypothesis of neuro-angiogenesis coupling, but also suggest coupling of survival responses to adhesion and migration in NPCs, since adhesion and migration are controlled by cytoskeleton remodeling. Induction of population expansion and targeted-migration of nestin-positive NPCs and doublecortin-positive neuroblasts by administration of Ang1 (Ohab et al., 2006) or chemical agonists targeted to the Ang1–Tie2–PI3K axis are promising approaches in developing new therapies against cerebral ischemia and hypoxia.

Acknowledgments—This work is supported by the National Natural Sciences Foundation of China (30600167). We thank Prof. Albert Cheung Hoi Yu (Neuroscience Research Institute, Department of Neurobiology, Peking University Health Science Center) for providing the anaerobic chamber.

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