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Signaling pathway involved in hypoxia-inducible factor-1 α regulation in hypoxic-ischemic cortical neurons in vitro

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Abstract

Hypoxia-inducible factor-1 α (HIF-1 α) is a key transcriptional regulator of cellular and systemic oxygen homeostasis. Previous studies have shown that the regulation of HIF-1 α is involved in the activation of PI3K/Akt pathway in some cells. However, whether this pathway plays a role in modulating HIF-1 α in cultured cortical neurons during hypoxia-ischemia (HI) remains unclear. We therefore investigated the relationship between phosphoinositid 3-kinase/Akt (PI3K/Akt) pathway and HIF-1 α expression in cultured neurons using an oxygen and glucose deprivation (OGD) model. In this study, cortical neurons cultured in vitro were subjected to OGD for 3 h followed by reperfusion. The expressions of HIF-1 α , VEGF, total Akt and phosphorelated-Akt (p-Akt) were detected by RT-PCR, Western blot and immunocytochemistry. We found that HIF-1 α and VEGF were increased at 4 h and peaked at 8 h after OGD. Meanwhile, p-Akt increased and peaked at 4h after reperfusion, preceding HIF-1 α expression. Pre-treatment with wortmannin, a PI3K/Akt pathway inhibitor, significantly inhibited p-Akt expression and further attenuated both transcription and translation of HIF-1 α and VEGF. Collectively, our findings suggested that PI3K/Akt signaling pathway might be involved in HIF-1 α regulation after OGD in cultured cortical neurons.

Keywords

HIF-1 α ; neurons; hypoxia-ischemia; signaling pathway

HIF-1, a key regulator of oxygen homeostasis, is a heterodimeric protein composed of the two subunits, HIF-1 α and HIF-1 β . These two subunits belong to the basic helix-loop-helix (bHLH)-PAS protein family. HIF-1 α is an inducible subunit regulated by hypoxia, HIF-1 β , a constitutive subunit which is not regulated by oxygen concentration [7]. In addition to hypoxia,

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HIF-1 α has been shown to be induced by other stimuli, such as deferoxamine (DFO), insulin, and epidermal growth factor [4,9]. Under normoxia, HIF-1 α is rapidly degraded by ubiquitin-proteasome pathway which is mediated by hydroxylation of the prolines 564 and 462 residues. This process is regulated by an O₂-sensitive enzyme called HIF-1 α prolyl-hydroxylase [5]. Under hypoxia, the degradation of HIF-1 α by hydroxylation and ubiquitination is inhibited and thus stabilized. Stabilized HIF-1 α , a cytosolic protein then translocates into the nucleus, where HIF-1 α subunit is phosphorylated and dimerizes with HIF-1 β subunit, a nuclear protein, to form functional HIF-1 [18]. Activated HIF-1 binds to hypoxia response elements (HRE) of its target genes and then triggers the transcription of the target genes such as vascular endothelial growth factor (VEGF) and erythropoietin (EPO) [12,14]. Protective effects of VEGF include promoting angiogenesis, neurogenesis, and neurite outgrowth in brain after HI [19].

Although the factors modulating HIF-1 α expression have been studied extensively, the signaling pathways for HIF-1 α regulation remain controversial. In the central nervous system, Akt is suggested to be necessary for neuronal survival, neurogenesis, angiogenesis, axon or dendrite formation, synaptogenesis, and synaptic transmission [1,11]. Recent studies in HepG2 cells show that HIF-1 α is regulated by various signaling pathways including PI3K/Akt [13]. However, this regulation seems to be cell type specific. In HEK293T cells, the activation of PI3K/Akt pathway does not affect the transcriptional activity of HIF-1 α under either normoxic or hypoxic condition [2]. Whether PI3K/Akt pathway is involved in HIF-1 α regulation in cultured cortical neurons exposed to HI remains unclear. Therefore, we aimed to elucidate the relationship between PI3K/Akt and HIF-1 α to address this question.

All animal research was approved by Sichuan University Committee on Animal Research. Pregnant Sprague-Dawley rats were sacrificed and embryos at 16-18 days of gestation were removed and kept in 4°C phosphate buffered saline (PBS, pH 7.4). The whole cerebral cortex was isolated from the fetuses and cells were dissociated in a trypsin solution (1.25mg/mL in Hank's Buffer salt solution) for 10min at 37°C. The cortex cell suspension was centrifuged and resuspended, then seeded in 6-well plates precoated with poly-D-lysine (Sigma), and grown in Neurobasal medium (NB) (Gibco) with 2% B27 supplement (Gibco) and 500 μ M glutamine (Gibco) in a humidified incubator with 5% CO₂ at 37°C. The following experiments were performed at day in vitro 5.

Cultures were exposed to HI for 3h followed by reoxygenation. To induce OGD, cultured cells were gently washed twice with PBS, and then placed in DMEM without glucose. Cells were exposed to hypoxia (95% N₂/5% CO₂) at 37°C in the airtight chamber for 3h. After 3h of OGD treatment, the cell culture medium was changed back to normal NB medium and then returned to 5% CO₂, 37°C incubator. To elucidate whether PI3K is involved in HIF-1 α regulation under OGD, cells were pretreated with wortmannin (an inhibitor of PI3K) at the concentration of 100 nM for 30 min before OGD treatment.

Total RNA of cortical neurons was extracted by using Tissue/Cell RNA Mini Kit (Watson Biotechnologies, INC). Complementary DNA (cDNA) was synthesized from isolated RNA using reverse transcription reagents (RevertAid First Strand cDNA Synthesis Kit, Fermentas). Reverse transcription was performed using random hexamer primer at 25°C for 10 min, 42°C for 60 min, and 70°C for 10 min. cDNA was stored at -20°C until use. PCR was conducted using PCR Master Mix (2 $\times$) (Fermentas) and the following primers: (a) HIF-1 α : sense primer 5'-AAGTCTAGGGATGCAGCAC-3', antisense primer 5'-CAAGATCACCAGCATCTAG-3'; (b) VEGF: sense primer 5'-GCTCTCTTGGGTGCACTGGA-3', antisense primer 5'-CACCGCCTTGGCTTGTGTCACA-3'; (c) β -actin: sense primer 5'-ACACTGTGCCCATCTAGGAGG-3', antisense primer 5'-AGGGGCCGGACTCGTCATACT-3'.

The PCR for HIF-1 α was performed in 37 cycles with following cycle profile: 94°C for 30 sec, 55°C for 30 sec and 72°C for 1.5 min. The PCR for VEGF was performed in 33 cycles with following cycle profile: 94°C for 1 min, 55°C for 1 min and 72°C for 1.5 min. The PCR products were separated on a 2% agarose gel and stained with ethidium bromide using 100bp ladder DNA maker as a size reference (BioTeke Corporation) and photographed under ultraviolet transillumination.

Western blot was performed as previously described [10]. Cells were lysed in CHAP lysis buffer. Equal amounts of protein samples (100 μ g) were loaded per lane and electrophoresed on 8% SDS/PAGE gels. After blocked in nonfat milk buffer, the membranes were separately incubated with mouse HIF-1 α monoclonal antibody (Santa Cruz Biotechnology, diluted 1:200), rabbit VEGF polyclonal antibody (Santa Cruz Biotechnology, diluted 1:300), rabbit Akt polyclonal antibody or mouse phospho-Akt (Ser473) monoclonal antibody (Cell Signaling, diluted 1:500) overnight at 4°C. Mouse β -actin monoclonal antibody (Santa Cruz Biotechnology, diluted 1:1000) was detected as a loading control. Following washes, the membranes were incubated with horseradish peroxidase conjugated secondary antibody, goat anti-mouse or goat anti-rabbit IgG (Santa Cruz Biotechnology, 1:3000) in blocking solution at room temperature (RT) for 1 h. The bands were developed using enhanced chemiluminescence (Millipore Corporation). NIH image was used to measure the densities of the protein signals.

For immunocytochemistry analysis, cultured cells were fixed with 4% paraformaldehyde and treated with 0.3% hydrogen peroxide for 10 min, then blocked with 10% serum at 37°C for 30 min. Cells were incubated with mouse HIF-1 α monoclonal antibody (1:100 dilution) in PBS, or rabbit VEGF (1:200), rabbit Akt (1:400) or mouse p-Akt (1:400) at 37°C for 1 h and overnight at 4°C. After washes in PBS, cells were then incubated with biotin-conjugated goat anti-mouse or goat anti-rabbit IgG (Santa Cruz Biotechnology, 1:200) at 37 °C for 1 h, and avidin-biotin complex for 1 h. DAB was used to visualize the positive signals, and then cells were counterstained with hematoxylin. After washing, cultures were mounted on gelatin-coated slides, dried, and coverslipped. As negative controls, alternate cultures were incubated without primary antibody. Images were observed using microscope (Leica, CM 2000).

Data were represented as mean $\pm$ standard deviation (SD). One-way ANOVA with Bonferroni/Dunnett post-hoc tests was performed for multiple comparisons. $p < 0.05$ was considered statistically significant.

To study whether HIF-1 α and VEGF expression were induced by OGD in cultured cortical neurons, an OGD model of exposure of cultured neurons without glucose in the media to hypoxia for 3h was used. HIF-1 α and VEGF mRNA levels were measured in both normoxic neurons and OGD treated neurons at 0, 2, 4, 8, 12 and 24 h after reperfusion using RT-PCR. HIF-1 α mRNA was induced at 2 h, peaked at 4 h, and gradually declined at 12 h. Two bands corresponding to VEGF isoforms, VEGF164 (563 bp) and VEGF120 (431 bp) were maximally increased at 2 h, maintained at 4 h and decreased at 8 h. VEGF164 was the most dominant transcript (Fig.1A).

To test HIF-1 α and VEGF protein expression, Western blot analysis was performed in this study. HIF-1 α expression (110 kDa) was very weak in normoxic cultured neurons, but was up-regulated in response to OGD. HIF-1 α and VEGF (42 kDa) expression were elevated at 4 h, peaked at 8 h and decreased at 12 h after reperfusion (Fig.1B). After quantification with β -actin, HIF-1 α and VEGF protein levels were respectively increased approximately 4-fold and 3-fold at 8 h compared to that in normoxia ($p < 0.001$) (Fig.1C, Table 1).

The expression of Akt and p-Akt protein at the same time points as indicated above was also detected using Western blot. There was no significant difference in Akt (60 kDa) expression

between normoxic and OGD treated neurons. However, OGD induced a rapid increase in p-Akt (60 kDa). The maximal induction of p-Akt appeared at 4 h, started to decrease at 8 h, and returned to baseline at 24 h (Fig.2A), which was earlier than that in HIF-1 α and VEGF. After normalization with β -actin, an approximate 7-fold, 3.5-fold, and 3-fold increase of p-Akt induction was respectively observed at 4 h, 8 h, and 12 h after reperfusion ($p<0.001$) (Fig.2B, Table 1). Since the maximum induction of p-Akt expression (at 4 h) preceded HIF-1 α and its target gene VEGF (at 8 h) in cortical neurons with OGD, we hypothesized that PI3K/Akt pathway might be involved in the regulation of HIF-1 α and VEGF. To address this question, a PI3K inhibitor, wortmannin (100 nM), was used to pretreat cortical neurons for 30 min, followed by OGD for 3 h. P-Akt but not total Akt was significantly inhibited by wortmannin compared to DMSO treated control neurons at 4 h and 8 h after reperfusion ($p<0.001$) (Fig. 3A, B, Table 2). Immunocytochemistry showed the similar findings as found by Western blots (Fig. 3C) and verified that the positive immunoreactions were localized in the cytoplasm of the neurons (Fig. 3C).

HIF-1 α and VEGF expression after inhibition of p-Akt were measured. Pretreatment with wortmannin attenuated OGD induced HIF-1 α and VEGF mRNA levels (Fig.4A). Similar findings were observed with protein levels by Western blot detection (Fig.4B). After quantification, there was 60% and 70% of HIF-1 α protein, 35% and 40% of VEGF protein decrease respectively at 4 h and 8 h after reperfusion compared to control groups ($p<0.001$) (Fig.4C, Table 2). These findings suggest that Akt phosphorylation is required for OGD induced HIF-1 α and VEGF expression. Furthermore, immunocytochemistry also showed that the expression of HIF-1 α and VEGF is attenuated by wortmannin treatment, which is consistent with the findings of Western blot (Fig.4D).

In this study, HI significantly increased the expression of HIF-1 α and VEGF both at transcriptional and translational levels. HIF-1 α mRNA was increased at 2 h, peaked at 4 h, and gradually declined at 12 h. HIF-1 α protein were elevated at 4 h, peaked at 8 h and decreased at 12 h after reperfusion which agreed with previous reports that HIF-1 α protein peaked at 8 h and declined at 24 h in injured cortex in a rat neonatal stroke model [15]. Our findings that both HIF-1 α mRNA and protein are upregulated in cultured neurons treated with OGD, suggests that HI regulates HIF-1 α on both the transcriptional and translational levels.

Previous reports suggested that hypoxic stimulation could induce HIF-1 α expression via PI3K/Akt signaling pathway [17,8]. In this study, p-Akt peaked at 4 h after reperfusion from OGD, earlier than HIF-1 α following OGD, suggesting that HIF-1 α may be one of the downstream effectors of PI3K/Akt signaling pathway. Studies on astrocytes and rat brains show that PI3K/Akt pathway is a positive regulator of HIF-1 α activation [17,6,8]. However, these findings are not consistent with other reports in vitro and in vivo [2,3]. These opposite findings suggest that the effects of PI3K/Akt on HIF-1 α regulation are cell and tissue type specific. Wortmannin strongly inhibited p-Akt as well as HIF-1 α and VEGF. These findings further support the hypothesis that PI3K/Akt pathway plays an important role in regulating HIF-1 α and its target genes in cultured neurons following OGD.

Interestingly, we found that wortmannin treatment inhibited HIF-1 α transcription since HIF-1 α mRNA was also decreased in this study, which is not in agreement with our previous study in an HI in vivo model. We previously found that wortmannin treatment down-regulated HIF-1 α protein but not the mRNA transcript [8]. These different findings may be due to the difference between cultured neurons in vitro and animal models in vivo since there might be more complicated mechanisms for HIF-1 α regulation in vivo compared to in vitro, such as regulation by other cell types like astrocytes [16,20].

Taken together, these findings suggest that PI3K/Akt pathway might play an important role in the regulation of HIF-1 α on both transcriptional and translational levels in cultured neurons following HI. Elucidating HIF-1 α regulatory mechanisms may lead to new therapy strategies based on ischemic tolerance.

Acknowledgments

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References

1. Akama KT, McEwen BS. Estrogen stimulates postsynaptic density-95 rapid protein synthesis via the Akt/protein kinase B pathway. *J Neurosci* 2003;23:2333–2339. [PubMed: 12657692]
2. Alvarez-Tejado M, Alfranca A, Aragonés J, Vara A, Landázuri MO, del Peso L. Lack of evidence for the involvement of the phosphoinositide 3-kinase/Akt pathway in the activation of hypoxia-inducible factors by low oxygen tension. *J Biol Chem* 2002;277:13508–13517. [PubMed: 11815624]
3. Arsham AM, Plas DR, Thompson CB, Simon MC. Phosphatidylinositol 3-kinase/Akt signaling is neither required for hypoxic stabilization of HIF-1 α nor sufficient for HIF-1-dependent target gene transcription. *J Biol Chem* 2002;277:15162–15170. [PubMed: 11859074]
4. Hamrick SE, McQuillen PS, Jiang X, Mu D, Madan A, Ferriero DM. A role for hypoxia-inducible factor-1 α in desferoxamine neuroprotection. *Neurosci Lett* 2005;379:96–100. [PubMed: 15823423]
5. Ivan M, Kondo K, Yang H, Kim W, Valiando J, Ohh M, Salic A, Asara JM, Lane WS, Kaelin WG Jr. HIF1 α targeted for VHL-mediated destruction by proline hydroxylation: implications for O₂ sensing. *Science* 2001;292(5516):464–468. [PubMed: 11292862]
6. Jiang BH, Jiang G, Zheng JZ, Lu Z, Hunter T, Vogt PK. Phosphatidylinositol 3-kinase signaling controls levels of hypoxia-inducible factor 1. *Cell Growth Differ* 2001;12:363–369. [PubMed: 11457733]
7. Lee JW, Bae SH, Jeong JW, Kim SH, Kim KW. Hypoxia-inducible factor (HIF-1) α : its protein stability and biological functions. *Exp Mol Med* 2004;36:1–12. [PubMed: 15031665]
8. Li L, Qu Y, Mao M, Mu D. The involvement of phosphoinositid 3-kinase/Akt pathway in the activation of hypoxia-inducible factor-1 α in the developing rat brain after hypoxia-ischemia. *Brain Res* 2008;1197:152–158. [PubMed: 18241842]
9. Li Y, Bi Z, Yan B, Wan Y. UVB radiation induces expression of HIF-1 α and VEGF through the EGFR/PI3K/DEC1 pathway. *Int J Mol Med* 2006;18:713–719. [PubMed: 16964427]
10. Mao M, Hua Y, Jiang X, Li L, Zhang L, D Mu. Expression of tumor necrosis factor α and neuronal apoptosis in the developing rat brain after neonatal stroke. *Neurosci Lett* 2006;403:227–232. [PubMed: 16797840]
11. Markus A, Zhong J, Snider WD. Raf and akt mediate distinct aspects of sensory axon growth. *Neuron* 2002;35:65–76. [PubMed: 12123609]
12. Milosevic J, Maisel M, Wegner F, Leuchtenberger J, Wenger RH, Gerlach M, Storch A, Schwarz J. Lack of hypoxia-inducible factor-1 α impairs midbrain neural precursor cells involving vascular endothelial growth factor signaling. *J Neurosci* 2007;27:412–421. [PubMed: 17215402]
13. Mottet D, Dumont V, Deccache Y, Demazy C, Ninane N, Raes M, Michiels C. Regulation of hypoxia-inducible factor-1 α protein level during hypoxic conditions by the phosphatidylinositol 3-kinase/Akt/glycogen synthase kinase 3 β pathway in HepG2 cells. *J Biol Chem* 2003;278:31277–31285. [PubMed: 12764143]
14. Mu D, Chang YS, Vexler ZS, Ferriero DM. Hypoxia-inducible factor 1 α and erythropoietin upregulation with deferoxamine salvage after neonatal stroke. *Exp Neurol* 2005;195:407–415. [PubMed: 16023639]

15. Mu D, Jiang X, Sheldon RA, Fox CK, Hamrick SE, Vexler ZS, Ferriero DM. Regulation of hypoxia-inducible factor 1 alpha and induction of vascular endothelial growth factor in a rat neonatal stroke model. *Neurobiol Dis* 2003;14:524–534. [PubMed: 14678768]
16. Oh YT, Lee JY, Yoon H, Lee EH, Baik HH, Kim SS, Ha J, Yoon KS, Choe W, Kang I. Lipopolysaccharide induces hypoxia-inducible factor-1 alpha mRNA expression and activation via NADPH oxidase and Sp1-dependent pathway in BV2 murine microglial cells. *Neurosci Lett* 2008;431:155–160. [PubMed: 18164813]
17. Pore N, Jiang Z, Shu HK, Bernhard E, Kao GD, Maity A. Akt1 activation can augment hypoxia-inducible factor-1alpha expression by increasing protein translation through a mammalian target of rapamycin-independent pathway. *Mol Cancer Res* 2006;4:471–479. [PubMed: 16849522]
18. Salceda S, Caro J. Hypoxia-inducible factor 1alpha (HIF-1alpha) protein is rapidly degraded by the ubiquitin-proteasome system under normoxic conditions. Its stabilization by hypoxia depends on redox-induced changes. *J Biol Chem* 1997;272:22642–22647. [PubMed: 9278421]
19. Shen F, Fan Y, Su H, Zhu Y, Chen Y, Liu W, Young WL, Yang GY. Adeno-associated viral vector-mediated hypoxia-regulated VEGF gene transfer promotes angiogenesis following focal cerebral ischemia in mice. *Gene Ther* 2008;15:30–39. [PubMed: 17960159]
20. Vangeison G, Carr D, Federoff HJ, Rempe DA. The good, the bad, and the cell type-specific roles of hypoxia inducible factor-1 alpha in neurons and astrocytes. *J Neurosci* 2008;28:1988–1993. [PubMed: 18287515]

Fig.1. Up-regulation of HIF-1 α and VEGF in cultured neurons following OGD

(A) RT-PCR was used to detect HIF-1 α and VEGF mRNA expression in cortical neurons at 0, 2, 4, 8, 12, 24 h after reperfusion. Total mRNA was isolated from cortical neurons at each time points (n=4). HIF-1 α (175 bp), VEGF164 (563 bp), VEGF120 (431 bp) and β -actin (621 bp) were respectively amplified. β -actin was used for loading control. HIF-1 α mRNA were increased at 2 h after reperfusion, peaked at 4 h, and subsequently declined by 8 h. Two isoforms of VEGF were also peaked at 4 h after reperfusion, then decreased at 8 h. (B) Western blot was used to detect HIF-1 α and VEGF protein expression. Equal amount of protein samples was analyzed with mouse anti-HIF-1 α , rabbit anti-VEGF antibodies. β -actin was used as a loading control. It showed that both proteins of HIF-1 α and VEGF were increased at 4 h, peaked at 8h,

and decreased at 12 h. (C) The relative expression levels of proteins were calculated as relative optical density normalized to β -actin internal controls and represented as mean $\pm$ SD (n=4). (**P < 0.001 vs. normoxia)

Fig.2. Activation of PI3K/Akt pathway in cultured neurons subjected to OGD

(A) Western blot showed that total Akt protein was not changed after OGD, but p-Akt was increased at 4 h, gradually declined at 8 and 12 h, and returned to baseline at 24h. (B) The relative expression levels of proteins were calculated as relative optical density normalized to β -actin internal controls and represented as mean $\pm$ SD (n=4).

(**P < 0.001 vs. normoxia)

Fig.3. Expression of Akt and p-Akt in cultured neurons with wortmannin treatment

(A) Western blot showed that p-Akt but not Akt was significantly decreased at 4 h and 8 h after reperfusion from OGD in wortmannin-treated neurons compared to the DMSO control treated neurons. (B) Akt and p-Akt relative expression levels were calculated as relative optical density normalized to β-actin internal controls and represented as mean ± SD (n=4). (**p<0.001 vs. control) (C) Immunostaining for Akt and p-Akt in OGD neurons with wortmannin treatment. It showed that positive immunoreactions were localized to the cytoplasm of neurons. P-Akt but not total Akt protein was significantly down-regulated in wortmannin-treated neurons compared with DMSO treated neurons. ×400. (W, wortmannin; HI, hypoxia-ischemia).

Fig.4. Expression of HIF-1α and VEGF with wortmannin treatment

(A) RT-PCR showed that HIF-1α (Left) and VEGF (Right) mRNA levels were decreased in wortmannin-treated neurons at 4 h after reperfusion. (B) Western blot showed HIF-1α and VEGF protein were also decreased at 4 h and 8 h after reperfusion in wortmannin-treated neurons compared to DMSO treated neurons (C) The relative expression levels of proteins were calculated as relative optical density using β-actin as internal controls, n=4. (**p<0.001 vs. control) (D) Immunoreactivity for HIF-1α and VEGF was much weaker in wortmannin treated neurons compared to DMSO treated neurons. ×400.

(HI 4h, hypoxia-ischemia after reperfusion at 4h; D, DMSO; W, wortmannin)

Table 1

Expression of HIF-1 α , VEGF, Akt, p-Akt Protein in Cultured Neurons with OGD at Different Times after Reperfusion (n=4)

Time points (h)	Protein (OD)			
	HIF-1 α	VEGF	Akt	p-Akt
N	0.14 $\pm$ 0.01	0.24 $\pm$ 0.03	0.83 $\pm$ 0.10	0.05 $\pm$ 0.01
0	0.16 $\pm$ 0.02	0.26 $\pm$ 0.05	0.83 $\pm$ 0.10	0.06 $\pm$ 0.01
2	0.21 $\pm$ 0.01	0.33 $\pm$ 0.04	0.82 $\pm$ 0.08	0.07 $\pm$ 0.01
4	0.58 $\pm$ 0.03 **	0.65 $\pm$ 0.10 **	0.84 $\pm$ 0.08	0.38 $\pm$ 0.06 **
8	0.72 $\pm$ 0.03 **	0.91 $\pm$ 0.11 **	0.84 $\pm$ 0.10	0.22 $\pm$ 0.02 **
12	0.60 $\pm$ 0.01 **	0.62 $\pm$ 0.10 **	0.84 $\pm$ 0.07	0.18 $\pm$ 0.02 **
24	0.36 $\pm$ 0.04 **	0.59 $\pm$ 0.07 **	0.83 $\pm$ 0.09	0.04 $\pm$ 0.01

OD: Relative optical density; N, normoxia

**
p<0.001 vs. normoxia.

Table 2Expression of Akt, p-Akt, HIF-1 α and VEGF in cultured neurons with wortmannin treatment (n=4)

Time Points (h)	Protein (OD)			
	Akt	p-Akt	HIF-1 α	VEGF
HI4	0.92 $\pm$ 0.18	0.41 $\pm$ 0.02	0.41 $\pm$ 0.09	0.76 $\pm$ 0.06
W/ HI4	0.92 $\pm$ 0.17	0.12 $\pm$ 0.01**	0.16 $\pm$ 0.04**	0.50 $\pm$ 0.05**
HI8	0.90 $\pm$ 0.15	0.27 $\pm$ 0.01	0.54 $\pm$ 0.08	0.84 $\pm$ 0.07
W/ HI8	0.91 $\pm$ 0.17	0.09 $\pm$ 0.01**	0.16 $\pm$ 0.04**	0.51 $\pm$ 0.08**

OD: Relative optical density; HI, hypoxia-ischemia; W, wortmannin

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p<0.001 vs. control.