Upregulation of HIF-1α protein induces mitochondrial autophagy in primary cortical cell cultures through the inhibition of the mTOR pathway

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Received December 30, 2013; Accepted June 26, 2014

DOI: 10.3892/ijmm.2014.1850

Abstract. Cerebral ischemia/reperfusion (I/R) can induce neuronal death, particularly in the hippocampal formation (HF). Molecular genetic studies have suggested that the activities of the transcription factor, hypoxia-inducible factor-1α (HIF-1α), are closely linked to ischemia-induced neuronal death. However, the mechanisms through which HIF-1α functions remain poorly understood. In this study, primary cortical neurons were subjected to oxygen-glucose deprivation (OGD) to establish a cell model of OGD/reperfusion (RP). HIF-1α mRNA and protein expression was measured by qRT-PCR and western blot analysis. Cell proliferation was detected by MTT assay. Flow cytometric analysis was used to detect cell apoptosis and changes in mitochondrial mass. The expression of LC3-I and LC3-II was examined by western blot analysis. We found that HIF-1α increased cell proliferation and decreased cell apoptosis in our cell model of OGD/RP using cultured neonatal rat cortical neurons. The overexpression of HIF-1α significantly induced changes in mitochondrial mass and mitochondrial autophagy in cortical neurons. Moreover, the inhibition of HIF-1α markedly suppressed cell proliferation and mitochondrial autophagy. We also demonstrated that the HIF-1α-induced mitochondrial autophagy was accompanied by the inhibition of the mTOR pathway. This study provides direct *in vitro* evidence that HIF-1α overexpression triggers mitochondrial autophagy, thereby increasing neuronal survival. Our results highlight a novel target molecule toward which anti-ischemic neuroprotective effects can be applied.

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Abbreviations: I/R, cerebral ischemia/reperfusion; HIF-1α, hypoxia-inducible factor-1α; OGD, oxygen-glucose-deprivation; HF, hippocampal formation; siRNA, small interference RNA

Key words: cortical cell culture, oxygen-glucose deprivation, hypoxia-inducible factor-1α, mitochondrial autophagy

Introduction

Ischemic brain injury is an important disorder that threatens human health and life (1). Ischemic brain injury can cause high mortality and is the third leading cause of mortality among Americans (2). In addition to high mortality, ischemic brain injury can also lead to long-term disability (3). Cerebral ischemia/reperfusion (I/R) can induce neuronal death, particularly in the hippocampal formation (HF) (4). Molecular genetic studies have suggested that the activities of the transcription factor, hypoxia-inducible factor-1α (HIF-1α), are closely linked to ischemia-induced neuronal death (5,6). Cerebral ischemia results in low oxygen delivery and a decrease in the adenosine triphosphate (ATP) concentration (7), and the lack of oxygen can lead to the activation of HIF-1. The HIF-1 transcriptional complex plays an important role in the regulation of oxygen homeostasis in mammalian cells (8-10). HIF-1 is a member of the basic helix-loop-helix-Per-Arnt-Sim superfamily, and is composed of an HIF-1α and an HIF-1β subunit (11). HIF-1α is regulated by oxygen levels and determines the level of HIF-1 activity, whereas HIF-1β is constitutively expressed, and its activity is not affected by hypoxia (12,13). HIF-1 can affect many cellular processes, such as energy metabolism, tumor invasion, erythropoiesis, cell migration, angiogenesis and pH regulation, by controling the transcription of hundreds of HIF-mediated cells (14). The constitutive activation of HIF-1 inhibits mitochondrial biogenesis and cellular respiration by the repression of c-Myc activity in von Hippel-Lindau protein-deficient renal carcinoma cells (15). In

addition, HIF-1 can affect tumor cell apoptosis (16). Zhang *et al* indicated that HIF-1 increased hypoxic cell survival under hypoxic conditions (17) and also suggested that HIF-1 activity may be involved in the protection of neuronal cells through the regulation of mitochondrial autophagy (17).

The mitochondria are replaced every 2-4 weeks in the rat liver, kidneys, heart and brain (18). Mitochondrial degradation has been implicated in the irreversible cell damage that may occur during cerebral ischemia and reperfusion (19). The destruction of the mitochondria may be the result of the process of autophagy, in which parts of the cytoplasm are sequestered in autophagosomes. A number of studies have suggested that autophagy, which is induced in the heart under hypoxic or ischemic conditions, plays either a protective or pathogenic role in heart disease (20-22). Moreover, autophagy may induce changes in mitochondrial mass. Mitochondrial mass can change either due to mitochondrial degradation or due to mitochondrial amplification, and it has been suggested that autophagy results in decreased mitochondrial mass through the accelerated mitochondrial degradation (23).

To the best of our knowledge, the present study provides the first direct evidence that the process of mitochondrial autophagy is dependent on the expression of HIF-1 under conditions of cerebral I/R. We demonstrate that mitochondrial autophagy protects neuronal cells, and the effects of HIF-1 on mitochondrial autophagy involve the inhibition of the mTOR signaling pathway.

Materials and methods

Animals. All procedures were carried out according to the protocols approved by the Ethics Committee for Animal Experimentation of the General Hospital of People's Liberation Army Chengdu Military Region (Chengdu, China). The Sprague-Dawley rats (210-240 g) that were used in this study were obtained from the Animal Center of the Institute of Field Surgery of the Third Military Medical University in China.

Primary cortical neuronal culture. Mouse cortical neurons were cultured according to a previously described method (24). Newborn (1-3 days old) Sprague-Dawley rats were anesthetized with halothane and sacrificed by cervical dislocation. The cortices obtained from the Sprague-Dawley rats were suspended in cold D-Hank's (HyClone, Salt Lake City, UT, USA) solution and dissected free of meninges and blood vessels. The cerebral tissues were digested in 0.125% trypsin for 30 min at 37˚C. The cell suspension was centrifuged at 3,000 x g for 10 min at 4˚C, and the precipitate was re-suspended in DMEM/F12 medium (HyClone) supplemented with 20% FBS (HyClone), 100 mg/l streptomycin and 100 kU/l benzylpenicillin. The cells were plated at $1x10^6$/ml on 96-well plates coated with 10 mg/l poly-L-lysine. After 72 h, 5 μg/ml arabinosylcytosine were added to the cells to prevent the growth of non-neuronal cells. Subsequently, the culture medium was replaced with normal medium after 24 h and was refreshed every 2-3 days (25). The cultures typically contained >95% neurons under these conditions.

In vitro simulation of I/R. Oxygen-glucose-deprivation (OGD) was induced according to a previously described method with minor modifications (24,26). Briefly, the cells were rinsed twice with PBS and glucose-free Earl's solution [116.4 mmol/l NaC_l, 5.4 mmol/l KCl, 1.8 mmol/l $CaCl_2$, 0.8 mmol/l $MgSO_4$, 2.6 mmol/l NaH_2PO_4, 26.2 mmol/l $NaHCO_3$ and 20.1 mmol/l HEPES (pH 7.4)] was added and the cells were cultured at 37˚C in an incubator in an atmosphere of 5% CO_2 and 95% N_2 (OGD) for 2 h. OGD was terminated by the replacement of stored medium (Earl's solution, with the addition of 5.6 mmol/l glucose into the glucose-free Earl's solution) and by returning the cultures to an atmosphere of 5% CO_2 and 95% O_2 for another 12 h.

Construction of clone and vector expressing HIF-1α. The Ad-CMV-HIF-1α vector was constructed as described in a previous study (27). Briefly, total RNA was extracted from the cultured OGD-treated neurons using TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. Approximately 5 μg of total RNA was reverse transcribed into cDNA using a PrimeScript RT reagent kit (Takara Bio, Inc., Shiga, Japan). The cDNAs were used as templates for the amplification of HIF-1α using 2 primers: forward, 5'-CGGTACCATGGAGGGCGCCGGCG GCGCG-3' and reverse, 5'-CGCGGCCGCTCAGTTAACTTG ATCCAAAGC-3'. The obtained sequences were fully sequenced (Shanghai Sangon Biotech Co., Ltd., Shanghai, China). The cloned HIF-1α was inserted into a pENTR11 vector with *Kpn*I and *Not*I restriction sites. Ad-CMV-HIF-1α was generated using the ViraPower™ Adenoviral Expression System (Invitrogen Life Technologies) according to the operating protocols. Cells were seeded in 6-well plates ($1.5x10^5$ cells/well) and 100 μl ($5x10^{10}$ IU/ml) Ad-CMV-HIF-1α were added and the cells were incubated at 37˚C with 5% CO_2 for the indicated periods of time.

qRT-PCR. The transcription of HIF-1α was detected using the RT-PCR method as previously described with minor modifications (28). The total RNA of primary neuronal cultures was isolated using TRIzol reagent according to the manufacturer's instructions (Biostar, Shanghai, China) and was then purified using the RNeasy mini kit (Qiagen, Palo Alto, CA, USA). Approximately 2 μg of total RNA was reverse transcribed into first-strand cDNA using a QuantiTect® reverse transcription (RT) kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. qRT-PCR was performed to analyze the levels of mRNA transcripts using specific primers, using the QuantiTect™ SYBR®-Green PCR kit (Qiagen) and a Smart Cycler® 1.2f Detection System (Cepheid, Sunnyvale, CA, USA). The primer sequences used for qRT-PCR were as follows: HIF-1α sense, 5'-CCAGCAGACTCAAATACAAGAACC-3' and antisense, 5'-TGTATGTGGGTAGGAGATGGAGAT-3'; β-actin sense, 5'-AAGCAGGAGTATGACGAGTCCG-3' and antisense, 5'-GCCTTCATACATCTCAAGTTGG-3'. These primers were all synthesized by Shanghai Sangon Biotech Co., Ltd. Cycling conditions were as follows: pre-incubation at 95˚C, 15 min; PCR: 95˚C, 15 sec and 56˚C, 30 sec, 40 cycles; final elongation: 72˚C, 10 min. β-actin was used as an internal control and the expression levels of the relative genes were calculated using the $2^{-\Delta\Delta CT}$ method, as previously described (29).

Western blot analysis. The total protein of the OGD-treated neurons was extracted using RIPA lysis buffer (Beyotime, Nantong, China) according to the manufacturer's instructions. A total of 40 μg of protein per lane was subjected to 10% SDS-PAGE

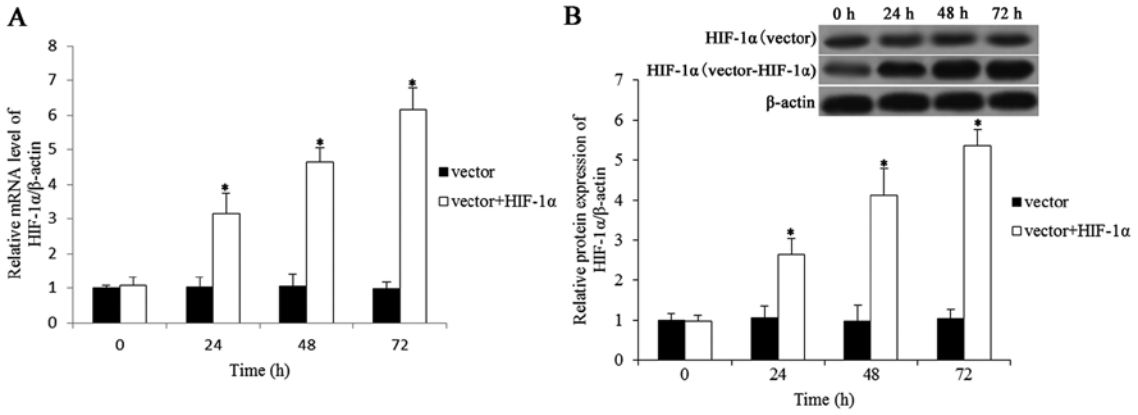

Figure 1. Hypoxia-inducible factor-1α (HIF-1α) mRNA and protein levels increased following transfection with Ad-CMV-HIF-1α. (A) The relative mRNA levels were determined by qRT-PCR and normalized to the housekeeping gene, β-actin. (B) Western blot analysis of HIF-1α expression in the cells transfected with Ad-CMV-HIF-1α. Data are normalized to β-actin values and are expressed as fold changes of β-actin in the cells transfected with Ad-CMV-HIF-1α relative to the control. The expression of HIF-1α in the cells transfected with Ad-CMV-HIF-1α groups was significantly higher compared with the cells transfected with the control vector. All experiments were repeated at least 3 times. Values are shown as the means ± SD (n=3). *P<0.05 vs. control.

and electroblotted onto nitrocellulose membranes (Amersham Pharmacia Biotech, Freiburg, Germany). The immunodetection of HIF-1α, LC3, p70S6 kinase and β-actin was carried out using HIF-1-α antibody (Abcam, Cambridge, MA, USA), LC3 antibody (Abcam), phospho-p70S6 kinase antibody (Thr-389; Cell Signaling Technology, Inc., Danvers, MA, USA) and β-actin antibody (Abcam). Goat anti-rabbit IgG (Abcam) was used as the secondary antibody. The bound antibodies were visualized using LumiGLO® reagent (Pierce Biotechnology, Inc., Rockford, IL, USA). All experiments were repeated 3 times.

Fluorescence-activated cell sorting (FACS) analysis. The analysis of mitochondrial mass was performed using a FACScan flow cytometer (BD Biosciences, San Jose, CA, USA) according to a previously described method with minor modifications. Briefly, the cells were stained with 1 μM dichlorodihydrofluorescein diacetate, 10 nM nonyl acridine orange (NAO), or 1 μM ER-tracker™ Green dye, and then cultured in PBS solution supplemented with 5% fetal bovine serum at 37˚C for 15 min. The stained cells were analyzed using a FACScan flow cytometer. Apoptosis was measured by flow cytometry using an Annexin V-FITC/PI kit (BD Pharmingen, San Diego, CA, USA) according to the manufacturer's instructions.

MTT cell proliferation assay. Cortical neuronal proliferation was measured by MTT assay as previously described (30). Briefly, the cortical neurons were seeded into 96-well culture plates for 24, 48 and 72 h, and then 5 mg/ml MTT (20 μl) were added to the cells at 37˚C for 4 h. A total of 200 μl of DMSO was added to solubilize the crystals. The OD value was measured at a wavelength of 490 nm using a spectrophotometer (Multiskan MK3; Thermo Scientific, Waltham, MA, USA). Data are expressed as the means ± standard error of the mean (SEM). Differences between the treatment groups were analyzed using the Student's t-test and a value of P≤0.05 was considered to indicate a statistically significant difference.

Measurement of ATP levels. ATP levels in the neurons were determined using recombinant firefly luciferase and its substrate, D-luciferin (Invitrogen Life Technologies) according to the manufacturer's instructions and as previously described (31). ATP levels were quantitatively detected, and luminescence was measured using a luminometer (emission maximum at 560 nm).

Transfection with small interfering RNA (siRNA). The neuronal cells expressing the HIF-1α protein were transfected with HIF-1α siRNA (siHIF-1α) or scrambled siRNA (siMock) using Lipofectamine 2000 (Invitrogen Life Technologies) according to the manufacturer's instructions. For the siRNA experiments, the HIF-1α overexpressing cells and the wild-type control cells were seeded on 60-mm plates. The cells were transfected and, after 20 h, were divided into 24-well plates. The cells were analyzed 3 days after transfection. The siRNA oligo sequences for HIF-1α were as previously described (32,33). The siHIF-1α target sequences were: sense, 5'-CUGAUGA CCAGCAACUUGAdTdT-3' and antisense, 5'-UCAAGUUGC UGGUCAUCAGdTdT-3'.

Statistical analysis. All values are expressed as the means ± SEM. Statistical analysis was performed using the Student's t-test. A value of P<0.05 was considered to indicate a statistically significant difference. All experiments were repeated at least 3 times.

Results

HIF-1α levels were increased in neurons transfected with Ad-CMV-HIF-1α. In order to induce the overexpression of HIF-1α in primary cortical neurons, we created an adenoviral vector that encodes HIF-1α under normoxic conditions. The expression of HIF-1α in the cortical neurons was determined by qRT-PCR and western blot analysis. The qRT-PCR results (Fig. 1A) showed that the HIF-1α mRNA levels in the neurons transfected with Ad-CMV-HIF-1α were markedly higher than those of the control cells 24 h later. The protein expression was also determined and the results revealed that the HIF-1α protein levels were upregulated in the neurons transfected with Ad-CMV-HIF-1α (Fig. 1B).

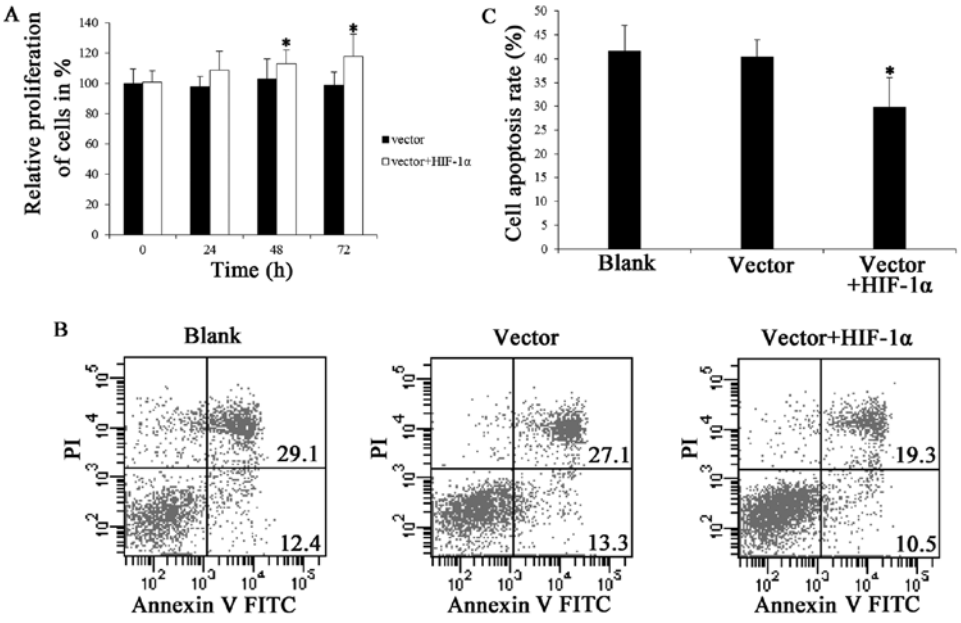

Figure 2. Hypoxia-inducible factor-1α (HIF-1α) overexpression increased cell proliferation and decreased cell apoptosis. (A) HIF-1α overexpression increased cell proliferation in a time-dependent manner. Cell proliferation was determined by MTT assay. Cells were transfected with Ad-CMV-HIF-1α or the control vector for 24, 48 or 72 h. (B) Effect of HIF-1α overexpression on cell apoptosis. Flow cytometric analysis of HIF-1α-overexpressing cells stained with Annexin V and PI. (C) Cell apoptotic rate. Flow cytometric analysis of HIF-1α-overexpressing cells stained with Annexin V and PI. Cells were transfected with Ad-CMV-HIF-1α or the control vector for 72 h. All experiments were repeated at least 3 times. The 'blank' group indicated untreated cells. The 'vector' group indicated cells infected with the empty plasmid packed in adenovirus. Values are shown as the means ± SD (n=3). *P<0.05 vs. control.

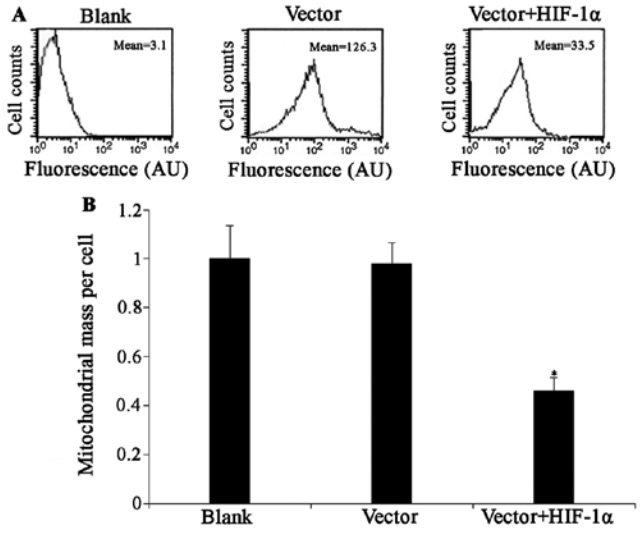

Figure 3. Evaluation of mitochondrial mass in the model of oxygen-glucose-deprivation (OGD)/RP using cultured neonatal rat cortical neurons by fluorescence-activated cell sorting (FACS) analysis. Cells were transfected with hypoxia-inducible factor-1α (HIF-1α)-expressing vector (Ad-CMV-HIF-1α) or with the control vector. (A) FACS analysis. Mitochondria were labeled with MitoTracker Green (MTG), and signals were measured using the FACS analyzer. Cells were analyzed at 72 h after transfection. (B) Summary of mitochondrial mass by FACS analysis. Cells were analyzed at 72 h after transfection. Mean values were converted to arbitrary units (AU) to make the results from different experiments directly comparable with each other. All experiments were repeated at least 3 times. Values are shown as the means ± SD n=3). *P<0.05 vs. control.

Effect of HIF-1α overexpression on neuronal cell proliferation and apoptosis. The effect of HIF-1α overexpression on neuronal cell proliferation was determined by MTT assay. The results indicated that there was an increase in the proliferation of cortical neurons which overexpressed HIF-1α and were subjected to OGD, and the difference was significant following transfection with HIF-1α for 48 h; however, there was no significant difference at 24 h (Fig. 2A). We found that HIF-1α overexpression led to an almost 18% increase in the number of cells. This indicated that HIF-1α exerts positive effects by increasing the number of cells.

It has recently been demonstrated that HIF-1 exerts an anti-apoptotic effect on p53-mediated apoptosis through a secreted neuronal tyrosinase (34). Moreover, HIF-1 can induce erythropoietin production under hypoxic and hypoglycemic conditions, and erythropoietin can prevent neuronal apoptosis following cerebral ischemia and metabolic stress (35,36). Thus, we examined whether the cell increase in our experiments was mediated by apoptosis. Apoptosis was first examined by flow cytometric analysis of the cells stained with Annexin V and PI. The results revealed that HIF-1α overexpression prevented cell apoptosis, and that the apoptotic rate decreased to 29.8% when the cells were transfected with Ad-CMV-HIF-1α for 72 h (Fig. 2B and C).

Effect of HIF-1α overexpression on mitochondrial mass in cortical neurons. The above-mentioned results suggested that HIF-1α overexpression protected neurons against apoptosis; however, the mechanisms involved remain unclear. The constitutive activation of HIF-1 inhibits mitochondrial biogenesis by repressing c-Myc activity in von Hippel-Lindau protein-deficient renal carcinoma cells (15). In addition, Zhang *et al* also suggested that HIF-1 activity may be involved in protecting neuron cells through the regulation of mitochondrial autophagy (17). We first evaluated mitochondrial mass using FACS analysis. Living cells

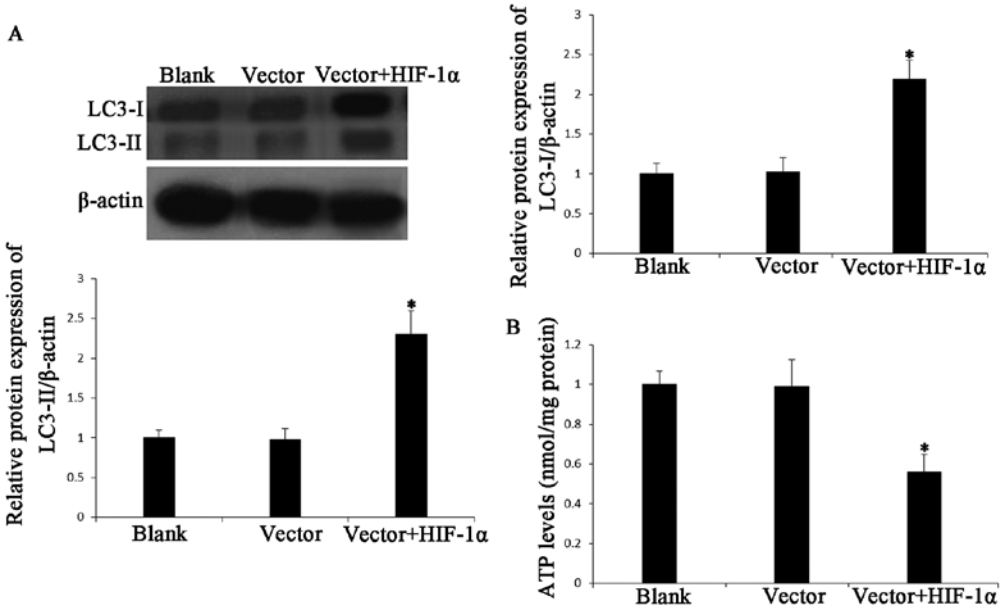

Figure 4. Hypoxia-inducible factor-1α (HIF-1α) overexpression induced mitochondrial autophagy. (A) Levels of LC3 (I and II) were increased after the induction of HIF-1α overexpression. Cells were transfected with HIF-1α vector carrying the HIF-1α fragment or with the control vector. Cells were analyzed at 72 h after transfection. Data were normalized to β-actin values and are expressed as fold changes of β-actin in HIF-1α overexpression relative to the control. (B) ATP levels were decreased after the induction of HIF-1α overexpression. All experiments were repeated at least 3 times. Values are shown as the means ± SD (n=3). *P<0.05 vs. control.

were stained with MitoTracker Green (MTG), a fluorescent vital stain specific for mitochondria (23). The cells were transfected using the HIF-1α adenoviral construct and analyzed at several time points following transfection. We found that the overexpression of HIF-1α for 24 h or longer caused a significant decrease in mitochondrial mass compared with the control group (Fig. 3).

Effect of HIF-1α overexpression on mitochondrial autophagy. Mitochondrial mass can change due to mitochondrial degradation, and autophagy results in a decrease in mitochondrial mass through accelerated mitochondrial degradation (23). We subsequently detected the effect of HIF-1α overexpression on mitochondrial autophagy. Certain studies have demonstrated that LC3 (ATG8 in yeast) plays a central role in the autophagy pathway and promotes the formation of autophagosomes (37,38). LC3 is known to exist in a soluble form, termed LC3-I and a lipidated form termed, LC3-II that is associated with autophagosomal membranes (39). LC3 levels in the cortical neurons following the induction of HIF-1α overexpression were detected by western blot analysis. The levels of LC3-I and LC3-II were significantly increased in the cortical neurons following the induction of the overexpression of HIF-1α for 24 h (data not shown), and maximally at 72 h (Fig. 4A). Certain studies have shown that mitochondrial autophagy is associated with a change in the ATP levels (17,23). Thus, we also determined whether HIF-1α regulates ATP levels. The overexpression of HIF-1α for 24, 48 (data not shown) and 72 h led to significantly decreased ATP levels (Fig. 4B). These results are consistent with the above-mentioned findings that HIF-1α leads to mitochondrial degradation.

Specific inhibition of HIF-1α mRNA and protein expression by HIF-1α-specific siRNA. The above-mentioned experiments were designed to examine the effect of the overexpression of HIF-1α in cortical neurons. We subsequently employed a complementary approach using RNA interference technology and examined the effects of suppressing the levels of HIF-1α in the HIF-1α-overexpressing cells. The cells were transfected with siHIF-1α or control siRNA (siMock) and were analyzed after 2 days of growth. Compared with the siMock-transfected cells, the levels of HIF-1α in the cells transfected with siHIF-1α were significantly decreased (Fig. 5A and B). We then determined the effect of HIF-1α silencing on cell proliferation and mitochondrial autophagy. As shown in Fig. 5C, there was a decrease in the growth of the siHIF-1α-transfected cells compared with the siMock-transfected cells. Moreover, the LC3 protein levels were also decreased in the siHIF-1α-transfected cells (Fig. 5D). These results indicated that the increase in cell growth and the activity of mitochondrial autophagy in cortical neurons was associated with the overexpression of HIF-1α.

Overexpression of HIF-1α is directly related to the inhibition of mTOR. There is evidence that the induction of autophagy is mediated by the inhibition of the mTOR pathway (40). Since HIF-1α induces autophagy, we examined whether it also inhibits mTOR signaling. The expression of p70S6 kinase was detected by western blot analysis. p70S6 kinase expression was significantly increased in the siHIF-1α-transfected cells compared with siMock group (Fig. 6). These results demonstrated that HIF-1α overexpression inhibited the mTOR pathway.

Discussion

The main findings of the present study were the following: i) the overexpression of HIF-1α improves cell survival in the OGD/RP model using cultured neonatal rat cortical neurons; ii) the overexpression of HIF-1α significantly induces mitochondrial autophagy and degradation in the OGD/RP model

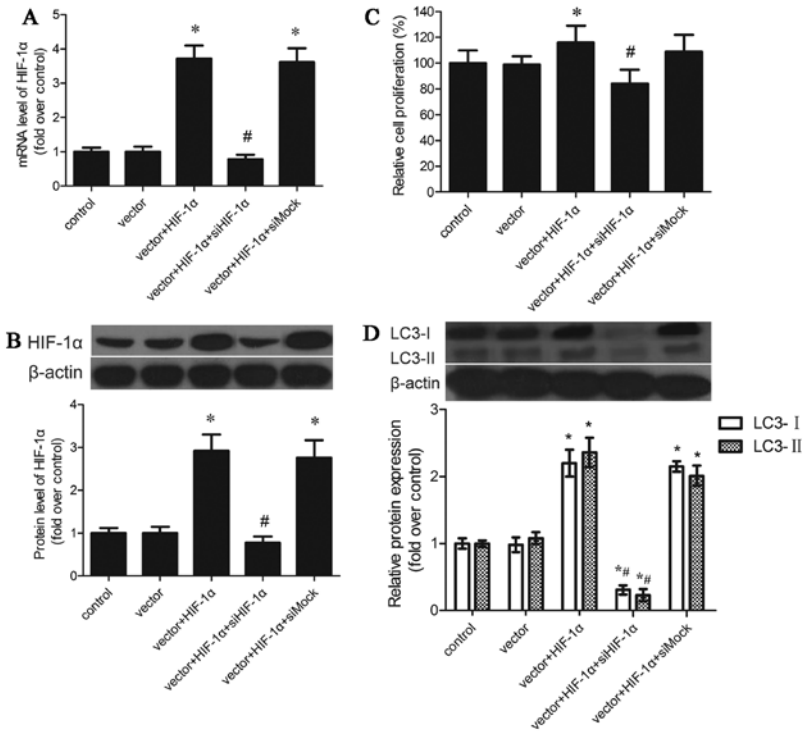

Figure 5. Effect of HIF-1α siRNA (siHIF-1α)-transfection on cell proliferation and mitochondrial autophagy. (A) The proliferation of cells transfected with siHIF-1α or scrambled siRNA (siMock) cells after the induction of hypoxia-inducible factor-1α (HIF-1α) overexpression. (B) Western blot analysis of LC3-I and LC3-II expression. LC3-I and LC3-II expression levels were normalized to β-actin. The 'control group' indicates wild-type untreated cells. The 'vector' group indicates cells infected with the empty plasmid packed in adenovirus. All experiments were repeated at least 3 times. Values are shown as the means ± SD (n=3). *P<0.05 vs. control; #P<0.05 vs. vector + HIF-1α.

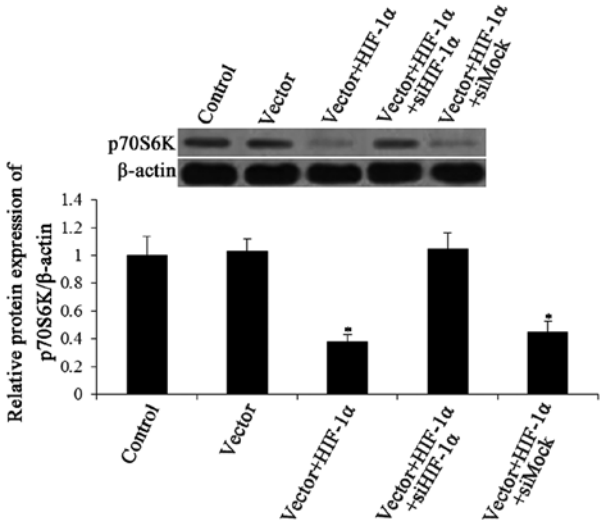

Figure 6. Hypoxia-inducible factor-1α (HIF-1α) overexpression inhibits mTOR activation. The expression of p70S6 kinase (p70S6K) was detected by western blot analysis. The expression of p70S6K was markedly reduced in the HIF-1α-overexpressing cells compared with the control cells, whereas the p70S6K level was significantly increased after HIF-1α silencing. Data were normalized to β-actin values and expressed as fold changes of β-actin. All experiments were repeated at least 3 times. Values are shown as the means ± SD (n=3). *P<0.05 vs. control.

using cultured neonatal rat cortical neurons; iii) the inhibition of HIF-1α markedly suppresses mitochondrial autophagy in rat cortical neurons; and iv) the overexpression of HIF-1α induced mitochondrial autophagy and this was accompanied by the inhibition of the mTOR pathway. To the best of our knowledge, these data demonstrate for the first time that the neuroprotective effects of the overexpression of HIF-1α in a model of OGD/RP using cultured neonatal rat cortical neurons involve an increase in mitochondrial autophagy. Thus, the results provide insight into the role of HIF-1α in cerebral I/R injury.

In the present study, we analyzed the effects of HIF-1α overexpression. HIF-1 is a major regulator of oxygen homeostasis (10). It is composed of 2 subunits, an α and a β subunit, but the regulation of HIF-1 activity mostly depends on the α subunit. The upregulation of HIF-1 and the increased HIF-1 binding activity have been shown to protect astrocytes from ischemic injury during 6 h of hyperthermia (38 or 40˚C) (41). Moreover, the upregulation of HIF-1α and the increased binding activity of HIF-1 are associated with the neuroprotective effects induced by hypoxia/ischemia pre-conditioning (42). The present study demonstrates that the overexpression of HIF-1α improves cell survival in a model of OGD/RP using cultured neonatal rat cortical neurons. We found that a high level of HIF-1α is associated with an increase in cell proliferation and a decrease in cell apoptosis. These data suggest that the upregulation of the level of HIF-1α protects cortical neurons from ischemia-reperfusion injury.

The present study also demonstrates that the overexpression of HIF-1α significantly induces changes in mitochondrial mass in cortical neurons. Changes in mitochondrial mass may be due to mitochondrial degradation, and autophagy results in a decrease in the mitochondrial mass through accelerated mitochondrial degradation (23). Thus, we also examined the

effect of HIF-1α overexpression on mitochondrial autophagy. LC3 (ATG8 in yeast) is a well-accepted marker of increased autophagy, plays a central role in the autophagy pathway and can promote the formation of autophagosomes (37,38). Our study demonstrated that HIF-1α overexpression is followed by increased LC3 protein levels, particularly LC3-II. Our data indicated that HIF-1α overexpression induced mitochondrial autophagy. The present study also demonstrated that the inhibition of HIF-1α expression markedly suppressed cortical neuron survival and mitochondrial autophagy. A previous study demonstrated that mitochondrial autophagy is dependent on the level of HIF-1 (17). We found that siHIF-1α suppressed cell survival and increased the apoptotic rate. Moreover, we demonstrated that HIF-1α silencing also inhibited mitochondrial autophagy. In addition, we found that HIF-1α induced mitochondrial autophagy through the inhibition of the mTOR pathway.

In conclusion, the present results indicate that HIF-1α overexpression increases cell survival in a model of OGD/RP using cultured neonatal rat cortical neurons. Moreover, HIF-1α overexpression increased mitochondrial autophagy. The inhibition of the expression of HIF-1α suppressed cell survival and decreased mitochondrial autophagy. It is, therefore, conceivable that HIF-1α targeting may have detrimental effects on cortical neurons subjected to OGD/RP. More importantly, our findings raise the possibility that HIF-1α may serve as a novel regulator of cerebral I/R injury.

Acknowledgements

This study was supported by the Project of General Hospital of the People's Liberation Army Chengdu Military Region (2013YG-A010).

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