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Hypoxia-induced iNOS expression in microglia is regulated by the PI3-kinase/Akt/mTOR signaling pathway and activation of hypoxia inducible factor-1α

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Abbreviations:
CNS, central nervous system
HIF-1α, hypoxia inducible factor-1alpha
IL-1, interleukin-1
iNOS, inducible nitric oxide synthase
LY294002, 2-(4-morpholinyl)-8-henyl-1(4H)-benzopyran-4-one
mTOR, mammalian target of rapamycin
NO, nitric oxide
PD980509, 2-(2-amino-3-methoxyphenyl)-4H-1-benzopyran-4-one
PI3-kinase, phosphatidylinositol 3-kinase
ROS, reactive oxygen species

ABSTRACT

Exposure to hypoxia induced microglia activation and animal studies have shown that neuronal cell death is correlated with microglial activation following cerebral ischemia. Thus, it is likely that toxic inflammatory mediators produced by activated microglia under hypoxic conditions may exacerbate neuronal injury following cerebral ischemia. The hypoxia-inducible factor-1 (HIF-1) is primarily involved in the sensing and adapting of cells to changes in the O_2 level, which is regulated by many physiological functions.

However, the role of HIF-1 in microglia activation under hypoxia has not yet been defined. In the current work, we investigate the signaling pathways of HIF-1α involved in the regulation of hypoxia-induced overexpression of inducible NO synthase (iNOS) in microglia. Exposure of primary rat microglial cultures as well as established microglial cell line BV-2 to hypoxia induced the expression of iNOS, indicating that hypoxia could lead to the inflammatory activation of microglia. iNOS induction was accompanied with NO production. Moreover, the molecular analysis of these events indicated that iNOS expression was regulated by the phosphatidylinositol 3-kinase (PI3-kinase)/AKT/ mammalian target of rapamycin (mTOR) signaling pathway and activation of hypoxia inducible factor-1α (HIF-1α). Thus, during cerebral ischemia, hypoxia may not only directly damage neurons, but also promote neuronal injury indirectly via microglia activation. In this study, we demonstrated that hypoxia induced iNOS expression by regulation of HIF-1α in microglia.

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SB203580, 4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)-1H-imidazole
TNF-α, tumor necrosis factor-alpha

1. Introduction

HIF-1 is a heterodimeric transcription factor composed of the basic helix–loop–helix-PAS-domain, containing the proteins HIF-1α and arylhydrocarbon receptor nuclear translocator (ARNT, HIF-1β) [1]. The availability of HIF-1 is determined primarily by HIF-1α, which is regulated at the protein level in an oxygen-sensitive manner, in contrast to HIF-1β, which is stably expressed [2,3]. During normoxia, HIF-1α is efficiently degraded through the VHL-dependent ubiquitin-proteasome pathway [3]. Under hypoxia, HIF-1α protein is markedly stabilized, translocates to the nucleus, heterodimerizes with HIF-1β, and activates gene transcription of VEGF, EPO, iNOS, and glycolytic enzymes that enhance cellular adaptation to hypoxia [4]. In the brain, HIF-1α expression seems to be induced by hypoxia in neurons, astrocytes, ependymal cells and possibly endothelial cells [5,6]. HIF-1 expression in microglia and oligodendrocytes awaits further study [4].

Microglia, the resident histiocytes of the CNS, are ontogenetically related to the mononuclear-phagocyte lineage and represent about 20% of the glial population in brain. After an insult, the ramified morphology of resting microglia is transformed into ''reactive microglia''. Activated microglia initially serve to phagocytize necrotic debris allowing for the eventual healing of the injured brain. Under pathological conditions, activated microglia have been implicated as the predominant cell type governing inflammation-mediated neuronal damage. Activated microglia exert cytotoxic effects by releasing inflammatory mediators, such as arachidonic acid metabolites, interleukin-1 (IL-1), nitric oxide (NO), reactive oxygen species (ROS), and tumor necrosis factor-alpha (TNF-α) [7,8]. Although these immunotoxic factors are necessary for normal function, the microglia response must be tightly regulated to avoid overactivation and disastrous neurotoxic consequences [9]. Overactivation of microglial cells may cause severe brain tissue damage in various neurodegenerative diseases [10]. Glial activation involves changes in cell phenotype and the expression of new proteins, such as the inducible isoform of nitric oxide synthase (iNOS). The mechanism by which activated glia induce neuronal death has been shown to involve NO [11,12], ROS and proinflammatory cytokines [13,14]. Glia become activated and express iNOS in response to hypoxia [15,16]. NO from iNOS is thought to mediate postischemic neuronal damage, as inhibitors of iNOS reduce the infarct volume [17,18] and iNOS knockout mice are strongly protected against ischemia-induced brain injury [19]. Systemic infection, inflammation, or prior strokes are known to be strong risk factors for subsequent strokes [20], suggesting the possibility that inflammation sensitizes the ischemia response.

In the present study, we investigated the effect and action mechanism of hypoxia on iNOS expression in microglia. Our results reveal that hypoxia-induced iNOS expression in microlgia is HIF-1α-dependent and involves the activation of PI3-kinase/Akt/mTOR signaling pathway.

2. Materials and methods

2.1. Materials

LY294002, PD98059, rapamycin, SB203580, and wortmannin were purchased from Sigma–Aldrich (St. Louis, MO). pHRE-luciferase was kindly provided by Dr. M.-L. Kuo (National Taiwan University, Taipei, Taiwan).

2.2. Microglia culture

Rat primary microglia were isolated from the glial cultures that were prepared from neonatal rats. Briefly, glial cells were cultured in 75 cm^2 flasks for 14 days in Dulbecco's modified Eagle's medium/F12 (DMEM/F12; Gibco, Grand Island, NY, USA) supplemented with 10% fetal calf serum (FCS; Hyclone, Logan, UT, USA), 100 U/ml penicillin and 100 mg/ml streptomycin. To separate microglia, flasks were shaken for 2 h at 180 rpm in a rotary shaker at 37 °C. Detached cells were plated into 24-well plates at a density of 2×10^5 cells/well. The purity of microglia cultures was assessed using CD11b antibody and more than 90% of cells stained positively. Cells were cultured for 2 days before treatment.

The murine BV-2 cell line was cultured in Dulbecco's modified Eagle medium (DMEM; Gibco, Grand Island, NY, USA) with 10% FCS at 37 °C in a humidified incubator under 5% CO_2 and 95% air. Confluent cultures were passaged by trypsinization.

2.3. Hypoxia and reoxygenation

For the generation of hypoxic condition, cells were carried out in serum-free DMEM and gassed with 95% N_2, and 5% CO_2 (Anaerobic System PROOX model 110; BioSpherix) and incubated at 37 °C within the chamber for different time intervals as described in Section 3. To reoxygenate hypoxic cultures, cells were transferred into a regular normoxic incubator (95% air, 5% CO_2) and incubated for further 24 h. Cell viability was only slightly reduced after exposure to hypoxia for 8 h.

2.4. Western blotting

BV-2 cells were plated on 6-well plates. Cells were subjected to hypoxia with or without drugs for 8 h (4 h for the detection of HIF-1α and phosphorylated Akt) and then washed with cold PBS, lysed for 30 min on ice with radioimmunoprecipitation assay buffer (RIPA buffer). Protein samples containing 30 μg

protein were separated on 8% sodium dodecyl sulphate-polyacrylamide gels and transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA, USA). The membranes were incubated for 1 h with 4% dry skim milk in PBS buffer to block nonspecific binding. The membranes were then incubated with rabbit antibodies against iNOS (1:1000; BD Transduction Lab, Lexington, KY), COX-2 (1:1000; Cayman Chemicals, Ann Arbor, MI), phosphoAkt (serine473; Santa Cruz Biotechnology) and phosphomTOR (serine2448; Cell Signaling Technology, MA) or mouse antibodies against HIF-1α (1:1000; Novus Biologicals, Littleton, CO, USA), α-tubulin and nucleolin (1:1000; Santa Cruz Biotechnology). The membranes were then incubated with goat anti-rabbit or anti-mouse peroxidase-conjugated secondary antibody (1:1000; Santa Cruz Biotechnology) for 1 h. The blots were visualized by enhanced chemiluminescence (ECL; Santa Cruz Biotechnology) using Kodak X-OMAT LS film (Eastman Kodak, Rochester, NY).

For the study of HIF-1α translocation, cells were rinsed with PBS and suspended in hypotonic buffer A (10 mM HEPES, pH 7.6, 10 mM KCl, 1 mM DTT, 0.1 mM EDTA, and 0.5 mM phenylmethylsulfonyl fluoride) for 10 min on ice and vortexed for 10 s. The lysates were separated into cytosolic and nuclear fractions by centrifugation at 12,000 × *g* for 2 min. The supernatants containing cytosolic proteins were collected. A pellet containing nuclei was resuspended in buffer C (20 mM HEPES, pH 7.6, 1 mM EDTA, 1 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride, 25% glycerol, and 0.4 M NaCl) for 30 min on ice. The supernatants containing nuclei proteins were collected by centrifugation at 12,000 × *g* for 20 min and stored at −70 °C. All protein concentration was determined by colorimetric assay using Bio-Rad assay kit (Bio-Rad, Hercules, CA).

Equal amounts (40 μg) of each protein from cytosolic or nuclei fractions were separated by 8% polyacrylamide-SDS gel and then electrotransferred to PVDF membranes. The blocked membranes were incubated overnight at room temperature with mouse anti-HIF-1α antibody. After washing with PBS, the blots were incubated for 1 h at room temperature with secondary antibody.

2.5. RT-PCR

Total RNA was extracted from BV-2 microglia using a TRIzol kit (MDBio, Inc., Taipei, Taiwan). Five micrograms of RNA from BV-2 cells were used for reverse transcriptase-polymerase chain reaction (RT-PCR) by using a commercial kit (Invitrogen; Carlsbad, CA). PCR was performed using an initial step of denaturation (5 min at 94 °C), 28 cycles of amplification (94 °C for 1 min, 58 °C for 30 s, and 68 °C for 30 s), and an extension (68 °C for 2 min). PCR products were analyzed on 2% agarose gels. The mRNA of GAPDH served as the internal control for sample loading and mRNA integrity. HIF-1α mRNA levels were normalized to levels of GAPDH. The oligonucleotide primers were: HIF-1α: 5′-TGAGGCTCACCATCAGTTAT-3′ and 5′-TAACCCCATGTATTTGTTC-3′; GAPDH: 5′-ACCACAGTCCATGCCATCAC-3′ and 5′-TCCACCACCCTGTTGCTGTA-3′.

2.6. Real-time PCR

Real-time PCR was carried out using Taqman one-step PCR Master Mix and analyzed with a model 7900 Sequence Detector System (Applied Biosystems. Foster City, CA). Quantitative PCR was performed for iNOS mRNA detection. β-Actin mRNA amplicon was used as an internal control for quantitation of the total amount of cDNA. The amplification reaction mixture (25 μl) contained 100 ng of the cDNA sample, PCR reaction buffer, 300 nM of each primer, and 200 nM TaqMan probe (Applied Biosystems). The thermal cycling conditions included 2 min at 50 °C and 10 min at 95 °C. Thermal cycling proceeded with 40 cycles of 95 °C for 15 s and 60 °C for 1 min. The threshold was set above the nontemplate control background and within the linear phase of target gene amplification to calculate the cycle number at which the transcript was detected (denoted as C_T).

2.7. Transfection and transcriptional activity assay

BV-2 microglia were co-transfected with 0.8 μg pHRE-luciferase plasmid, 0.4 μg β-galactosidase expression vector and either the dominant-negative mutants (DN), including DN-p85 (△p85; deletion of residues 479-513 from p85), DN-Akt (Akt K179A) (a gift from RH Chen, National Taiwan University, Taipei, Taiwan) or DN-HIF-1α (HIF-1α 3′ basic domain deleted) (American Type Culture Collection; Manassas, VA). BV-2 microglia were grown to 80% confluent in 6 well plates and were transfected on the following day by Lipofectamine 2000 (10 μg/ml) (LF2000; Invitrogen). DNA and LF2000 were premixed for 20 min and then applied to the cells (1 ml/well). DMEM containing 20% FCS (1 ml) was added 4 h later. After 24 h transfection, the cells were replaced with fresh serum-free DMEM medium and exposed to hypoxia for another 8 h. To prepare lysates, 100 μl reporter lysis buffer (Promega, Madison, WI) was added to each well, and cells were scraped from dishes. The supernatant was collected after centrifugation at 13,000 rpm for 2 min. Aliquots of cell lysates (20 μl) containing equal amounts of protein (20–30 μg) were placed into wells of an opaque black 96-well microplate. The luciferase activity was determined using a dual-luciferase reporter assay system (Promega) and activity value was normalized to transfection efficiency monitored by the cotransfected β-galactosidase expression vector.

2.8. Electrophoretic mobility shift assay (EMSA)

The following oligonucleotide sequence to what HIF should bind was used for electrophoretic mobility shift assay: 5′-GTGACTACGTGCTGCC TAG-3′ [21]. The consensus oligonucleotide probes were end-labeled with [γ-^{32}P] ATP according to the manufacturer's description. For the binding reaction, 2 ng labeled oligonucleotide (approximately 20,000 cpm) and 2 μg poly(dIdC) (Amersham Biosciences) carrier were incubated with 10 μg nuclear protein in a binding buffer (10 mM HEPES, 60 mM KCl, 1 mM DTT, 1 mM EDTA, and 7% glycerol, pH 7.6) for 30 min at room temperature. Protein-DNA complexes were separated by electrophoresis on a nondenaturing 6% polyacrylamide gel and visualized by autoradiography.

2.9. Assay of nitric oxide

BV-2 cells (2×10^5 cells in 500 μl/well) were exposed to hypoxia-reoxygenation in 24-well plates. Production of NO

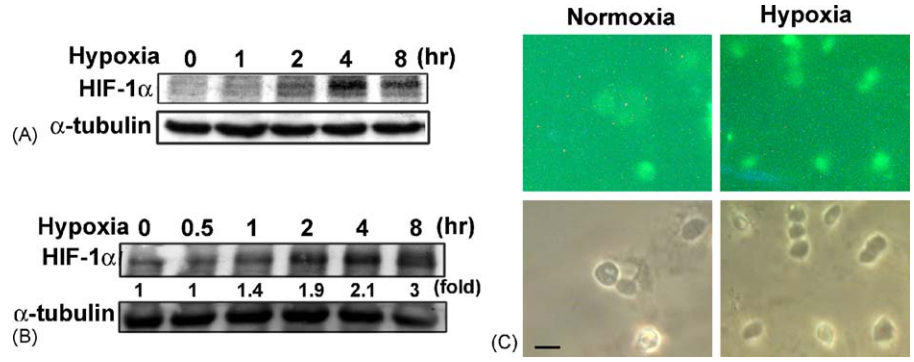

Fig. 1 – Hypoxia enhances HIF-1α expression in microglia. Primary microglia culture (A) or BV-2 cell line (B) were exposed to hypoxia for indicated time intervals. Cellular extracts were prepared for the determination of protein levels of HIF-1α and normalized to α-tubulin. Compared with normoxia, hypoxia increased the protein levels of HIF-1α in a time-dependent manner. (C) HIF-1α immunofluorescence increased in response to hypoxia (upper panels). Phase-contrast images are shown in lower panels. Scale bar, 10 μm. Data are the representative of three independent experiments.

was assayed by measuring the stable metabolite of nitrite levels in culture medium. Sample aliquots (100 μl) were mixed with 100 μl Griess reagent (1% sulfanilamide/0.1% naphthylethylene diamine dihydrochloride/2% phosphoric acid) in 24-well plate and incubated at 25 °C for 10 min. The absorbance at 550 nm was measured on a microplate reader.

2.10. *Statistical evaluation*

Values are expressed as the mean ± S.E.M. of at least three experiments. Student's *t* test was used to assess the statistical significance of the differences; $p < 0.05$ was considered statistically significant.

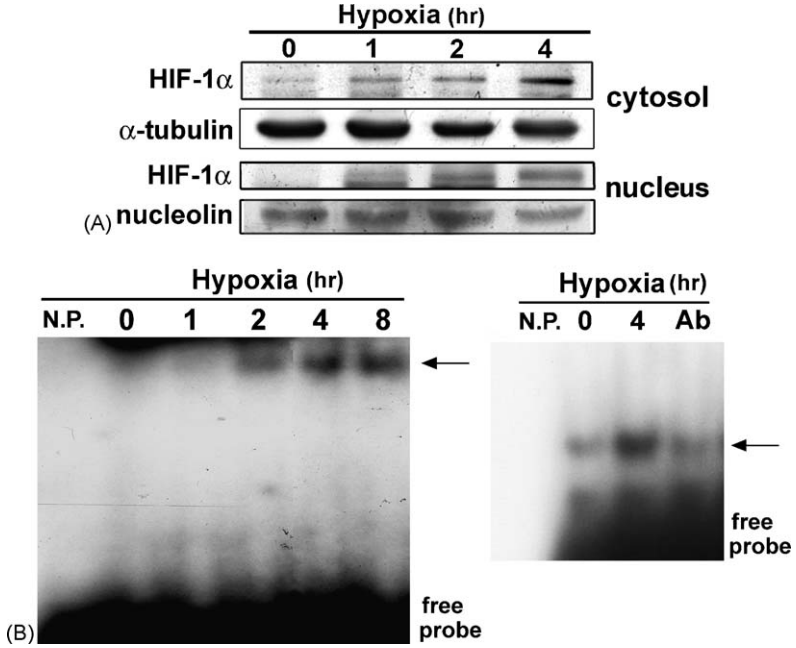

Fig. 2 – Hypoxia promotes HIF-1α translocation into nucleus and binding to HRE binding site. (A) Cytosolic and nuclear fractions were prepared from BV-2 cells exposed to hypoxia for various time intervals. Cytosolic and nuclear fractions were then subjected to Western blot analysis. α-Tubulin and nucleolin were used as the internal control for cytosolic and nuclear fractions, respectively. Note that hypoxia-induced HIF-1α accumulation in cytosol and translocation into nucleus is time-dependent. (B) Nuclear extracts from BV-2 microglia exposed to hypoxia for various time intervals, incubated with or without HRE probe and then analyzed by EMSA. The HIF-1α-specific complex is indicated by an arrow. Results are the representative of at least three independent experiments. Preincubation of HIF-1α antibody (Ab, 5 μg) with nuclear extracts isolated from cells exposed to 4 h hypoxia abolished the oligonucleotide binding (right panel).

3. Results

3.1. *Hypoxia enhances HIF-1α expression and nuclear translocation in microglia*

As shown in Fig. 1, primary microglia culture (Fig. 1A) and BV-2 cells (Fig. 1B) were exposed to hypoxia for various time intervals. Up-regulation of HIF-1α by hypoxia was observed both in primary culture and BV-2 microglia. HIF-1α expression reached peak earlierly in primary cultured than that in cell line (4 and 8 h for primary culture and cell line, respectively). In addition, immunofluorescent staining revealed an increase of HIF-1α in hypoxia-exposed cells in comparison with normoxic condition (Fig. 1C). As shown in Fig. 2A, hypoxia enhanced the accumulation of HIF-1α in cytosol and the translocation into the nucleus in a time-dependent manner. We then performed EMSA to examine the binding of HIF-1α to iNOS-HRE. EMSA was performed with nuclear extract prepared from BV-2 microglia exposed to hypoxia, using a radiolabeled probe of 19-base pair oligonucleotide containing the HIF-1 binding site of the iNOS promoter. As shown in Fig. 2B, nuclear extracts obtained from hypoxia-exposed BV-2 microglia increased the DNA binding of HIF-1α, which was dependent on hypoxia duration. HIF-1 binding to the probe was initially detected at 2 h after exposure to hypoxia, and reached peak at 8 h. There was no any detectable radioactivity without probe (N.P.). In addition, preincubation of HIF-1α antibody (5 μg) with nuclear extracts isolated from cells exposed to 4 h hypoxia abolished the oligonucleotide binding (Fig. 2B, right panel), suggesting the selectivity of HIF-1α-probe interaction. Based upon above findings, we suggest that hypoxia increased the stability of HIF protein and thus the nuclear HIF-1 binding activity of iNOS-HRE. We then examined whether hypoxia up-regulated HIF-1α protein in microglia via the increase of mRNA level. Our results confirmed the previous studies [3,22] that hypoxia did not affect the mRNA level of HIF-1α (Fig. 3). Therefore, the up-regulation of HIF-1α seems to occur mainly at the protein level by the stabilization of HIF-1α.

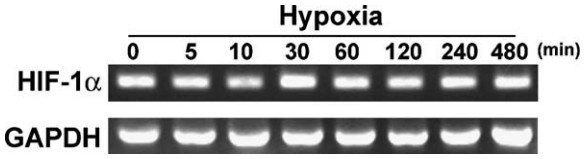

Fig. 3 – Hypoxia does not affect the mRNA expression of HIF-1α. BV-2 microglia were exposed to hypoxia for various time periods for the examination of the mRNA levels of HIF-1α by RT-PCR. Results are representative of at least three independent experiments.

3.2. *Hypoxia enhances iNOS expression in the primary cultured microglia and BV-2 cell line*

Both primary microglia cultures (Fig. 4A) and BV-2 cells (Fig. 4B) displayed an increase of iNOS expression in a time-dependent manner following the exposure to hypoxia. The induction of iNOS mRNA in BV-2 microglia after exposure to hypoxia was assessed by real time-PCR. As shown in Fig. 4C, iNOS mRNA induction was increased time-dependently when microglia incubated in hypoxia condition. We then transfected the microglia with dominant-negative mutant of HIF-1α (DN-HIF-1α) for 24 h and it was found that iNOS protein induction was inhibited after 8 h exposure to hypoxia compared with empty vector's transfection. These results suggest that hypoxia-induced iNOS expression is HIF-1α-dependent.

3.3. *Role of PI3-kinase/Akt/mTOR activation in hypoxia-induced iNOS expression*

To examine whether PI3-kinase signaling pathway is involved in hypoxia-induced iNOS up-regulation, primary cultured microglia (Fig. 5A and C) and BV-2 cell line (Fig. 5B and D) were exposed to hypoxia and the total cellular protein extracts were used to determine the levels of phosphoAkt in Ser473 and

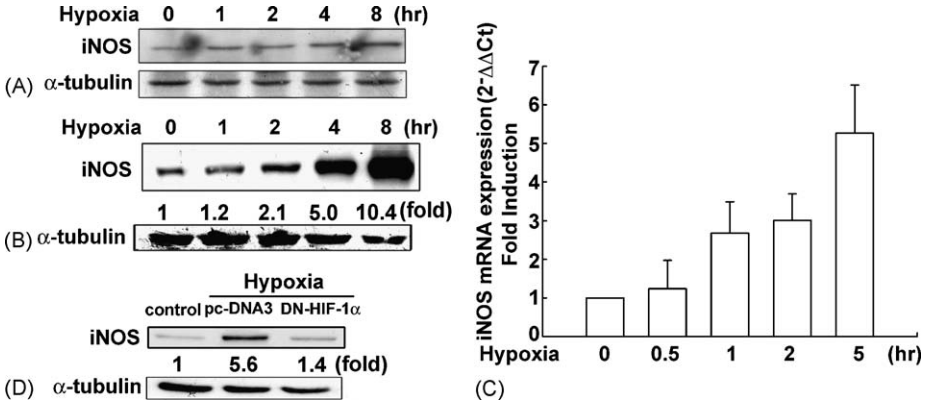

Fig. 4 – Hypoxia enhances iNOS expression in microglia. Primary microglia culture (A) and BV-2 cell line (B) were exposed to hypoxia as indicated time intervals. Cell lysate were used to examine the protein level of iNOS by Western blotting, and the results were normalized to α-tubulin. C, BV-2 microglia cells were exposed to hypoxia for various time intervals, and total cellular mRNA was extracted and subjected to real-time PCR. β-Actin mRNA levels were used as an internal control. (D) BV-2 microglia cells were transfected with empty vector (pcDNA3) or DN-HIF-1α for 24 h followed by exposure to hypoxia for 8 h. Note that the induction of iNOS was inhibited by DN-HIF-1α transfection.

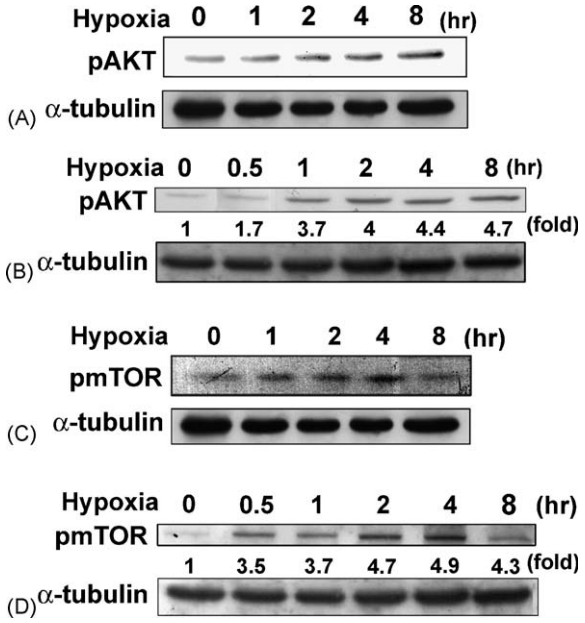

Fig. 5 – Hypoxia potentiates the activation of Akt and mTOR in microglia. Primary microglia culture (A and C) and BV-2 cell line (B and D) were exposed to hypoxia as indicated time intervals. The total protein extracts were prepared and subjected to Western blotting using antibodies specific for pAkt (Ser473) (A and B) or pmTOR (Ser2448). Compared with control, hypoxia increased the activation of Akt and mTOR in a time-dependent manner.

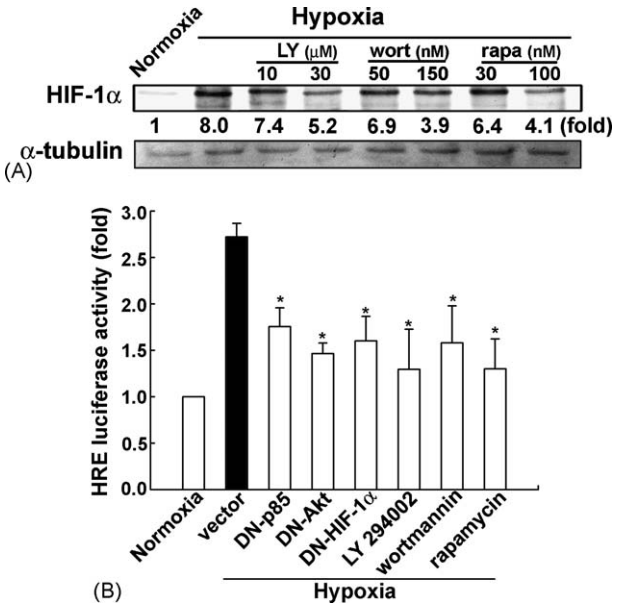

Fig. 6 – Involvement of PI3-kinase/Akt/mTOR pathway in hypoxia-induced HIF-1α accumulation in microglia. (A) BV-2 microglia were pretreated with LY294002, wortmannin or rapamycin at indicated concentrations for 30 min and then exposed to hypoxia for 4 h. Cells were lysed for measuring the protein level of HIF-1α by Western blotting, and the results were normalized to α-tubulin. All three inhibitors reduced hypoxia-induced HIF-1α expression. (B) BV-2 microglia cells were co-transfected with HRE-luciferase reporter gene, SV-β-galactosidase vector and empty vector pc-DNA3 or DN-Akt, DN-p85, DN-HIF-1α for 24 h followed by exposure to hypoxia for 8 h. Luciferase activity was normalized to the transfection efficiency with β-galactosidase expression vector. The data represent the mean ± S.E.M. from at least three independent experiments. $^{*}p < 0.05$ as compared with hypoxia alone.

phosphomTOR in Ser2448 using immunoblotting. It was found that hypoxia-induced both Akt and mTOR phosphorylation in a time-dependent manner. We then examined whether PI3-kinase/Akt/mTOR pathway is involved in hypoxia-induced enhancement of HIF-1 activity by using pHRE-Luc reporter plasmids. The cells were transiently transfected with the reporter plasmids and were subjected to hypoxic conditions for 8 h in the presence or absence of inhibitors. Cell lysates were then prepared, and the luciferase activities were analyzed. As shown in Fig. 6A, HIF-1α was significantly increased when the cells exposed to hypoxic conditions, whereas, treatmeat of PI3-kinase inhibitors of LY294002 and wortmannin or mTOR inhibitor rapamycin significantly inhibited hypoxia-enhanced the expression of HIF-1α in both protein levels (Fig. 6A) and transcriptional activity (Fig. 6B). Furthermore, we determined the involvement of PI3-kinase/Akt/mTOR pathway in HIF-1α enhancement by co-transfection of dominant-negative mutants of PI3-kinase (DN-p85), Akt (DN-Akt) or HIF-1α (DN-HIF-1α). As shown in Fig. 6B, hypoxia-enhanced HRE promoter activity was also reduced by DN-Akt, DN-p85 or DN-HIF-1α. These results suggest that HIF-1α expression in hypoxia was regulated by PI3-kinase/Akt/mTOR signaling pathway. We further examined the role of PI3-kinase/Akt/mTOR in hypoxia-induced iNOS expression. Pretreatment with PI3-kinase inhibitors of LY294002 and wortmannin or mTOR inhibitor rapamycin inhibited hypoxia-induced iNOS expression in a concentration-dependent manner (Fig. 7A). Transfection of BV-2 microglia with the DN-p85 or DN-Akt for 24 h also inhibited hypoxia (8 h)-induced iNOS up-regulation (Fig. 7B). Furthermore, we determined whether hypoxia-induced production of NO was affected by the same signaling pathway in microglia. BV-2 cells were exposed to hypoxia for 8 h continued with reoxygenation for 24 h, the amount of NO produced in supernatant was measured. Compared to normoxic cells, hypoxia and reoxygenation markedly induced NO production in BV-2 microglia cells (6.1 ± 2.5 and 60.3 ± 4.4 μM, respectively). Pretreatment with MAPK inhibitors of PD98059 (30 μM) and SB203580 (10 μM) or PI3-kinase inhibitors of LY294002 (10 μM) and wortmannin (150 nM) significantly inhibited hypoxia-induced NO production.

4. Discussion

Several molecular pathways have recently been shown to mediate hypoxia sensing at the cellular and molecular levels. The ability of cells and an organism to adapt to periods of hypoxia is important for their survival in both physiological

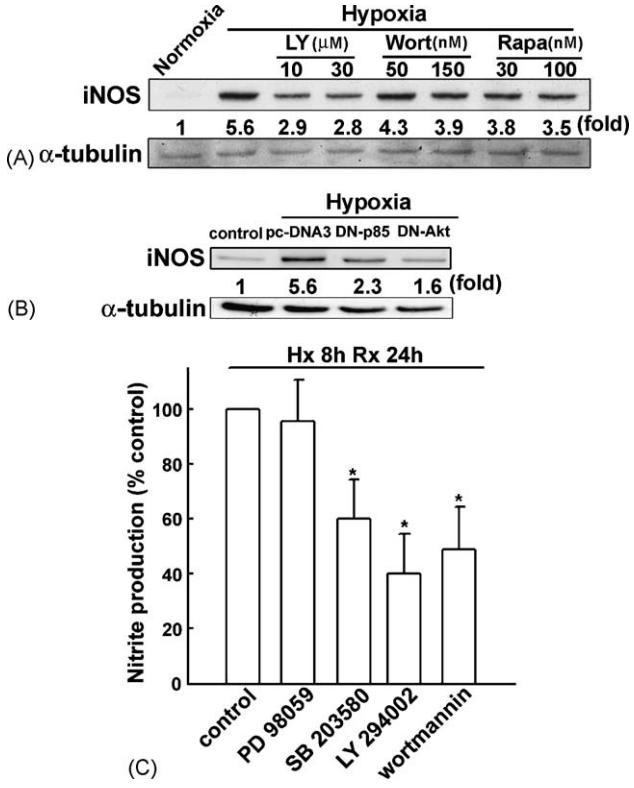

Fig. 7 – Involvement of PI3-kinase/Akt/mTOR in hypoxia-induced iNOS expression in microglia. (A) BV-2 microglia were pretreated with LY294002, wortmannin or rapamycin at indicated concentrations for 30 min and then exposed to hypoxia for 8 h. Cell lysates were used for measuring the protein levels of iNOS by Western blotting, and the results were normalized to α-tubulin. All of the three inhibitors decreased hypoxia-induced iNOS expression. (B) BV-2 microglia cells were transfected with either empty vector pcDNA3 or DN-p85, DN-Akt for 24 h followed by exposure to hypoxia for 8 h. The iNOS expression was normalized to α-tubulin. (C) NO production induced by hypoxia in BV-2 microglia. Cells were pretreated with PD98059 (30 μM), SB203580 (10 μM), LY294002 (30 μM), or wortmannin (150 nM) for 30 min and then exposed to hypoxia (Hx) for 8 h following by reoxygenation (Rx) for 24 h. The production of NO was evaluated by Griess reaction by measuring the level of nitrite. The data represent the mean ± S.E.M. of at least three independent experiments. $^{*}p < 0.05$ compared with hypoxia alone.

and pathophysiological states [23]. HIF-1 is thought to play a major role in response to hypoxia [24]. Preconditioning hypoxia increases target genes expression in response to HIF-1α, which produces ischemic tolerance to longer duration of focal ischemia [25]. HIF-1 expression by treatment with hypoxia-mimetic agents, such as $CoCl_2$ and desferrioxamine could induce several neuroprotection mechanisms, such as increased glucose transport, glycolysis, and vascular growth, all of which may contribute to protect the brain during ischemia [26]. Recently, it is reported that brain-specific knock-out of HIF-1α significantly decreases neuronal cell loss in response to hypoxia [27]. It is possible that the expression of HIF-1 in cerebral ischemia plays an important role in neuronal function. In the brain, HIF-1α expression seems to be induced by hypoxia in neurons, astrocytes, ependymal cells and possibly endothelial cells [5,6]. The expression of HIF-1 in microglia and oligodendrocytes is still not clear [4]. This is the first study to detect the HIF-1α expression in microglia and to explore the role of HIF-1α in the regulation of hypoxia-induced iNOS expression in microglia. As shown in Fig. 1, both primary cultured microglia and BV-2 cell line up-regulate HIF-1α in response to hypoxia compared with normoxia control and reveals a time-dependent manner. It has been reported that HIF-1β is not significantly affected by cellular oxygen tension [1,28]. HIF-1α protein is rapidly degraded under normoxic condition by the ubiquitin-proteasome system [29,30], whereas hypoxia induces both the stabilization and transactivation of HIF-1α [3,22]. Our data suggest that hypoxia up-regulates HIF-1α by stabilizing the protein. RT-PCR analysis revealed that HIF-1α mRNA levels remained unchanged in response to hypoxia.

HIF-1α has been reported to be regulated by a mTOR-dependent pathway [9]. Numerous studies reported that hypoxia induced HIF-1α via the PI3-kinase/AKT/mTOR pathway [31–33]. The present study also indicates that the PI3-kinase/AKT/mTOR signaling pathway is required for the enhanced expression of HIF-1α and iNOS induced by hypoxia in microglia. The following evidences support this conclusion: (a) hypoxia caused up-regulation of HIF-1α protein in a time-dependent manner; (b) PI3-kinase inhibitors, LY294002 and wortmannin, and mTOR/FRAP inhibitor, rapamycin, inhibited HIF-1α expression induced by hypoxia; (c) LY294002, wortmannin and rapamycin decreased the protein level of iNOS induced by hypoxia in a concentration-dependent manner; (d) pretreatment with PI3-kinase inhibitors or mTOR inhibitor or transfection with DN-p85 or DN-Akt inhibited hypoxia-induced HRE activity; (e) transfection of DN-Akt and DN-p85 decreased the protein level of iNOS induced by hypoxia. These data suggest that PI3-kinase/Akt/mTOR/HIF-1α is involved in the regulation of iNOS expression in microglia subjected to hypoxia.

Hypoxic condition used in the current study can be regarded like an ischemic insult, because glucose was present in culture media during a relatively short period of hypoxic exposure. Recent studies indicated that primary microglia and BV-2 microglia exposed to hypoxia caused an increase of NO [34] and IL-1β [35]. NO produced by activated glial cells is known to participate in both neuroprotection and neurodegeneration depending on the time and amounts of NO produced [36]. In general, reactive microglia may exert a bi-directional role after cerebral ischemia, a long-term beneficial effect due to involvement in the removal of neuronal debris, and a short-term injurious effect due to the release of proinflammatory cytokines and reactive oxygen metabolites after reperfusion.

It has been reported that C/EBP-β mediates iNOS induction by hypoxia in rat pulmonary microvascular smooth muscle cells [37]. In addition, Kruppel-like factor 6 enhanced the transcriptional activation of human iNOS [38]. In this work, we demonstrated that HIF-1α expression in microglia subjected to hypoxia upregulated iNOS. In addition, we have shown that

hypoxia-induced NO and iNOS production is regulated by PI3-kinase signaling pathway. NO production by microglia under hypoxia may play an important role in neuronal injury during cerebral ischemia. Upon occlusion of blood vessels in CNS, a number of pathological mechanisms are set in motion to kill neurons. Excitotoxicity, oxidative stress, and inflammation are some of those mechanisms involved in ischemic neuronal cell death [39–41]. The inflammatory mediators also amplify the local inflammatory responses to worsen the situation. As shown in Fig. 4, primary microglia culture and BV-2 cell line exposed to hypoxia increased iNOS expression, suggesting that hypoxia may also cause inflammation by the direct activation of microglia, which is independent to neuronal cell death, as mentioned by previous reports [34,42]. The iNOS inhibitor, aminoguanidine, effectively reduced focal cerebral ischemic damage in rat model of MCA occlusion [18]. The infarct volume and the motor deficits produced by MCA occlusion were smaller in iNOS knockout mice [43]. Thus, hypoxia seems to influence both neurons and microglia and leads to pathological neuronal death and inflammatory activation.

In conclusion, we here demonstrated that HIF-1α expression increased in primary microglia culture and BV-2 cell line under hypoxia condition. We also found that hypoxia was able to up-regulate iNOS in microglia via the activation of HIF-1 and PI3-kinase/Akt/mTOR signaling pathway. Our study provides a novel regulatory action of HIF-1α in neuroinflammation.

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